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Use of rapeseed, coffee and breadcrumbs waste by-products as substrates for microbial production of bioethanol

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Thesis to obtain the Master of Science Degree in

Biological Engineering

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PREFACE

The work presented in this document was developed for a Master Dissertation in Biological Engineering in Instituto Superior Técnico de Lisboa (IST), in order to obtain 30 ECTS.

The research was carried at the Faculty of Chemistry, Institute of Food Science and Biotechnology, at Brno University of Technology (BUT), from February to July of 2016, under the supervision of Professor Ivana Márová and Viliam Hlaváček (PhD student).

This thesis consists of a literature-based theoretical part related to the project, a description of the materials and methods used in this work, a chapter with the results obtained and its discussion, and finally a conclusion and future prospects related to the project developed.

ACKNOWLEDGEMENTS

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To my friends who were always there regardless the distance. And, my BFFs, specially Inês, Lia, Marlene, Sara and Tatiana, I don't know how to thank you for being the friends you are, it's been a hell of a journey and many more are to come!

Above all, I want to thank my family for always looking out for me and teaching me how to be the person I am today. To my cousin João for all the advice and constructive corrections related to my work.

"Aos meus avós que SEMPRE me mostraram o que é amar alguém incondicionalmente, tendo sempre uma palavra querida e um abraço apertado, sem nunca pedir nada em troca. À minha irmã por ser a minha melhor amiga, mesmo quando é a pessoa mais irritante que conheço. Aos meus pais por tudo o que fazem dia após dia por mim, por tudo o que me ensinam, por todas as ausências que me desculpam, por terem sempre tanto orgulho em mim e por estarem lá, sempre! A vocês um abraço apertadíssimo! São a minha força!"

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Thank you all! Děkuji vám všem! Obrigada a todos!

Joana, October 2016

RESUMO

A produção de biocombustíveis e químicos capazes de sustentar a dependência energética do mundo está a trazer novas descobertas neste campo científico. Muitas linhas de investigação estão atualmente direcionadas para a conversão de materiais lenhocelulósicos em açúcares, com o principal objetivo de descobrir quais os resíduos a usar como substrato na produção de biocombustíveis.

Neste trabalho estudou-se o uso de resíduos de colza, café e pão, como substratos no processo de sacarificação e fermentação a bioetanol, tendo sido usadas diferentes concentrações de ácido clorídrico (HCl) na sua hidrólise química. Foram também usadas enzimas comerciais da Novozymes ou Sigma-Aldrich e enzimas produzidas a partir de *Phanerochaete chrysosporium*.

Para o mesmo resíduo, a produção de bioetanol obtida dependeu das condições de hidrólise usadas. A maior produção de bioetanol foi obtida com hidrólise química recorrendo a 0,6 M HCl quando o substrato em uso eram os resíduos de pão (30,6 g/L de etanol). A hidrólise química foi a condição que demonstrou maiores produções de bioetanol quando comparada com as duas outras condições em estudo (uso de enzimas comerciais e enzimas produzidas a partir de um fungo), obtendo-se 6,86 e 7,42 g/L de etanol, para os substratos colza e café, respetivamente.

No processo de sacarificação e fermentação simultânea com recurso a enzimas comerciais obteve-se 4,28, 3,04 e 26,8 g/L de etanol para os resíduos de colza, café e pão, respetivamente.

No uso de enzimas produzidas pelo fungo *Phanerochaete chrysosporium* o máximo de etanol produzido foi 14,8 g/L para resíduos de pão. Para os resíduos de colza e café obteve-se 2,44 e 3,16 g/L, respetivamente.

Palavras-chave: Bioetanol, hidrólise química, hidrólise enzimática, resíduos lenhoceluloses, enzimas comerciais, fermentação alcoólica.

ABSTRACT

The promise of biofuels as a sustainable energy source required to meet the world's increasing energetic demand is leading to new discovers in this scientific field. One of the main research topics in this moment concerns the conversion of lignocellulosic materials into fermentable sugars. The main goal is to discover waste substrates that lead to higher biofuel productivities.

In this work three waste substrates, rapeseed, coffee and bread, were used, together with different concentrations of hydrochloric acid (HCl) to find the optimal conditions for chemical hydrolysis. Commercial enzymes from Novozymes or Sigma-Aldrich and enzymes produced by *Phanerochaete chrysosporium* were also used.

For the same feedstock, bioethanol production obtained varied with hydrolysis conditions. The highest ethanol production was obtained for chemical hydrolysis performed with 0.6 M HCl using bread waste as substrate (30.6 g/L). Chemical hydrolysis led to the highest productions when compared to the other two conditions in study. 6.86 and 7.42 g/L, of ethanol were obtained when using rapeseed and coffee waste, respectively.

The simultaneous saccharification and fermentation process recurring to the use of commercial enzymes led to 4.28, 3.04 or 26.8 g/L of ethanol when rapeseed, coffee or bread waste were used, respectively.

When using enzymes produced by *Phanerochaete chrysosporium*, the maximum ethanol production was 14.8 g/L for bread waste as substrate. Rapeseed or coffee waste led to 2.44 and 3.16 g/L of ethanol, respectively.

Keywords: Bioethanol, chemical hydrolysis, enzymatic hydrolysis, lignocellulosic wastes, commercial enzymes, alcohol fermentation.

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NOMENCLATURE

APS	Ammonium Persulfate
C_{cont}	Cellulose content
CFU	Colony-forming unit
GDP	Gross Domestic Product
GHG	Greenhouse gas
H_{cont}	Hemicellulose content
Hex_{cont}	Hexoses content
HMF	5-hydroxymethlyfurfural
HPLC	High performance liquid chromatography
IEA	International Energy Agency
IPCC	Intergovernmental Panel on Climate Change
LCA	Life Cycle Assessment
η (%)	Efficiency of sugars conversion to ethanol
OD	Optic density
$Pent_{cont}$	Pentose content
PTFE	Polytetrafluoroethylene
Q_P (g/L·h)	Ethanol volumetric productivity
SDS	Sodium dodecyl sulfate
SHF	Separate hydrolysis and fermentation
SSF	Simultaneous saccharification and fermentation
Tris	Tris(hydroxymethyl)aminomethane
TS	Total Solids
UV-VIS	Ultraviolet and visible
W (g)	Weight
w/v	Weight per volume
$Y_{P\|S}$ (g/g)	Ethanol yield factor

1 INTRODUCTION

1.1 ENERGETIC SCENARIO

The increasing industrialization of previously undeveloped nations along with the constant need to fuel the westernized world economies is steadily increasing the world's energy consumption. This demand has raised concerns in the last decades due to the intensive use and dependence on fossil fuels. The global climate change and the increasing scarcity of fossil resources has led to the urge to develop and deploy renewable energy solutions and to consider a gradual transition towards a bio-based economy (Jönsson and Martín, 2016; Tesfaw and Assefa, 2014).

The Intergovernmental Panel on Climate Change (IPCC) Fourth Assessment Report highlighted that the world's growing population and per capita energy demand are leading to the rapid increase in greenhouse gas (GHG) emissions. In particular, over the past 10 years, the transport industry has shown higher rates of growth in GHG emissions than any other sector. It is also important to note that the primary source of energy for this sector is oil (Cherubini, 2010).

Accordingly to International Energy Agency (IEA), since 1971, each 1% increase in global Gross Domestic Product (GDP) has been accompanied by a 0.6% increase in primary oil demand (Teske *et al.*, 2013). Therefore, the energy demand and the increase in the monetary value of final goods and services is leading to an energy revolution.

A way to tackle this dependence on fossil fuels is to consider and study fuel production from plant-based raw materials. This process should be driven by well-developed integrated biorefining systems (Cardona and Sánchez, 2007; Jong and Jungmeier, 2015).

According to IEA Bioenergy Task 42, the definition of Biorefinery is "…the sustainable processing of biomass into a spectrum of marketable products and energy". By other words, it consists in the whole course of upstream, midstream and downstream processing of biomass into a range of products, where sustainability is the main driving aspect (Jong and Jungmeier, 2015).

Nowadays, it is known that the burning of fossil fuels is a key contributor to global warming (Naik *et al.*, 2010), and as such, it became urgent to find sustainable and environmentally friendly sources of energy, leading to the interest in the production of fuel from plants and organic waste.

Among the dominant renewable biofuels: bioethanol, biodiesel and biogas, the most common is ethanol from corn grain (starch) and sugar cane (sucrose) (Gray *et al.*, 2006). Nevertheless, these conventional crops are unable to meet the global demand of bioethanol production due to their primary value as food and feed (Sarkar *et al.*, 2012). Lignocellulosic biomass, such as agricultural and forestry wastes, are increasingly seen as an alternative source where the existing competition between food versus fuel is avoided.

Lignocellulosic materials are renewable, abundant and low cost. Their utilization in biorefineries requires proper cellulose uncovering pretreatment, increasing concentrations of fermentable sugars and therefore improving the efficiency of the whole process (Jönsson and Martín, 2016).

The complexity of lignocellulose makes it more difficult than starch to enzymatically degrade to fermentable sugars, making it necessary to study the major steps in the conversion process: thermochemical pretreatment, enzymatic saccharification and the fermentation process.

1.2 BIOFUELS

The world's dependence on oil and CO_2 production could be reduced by the use of biofuels produced from biomass.

Currently, fuels based on biomass are known as 1st and 2nd generation fuels. First generation biofuels are those produced from raw materials that compete with food and feed industries (Cherubini, 2010; Naik *et al.*, 2010). To overcome the issues about land use and competition with food crops, interest in the production of second generation biofuels has increased, as they use non-food source materials. Figure 1.1 depicts a comparison between the use of these two types of biofuels.

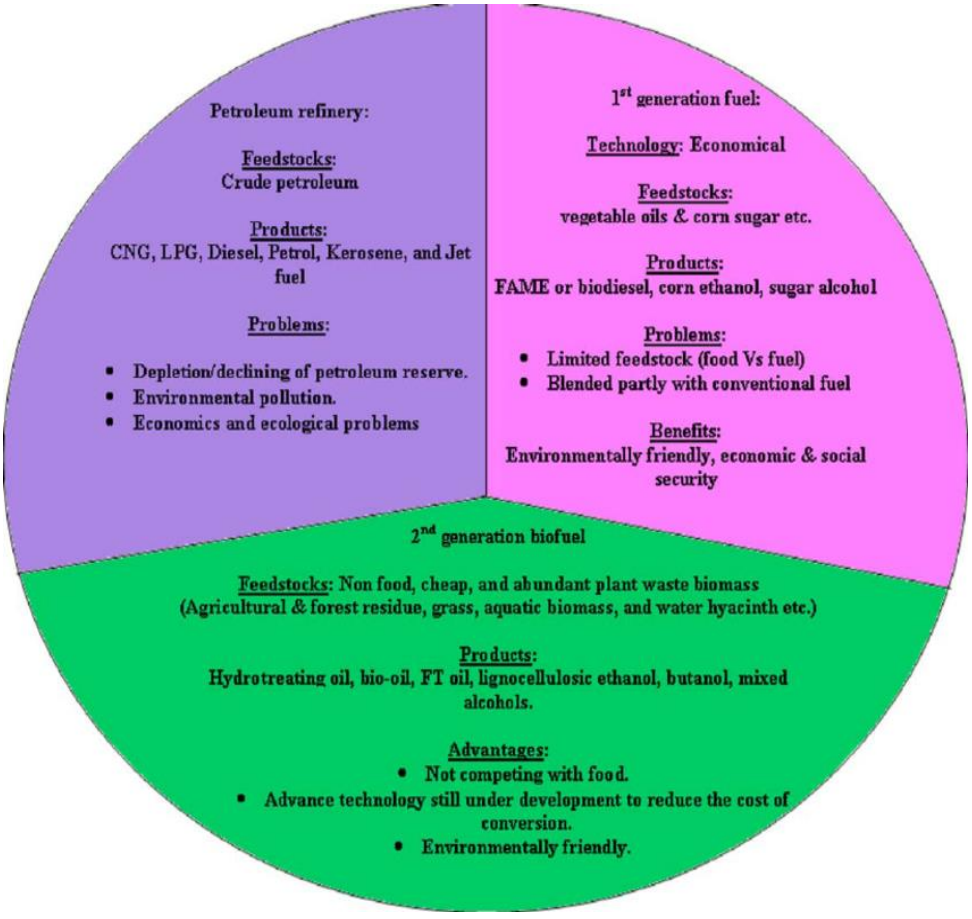

Figure 1.1 Comparison between first and second generation biofuels and petroleum refinery (Naik *et al.*, 2010).

1.2.1 First generation biofuels

First generation biofuels are being commercialized nowadays, with almost 50 billion litres produced annually (Naik *et al.*, 2010).

The high sugar or oil content of the raw materials used to produce these kind of biofuels are pointed as the main advantages, along with its capacity to be combined with petroleum-based fuels.

Most of these biofuels use crop plant components as an energy source, such as sugars, oils and cellulose (Aro, 2016). Life Cycle Assessment (LCA) analysed that when the conventional transportation fuels (diesel and gasoline) are replaced by biofuels (bioethanol and biodiesel), there is a net reduction in global warming emissions and fossil energy consumption (Cherubini, 2010).

However, there are some disadvantages to these first generation biofuels, especially the competition with food and feed industries for the use of biomass and crops. As a result of this problem, the food prices are rising because of the use of feedstock and land. Also, the potential of these biofuels is limited by soil fertility and the CO_2 emissions savings and fossil energy consumption reduction are limited by the high energy required for cultivation and conversion of crops (Cherubini, 2010; Naik *et al.*, 2010).

Some of these problems are expected to be surpassed with the use of second generation of biofuels.

1.2.2 Second generation biofuels

The second generation biofuels are produced from non-food crops such as residues containing lignocellulosic materials obtained from agriculture, forestry and industry. These type of biofuels are considered an optimum situation, since their raw materials are widespread, relatively cheap and easily available.

The use of the biomass crops could lead to the coproduction of valuable biofuels, chemicals, electricity and heat, as well as developing better energy and environmental performances (Cherubini, 2010).

Some further work is still needed in order to maximise the amount of renewable carbon and hydrogen that can be converted into fuels from the feedstock of lignocellulose (Aro, 2016).

1.3 Lignocellulosic materials

When using lignocellulosic materials as feedstock it is necessary to convert lignocellulosic biomass into intermediates (cellulose, hemicellulose, lignin) before conversion into the final products (Jong and Jungmeier, 2015).

Regarding the potential of plant biomass to produce liquid biofuels, most of its composition consists of cell walls, where 75% of it are polysaccharides (Naik *et al.*, 2010). This makes it necessary to disrupt the cell wall before further processing.

According to the composition of the lignocellulosic complex, the pre-step is when cellulose, hemicellulose and lignin are released. They are further converted mainly into glucose, mannose, and xylose, in a hydrolysis step (Cardona and Sánchez, 2007; Jong and Jungmeier, 2015).

1.3.1 Composition of lignocellulosic materials

Lignocellulose is a carbohydrate polymer composed of cellulose (40-50%), hemicellulose (20-40%) and lignin (20-30%). These polymers are interlinked in a hetero-matrix (Agbor *et al.*, 2011; Horn *et al.*, 2012).

Whereas cellulose is a linear and crystalline homopolysaccharide, hemicellulose is a short, highly branched and non-cellulosic polysaccharide and lignin is an intricate polyphenolic structure, tightly bound to these two carbohydrate polymers (Claassen *et al.*, 1999; Sarkar *et al.*, 2012). Figure 1.2 shows the crystalline structure referred as lignocellulose.

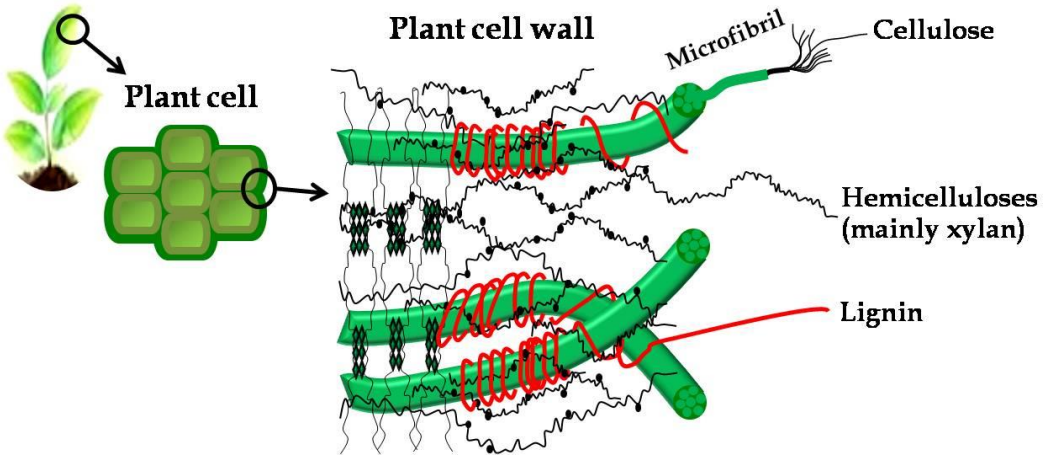

Figure 1.2 Structural organisation of the lignocellulose components in the plant cell wall (Ratanakhanokchai *et al.*, 2013).

Pretreatment methods are being developed in order to easily access the cellulose present in the lignocellulose matrix. These mechanisms must be studied in order to impede undesirable phenomena, such as the irreversible binding of the enzymes to lignin structure or the inhibition of the enzymes by hydrolysis products (Brummer, Jurena, *et al.*, 2014).

1.3.1.1 Cellulose

Unlike hemicelluloses and lignin, cellulose is a uniform component of most types of cellulosic biomass, as it is the structural base of plant cells.

Cellulose is a homopolysaccharide composed of anhydroglucose units linked by β-1,4-glycosidic bonds. The 24 to 36 chains aggregate into microfibrils via hydrogen bonding and van der Waals forces, thus making the macromolecule highly crystalline and difficult to hydrolyse. The hydrogen bonds determine the strength of the chain, while on the other hand interchain hydrogen bonds might introduce crystalline (order) or amorphous regions, which introduce disorder into the structure of the cellulose. Consecutive glucose monomers are rotated 180 degrees, what means that the repeating unit of this polymer is the disaccharide (cellobiose) (Agbor *et al.*, 2011; Horn *et al.*, 2012; Jönsson and Martín, 2016).

In order to be used, these tightly packed nanofibers, as seen in Figure 1.3, must be broken down through chemical or enzymatic hydrolysis to create sugar, being later converted into ethanol or other liquid fuels (Naik *et al.*, 2010). Its stability it is also due to the presence of hemicellulose and lignin.

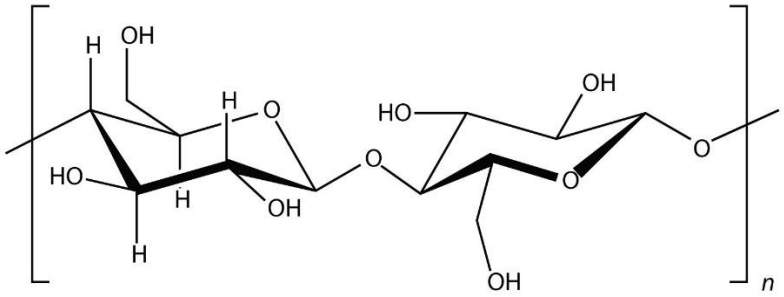

Figure 1.3 Structure of the cellulose polymer. Adapted from (Jönsson and Martín, 2016).

1.3.1.2 HEMICELLULOSE

Hemicellulose differs from cellulose as they are heteropolymers of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose) and acetylated sugars (Agbor *et al.*, 2011). Usually these polymers have low degree of polymerization, and are easy to hydrolyse.

The principal hemicellulose components are xylans and glucomannans, with xylans being the most abundant. This is the principal component of secondary cell walls of hardwoods and herbaceous plants, about 20-30% of its composition (Gírio *et al.*, 2010). In herbaceous plants, xylan has a β-1,4-linked xylose backbone with a high amount of acetylesterifications and a lesser amount of α-1,2 linked glucuronic acid/4-O-Methyl-glucuronic acid substituents (Horn *et al.*, 2012). The composition is variable, depending on the nature and source of each feedstock.

The most common hemicelluloses are composed of a main backbone of xylose linked by β-1→4 bonds, where the structural units are often substituted at positions C2 or C3 with arabinofuranosyl, 4-*O*-methylglucuronic acid, acetyl or phenolic substituents (Moure *et al.*, 2006).

While xylan can be easily extracted in an acid or alkaline environment, glucomannan requires strong alkaline environment. Nevertheless, the process should be controlled because hemicellulose sugars may be degraded to weak acids and furan derivatives which can act as

inhibitors of the ethanol fermentation (Agbor *et al.*, 2011; Gírio *et al.*, 2010). Figure 1.4 shows an example of xylan and xylanase specificity.

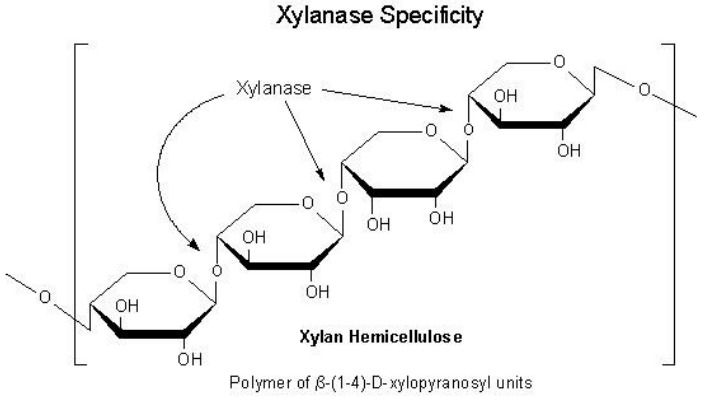

Figure 1.4 An example of xylan hemicellulose showing the xylanase specificity (Sigma-Aldrich, 2016a).

1.3.1.3 LIGNIN

Lignin is a relatively hydrophobic and complex aromatic heteropolymer composed of phenylpropanoid units (phenyl ring, C6, and propane, C3, side chain) kept together by different linkages (Horn *et al.*, 2012; Jönsson and Martín, 2016). This polymer constitutes a problem because of its rigidity, impermeability and resistance to hydrolytic enzymes (cellulase, β-glucosidase) from accessing the sugars because of its close association with cellulose microfibrils that give it structural integrity (Achyuthan *et al.*, 2010; Agbor *et al.*, 2011).

It is one of the most abundant natural polymers, comprising 15 to 25% of biomass. Lignin is composed of three main phenylpropane units namely, *p*-coumaryl, coniferyl and sinapyl alcohol, linked through different types of ether and ester bonds, and minor phenolic compounds (Achyuthan *et al.*, 2010). Its composition is also changed according to external and natural factors.

Due to lignin's aromatic compounds is difficult to find enzymes capable of degrading it, only oxidoreductases are known to be able to depolymerize lignin. Some of these phenols are shown in Figure 1.5.

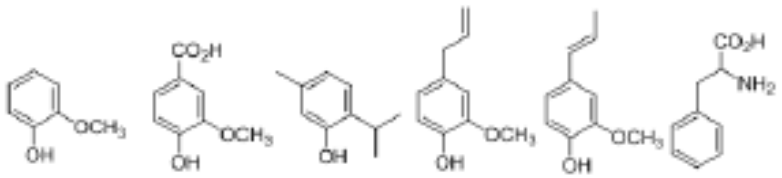

Figure 1.5 Some relevant structures of phenols occurring in lignin. Adapted from (Achyuthan *et al.*, 2010).

1.3.2 WASTE MATERIALS

An important aspect in biorefinery is the delivery of a renewable, consistent and regular supply of feedstock. Renewable carbon-based raw materials are provided from four different sectors: agriculture, forestry, industry and aquaculture.

In this work, rapeseed, coffee and bread were studied as substrates for bioethanol production since they are available and abundant waste materials.

1.3.2.1 RAPESEED

Rapeseed (*Brassica napus*) straw (Figure 1.6) is a lignocellulosic agricultural residue with high sugar content (approximately 60%) (López-Linares *et al.*, 2014), namely 44.6±0.2% cellulose and 20±0.2% hemicellulose (Tomás *et al.*, 2013).

Figure 1.6 Example of rapeseed straw aspect (Span, 2016).

Nowadays, rapeseed is one of the most cultivated oil plants for production of biofuels. However, after seed harvesting, the rapeseed straw left behind in the fields needs to be disposed. Relating to the concept of biorefinery, these residues can be used to produce bioethanol (Díaz *et al.*, 2010; Luo *et al.*, 2011).

The rapeseed straw composition is presented in Table 1.1. According to the rapeseed source used in this work the lipids composition was 2.55% and the water content was 12.29%.

Table 1.1 Composition of rapeseed straw (Díaz *et al.*, 2010).

Composition	Dry matter (%)
Cellulose (as glucose)	36.59±0.82
Hemicellulosic sugars	24.11±1.43
Xylose	18.37±0.47
Galactose	2.54±0.84

Arabinose	1.44±0.08
Mannose	1.76±0.04
Acid-insoluble lignin (AIL)	15.55±0.44
Acetyl groups	3.65±0.55
Acid soluble lignin (ASL)	1.58±0.14
Extractives	14.55±1.01
Ashes	5.73±0.38

1.3.2.2 COFFEE

Coffee is one of the most popular beverages around the world and has grown in commercial importance during the last years (Gouvea *et al.*, 2009; Mussatto *et al.*, 2011; Yadira *et al.*, 2014). It is the second largest traded commodity in the world and generates a large number of by-products during its processing from the fruit. These by-products can be given further use as solid residues.

The residues obtained consist of pulp, hull, husks and other spent parts of the coffee fruit, depending on the coffee processing. They present a high concentration of carbohydrates, and thus can be used for extraction of caffeine and polyphenols, and as a raw material for bioethanol production (Gouvea *et al.*, 2009; Murthy and Madhava Naidu, 2012). Besides carbohydrates, coffee bean is composed of cellulose, minerals, sugars, lipids, tannin and polyphenols (Murthy and Madhava Naidu, 2012). An example of a spent coffee sample can be seen in Figure 1.7.

Figure 1.7 Spent coffee grounds (Murthy and Madhava Naidu, 2012).

Spent coffee constitutes almost 50% of the worldwide production of soluble coffee preparation, it is rich in sugars containing mannose and galactose. The chemical composition of the coffee spent is presented in Table 1.2.

Table 1.2 Chemical composition of coffee by-products (Murthy and Madhava Naidu, 2012).

Composition (%)	Coffee pulp	Coffee husk	Silver skin	Coffee spent
Cellulose	63.0±2.5	43.0±8.0	17.8±6.0	8.6±1.8
Hemicellulose	2.3±1.0	7.0±3.0	13.1±9.0	36.7±5.0
Protein	11.5±2.0	8.0±5.0	18.6±4.0	13.6±3.8
Fat	2.0±2.6	0.5±5.0	2.2±1.9	ND
Total fiber	60.5±2.9	24±5.9	62.4±2.5	ND
Total polyphenols	1.5±1.5	0.8±50	1.0±2.0	1.5±1.0
Total sugars	14.4±0.9	58.0±20.0	6.65±10	8.5±1.2
Pectic substance	6.5±1.0	1.6±1.2	0.02±0.1	0.01±0.005
Lignin	17.5±2.2	9.0±1.6	1.0±2.0	0.05±0.05
Tannins	3.0±5.0	5.0±2.0	0.02±0.1	0.02±0.1
Chlorogenic acid	2.4±1.0	2.5±0.6	3.0±0.5	2.3±1.0
Caffeine	1.5±1.0	1.0±0.5	0.03±0.6	0.02±0.1

1.3.2.3 BREAD

One of the largest portions of municipal solid waste is attributed to food waste, which causes severe environmental problems. One possible application for food waste can be the production of bioethanol, more precisely, the use of bread waste since a few percent of the bread residues are moldy and can't be implemented as animal feed due to raise of health problems (Ebrahimi *et al.*, 2008).

The nutrients contained in bread waste are stored as macromolecules, such as starch and proteins, being starch the main constituent of bread dry weight. In order to use these macromolecules, they have to be broken down into sugars and amino acids. Usually the enzymatic hydrolysis of bread uses α-amylases and proteases that facilitate this process (Leung *et al.*, 2012).

Mold growth seems to be accompanied by consumption of valuable substrates and release of heat-resistant mycotoxins and other metabolites that pollute the feedstock, creating heterogeneity in bread composition.

Figure 1.8 shows the breadcrumbs before being used in the enzymatic hydrolysis.

Figure 1.8 Breadcrumbs before using as feedstock for the bioethanol production.

1.4 BIOMASS CONVERSION FOR BIOETHANOL PRODUCTION

Biochemical conversion of lignocellulosic biomass for bioethanol production is made through three major processes: a pretreatment step aiming to release cellulose and hemicellulose before hydrolysis; hydrolysis of these components to produce fermentable sugars and finally, the fermentation of the resulting sugars to bioethanol.

1.4.1 PRETREATMENT PROCESS

Pretreatment is an essential step because it facilitates the access to the solid biomass for further chemical or biological treatment. Due to the low efficiency of hydrolysing biomass to fermentable sugars using enzymes, the pretreatment is used to solubilize and separate the lignocellulosic components (Ertas *et al.*, 2014; Sarkar *et al.*, 2012).

During pretreatment the lignocellulosic matrix is broken with the goal of reducing the cellulose crystallinity, as most of the sugars are sheltered in the cellulose microfibrils, and increasing the amorphous fraction of cellulose, in order to release the fermentable sugars for enzymatic attack (Cardona and Sánchez, 2007; Obruca, 2015; Sarkar *et al.*, 2012).

Additionally, most of hemicellulose should be hydrolysed and lignin released or degraded in a process also called delignification (Gomez *et al.*, 2008; Obruca, 2015). It should be considered that cellulose hydrolysis is affected by the porosity of these materials. The yield of cellulose hydrolysis is less than 20% of the maximal theoretical value when pretreatment is not carried out, whereas the yield after pretreatment often exceeds 90% of the that value (Cardona and Sánchez, 2007).

Therefore, the goals of the pretreatment process are the formation of sugars or the ability to form them by hydrolysis, to avoid degradation of sugars formed, and to minimize the formation of inhibitory by-products (Cardona and Sánchez, 2007; Sarkar *et al.*, 2012).

Due to the differences in the composition of each feedstock, it is still not possible to design a suitable pretreatment method for all feedstocks. Also, the method must be chosen taking into

account the effects on polymers present in the lignocellulosic feedstock (Paris *et al.*, 2014). There are four pretreatment techniques used in this process: chemical, physical, physicochemical and biological. These techniques can be used individually or combined.

1.4.1.1 CHEMICAL PRETREATMENT

Chemical pretreatment can consist on the use of different chemical agents such as ozone, acids, alkalis, peroxide and organic solvents.

One of the most cost-effective pretreatments is the use of dilute acids. This technique is performed by soaking the material in dilute acid solution (HCl or H_2SO_4) and then heating to temperatures between 140 and 200ºC for a certain period of time. This cycle hydrolyses the hemicellulose, recovering most of its monomer sugars (Behera *et al.*, 2014; Cardona and Sánchez, 2007; Viikari *et al.*, 2007). This method is supposed to be the more efficient for commercial scale.

Organic acids (oxalic acid, acetylsalicylic acid and salicylic acid) can be used as catalysts when mixed with inorganic acids, which are used to break the internal lignin and hemicellulose bonds. Usually in these methods the hydrolysed lignin is dissolved and recovered in the organophilic phase. However, the problem is that concentrated acids are corrosive and must be recovered (Aditiya *et al.*, 2016; Behera *et al.*, 2014). In the chemical pretreatment, the chemicals degrade the lignocellulose's walls and the carbohydrate chain of starch through direct chemical reaction.

1.4.1.2 PHYSICAL PRETREATMENT

Enzymatic hydrolysis can be easier if biomass is chipped, milled and grinded into a fine powder, since this processing increases the cellulose's surface area (Behera *et al.*, 2014; Viikari *et al.*, 2007).

The goal in a mechanical pretreatment is related to the size reduction of the particle and decrease of the crystallinity. This size reduction leads to an increase of available surface area and a reduction of the degree of polymerization. It is known that the total hydrolysis yield of the lignocellulose in this process is 5-25% (Hendriks and Zeeman, 2009).

This method is not economically attractive due to their high energy costs. The energy required depends on the final particle size and biomass characteristics. In this context, extrusion would be more promising, a process where the materials are subjected to heat, mix and shear (Behera *et al.*, 2014).

1.4.1.3 PHYSICOCHEMICAL PRETREATMENT

Physicochemical pretreatment combines physical and chemical processes, which are considered more efficient than only using a physical pretreatment with the goal of altering lignin structure and

solubilizing hemicellulose. Most of the pretreatment methods belong to this category, such as steam explosion, liquid hot water, ammonia fibre/freeze explosion, wet oxidation, ammonia recycle percolation, aqueous ammonia, organic solvents and CO_2 explosion (Behera *et al.*, 2014).

The most widely used method in treating the biomass is steam explosion, where a combination of hydrothermal treatment and sudden pressure change is applied. Usually the raw material is treated with high-pressure saturated steam at a temperature typically between 160 and 240ºC, which is maintained for a few minutes (Aditiya *et al.*, 2016; Viikari *et al.*, 2007). After depressurisation the biomass fibres explode.

However, this method is prone to the formation of by-products during the steam explosion, which show inhibitory effects to fermentation.

1.4.1.4 BIOLOGICAL PRETREATMENT

Biological pretreatment comprises the use of microorganisms capable of producing enzymes that degrade lignin, hemicelluloses and polyphenols present in the biomass. These microorganisms need to possess the capacity of degrade the lignocellulosic components of the feedstock to their amorphous form (Aditiya *et al.*, 2016; Alvira *et al.*, 2010).

Brown, white and soft-rot fungi are the most effective biological pretreatment agents. Their ability depends on the hydrolytic and ligninolytic system, which aim to produce hydrolases for polysaccharide liberation and breaking the lignin structure (Aditiya *et al.*, 2016).

High delignification efficiencies have been demonstrated for several white-rot fungi, such as *Phanerochaete chrysosporium*, *Ceriporia lacerata*, *Cyathus stercolerus*, *Ceriporiopsis subvermispora*, *Pycnoporus cinnarbarinus* and *Pleurotus ostreaus* (Alvira *et al.*, 2010; Behera *et al.*, 2014). In fact, *Phanerochaete chrysosporium* has been proposed to be able to degrade the lignin into biomass which can be later transformed into ethanol, involving the separate fermentation of pentoses and hexoses (Alvira *et al.*, 2010; Cardona and Sánchez, 2007).

Biological pretreatment methods are environmentally friendly and save energy as they are executed at low temperature with no addition of chemicals. However, the rate of the process is too low for industrial use (Singh *et al.*, 2014; Viikari *et al.*, 2007).

1.4.2 HYDROLYSIS OF DELIGNIFIED MATERIALS

After the pretreatment, the cellulose from the lignocellulosic materials needs to be converted into sugars. This is the critical step of the process, saccharification, where the complex cellulose's carbohydrates are converted into simple monomers. Hydrolysis can consist of a chemical reaction in which acid (H_2SO_4 or HCl) is used, or an enzymatic reaction.

1.4.2.1 DILUTE ACID HYDROLYSIS

Sulphuric and hydrochloridric acids are the most common used diluted acids for the hydrolysis of lignocellulose biomass. When diluted acids (concentrations of 1-10%) are employed, high temperatures (in the range of 100-150ºC) are required in order to hydrolyse the crystalline cellulose of lignocellulose (Cardona and Sánchez, 2007; Obruca, 2015).

The acid can penetrate lignin without pretreatment at a fast rate. In this way, the acid catalyses the breakdown of long carbohydrate chains, forming shorter chain oligomers, and then, sugar monomers (Lenihan *et al.*, 2010; Obruca, 2015). Such severe conditions are not employed for the hydrolysis of hemicellulose due to its amorphous form in comparison to crystalline cellulose.

On the other hand, the high temperatures employed to hydrolyse the lignocellulose lead to a lower formation of hexoses. This is mainly due to the formation of toxic compounds during the decomposition of monosaccharides which decreases the ethanol yield (Lenihan *et al.*, 2010). This problem occurs when higher temperatures and higher acid concentrations are used.

One way to solve this problem is to pretreat the biomass material with alkaline hydrolysis, which is believed to enhance the removal of the xylan's crosslinks and to reduce the formation of inhibitory components (Verardi *et al.*, 2012).

1.4.2.2 ENZYMATIC HYDROLYSIS

Comparing acid hydrolysis with enzymatic hydrolysis, the latter requires less energy and mild environment conditions, and no inhibitory products (or in a low concentration) are formed during the cellulose hydrolysis process.

The mechanism of cellulose hydrolysis involves physical disruption of insoluble cellulose in addition to endo- and exo-acting enzymes (Zhang and Lynd, 2004). This process can be described in three steps. First, the glucan chains of repeating β-(1,4)-D glucose units are split by endo-1,4-β-D-glucanases (EC 3.2.1.4, cellulase enzymes). Second, cellobiose is generated by hydrolysis of the glucan chain with exo-1,4-β-D-glucanases, also called cellobiohydrolases (EC 3.2.1.91). In the last step, cellobiose is converted into glucose by β-glucosidases (EC 3.2.1.21) (Paris *et al.*, 2014). Hemicellulolytic enzymes are more complex, involving a mixture of eight enzymes (Sarkar *et al.*, 2012). Cellulose is hydrolysed to glucose whereas hemicellulose gives rise to several pentoses and hexoses. These enzymes benefit from the synergism of using an "enzyme cocktail" which leads to a higher performance and are highly substrate specific.

Most of the enzymes used in the industry are originated from microorganisms. Due to the high stability, fungi are the main producers of cellulolytic enzymes capable of degrading biomass (Obruca, 2015; Paris *et al.*, 2014). *Trichoderma reesei* is a saprobic fungus (fungi that colonize rotting wood and dead organic matter found in soil), known for efficient production of extracellular

enzymes, most commercial cellulotic enzymes are obtained from this aerobic microorganism (Cardona and Sánchez, 2007; Verardi *et al.*, 2012).

Yields of the enzymatic hydrolysis vary according to the material and depend on the accessibility of the cellulose, the degree of mechanical disruption and physical-chemical factors. The optimal temperature ranges from 45 to 50ºC and pH between 3 and 7, since these conditions lead to less degradation of sugar and higher yields comparing to chemical hydrolysis (Brummer, Jurena, *et al.*, 2014; Paris *et al.*, 2014; Sarkar *et al.*, 2012; Verardi *et al.*, 2012).

1.4.3 ALCOHOL FERMENTATION

One of the processes employed for biomass hydrolysates fermentation involves a sequential process where the hydrolysis of cellulose and the fermentation occur in different parts, called separate hydrolysis and fermentation process (SHF). When the microbial hydrolysis of the biomass and fermentation are carried out in a single step, the process is named simultaneous saccharification and fermentation (SSF). The microorganism most commonly used for fermentation of the saccharified biomass is *Saccharomyces cerevisiae*, which can efficiently ferment the hexoses present in the hydrolysates.

Ethanol can be produced by catalysed reactions. As for sugar, they can be extracted from sugar crops and fermented into ethanol. On the other hand, starch products first need to be broken down to simple glucose by acids or amylases so that they can then be fermented (Neves *et al.*, 2007).

As shown in equations (1.1) and (1.2), the carbohydrates fermented to ethanol under oxygen-free conditions, can yield a theoretical maximum of 0.51 kg ethanol and 0.49 kg carbon dioxide per kg of sugar (Hamelinck *et al.*, 2005).

$$3C_5H_{10}O_5 \rightarrow 5C_2H_5OH + 5CO_2 \quad (1.1)$$

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \quad (1.2)$$

1.4.3.1 *SACCHAROMYCES CEREVISIAE*

The microorganism used for the production of bioethanol needs to withstand high concentrations of ethanol and high temperature, be tolerant to inhibitors present in the hydrolysates and have cellulolytic activity, all said, *S. cerevisiae* is the most common used microorganism for ethanol production (Sarkar *et al.*, 2012). This yeast is capable of converting 90 to 93% of the sugars into ethanol (Paris *et al.*, 2014).

S. cerevisiae presents some advantages over the use of other yeasts, such as, its metabolic flux to ethanol is hardly affected by the presence of oxygen under excess conditions, this yeast is able to grow under anaerobic condition and it has a high ethanol tolerance (Claassen *et al.*, 1999).

The problem with *S. cerevisiae* is that this organism can only ferment monosaccharides and disaccharides, as glucose, fructose, maltose and sucrose, and can't use the main C-5 sugar, xylose.

1.4.3.2 SEPARATE HYDROLYSIS AND FERMENTATION

In this process, the fermentation is carried out after the hydrolysis in separated steps for starch hydrolysis and glucose fermentation. This allows the use of the optimum temperature in each process.

For the conversion of starch-containing material into ethanol, first the starch molecule is initially hydrolysed by the action of amylolytic enzymes and after complete hydrolysis, the fermentation is carried out (Neves *et al.*, 2007).

This process is long, taking up to one to four days, which can increase the contamination risks. One of the motives for this long process may be related to the slow xylose consumption during the fermentation, a consequence of the presence of toxic compounds which inhibit the growth and fermentation activity of the microorganism (Sarkar *et al.*, 2012).

1.4.3.3 SIMULTANEOUS SACCHARIFICATION AND FERMENTATION

In this type of fermentation, hydrolysis and fermentation are carried out at the same time, in the same vessel. One of the advantages is the fact that the sugar produced during the hydrolysis of polysaccharides can be consumed immediately by the microorganism used in the fermentation. In this way, the ethanol yield is improved, eliminating the end-product inhibition. In addition, this process is shorter and requires lower concentrations of enzymes when compared with SHF (Neves *et al.*, 2007). Besides the presence of the yeast, addition of enzymes reduces the sugar accumulation conducting to higher rates, yields and ethanol concentrations, because the sugar produced during the starch breakdown slows down α-amylase action.

This process has a higher ethanol yield coefficient when compared to SHF, since it is capable of converting xylose to xylitol more efficiently.

However, this process presents some disadvantages, such as use of different optimum temperatures for hydrolysis and fermentation, low substrate to liquid ratio due to the insoluble nature of the substrate and difficult control and optimisation of process parameters (Claassen *et al.*, 1999). One solution may be the use of thermo-tolerant microorganisms like *Kluyveromyces marxianus* which withstands the higher temperatures needed for enzymatic hydrolysis (Sarkar *et al.*, 2012).

1.5 AIMS OF THIS THESIS

The aim of this thesis was to compare the production of bioethanol using three different waste substrates, rapeseed, coffee and breadcrumbs, the latter of which will from now on be named as bread waste.

Another goal was to increase the productivity of the process by focusing on the chemical hydrolysis of the lignocellulosic material, in shake flasks.

Finally, the process was implemented in a 2 L bioreactor and used to compare the production of bioethanol using commercially available and in-house purified enzymes.

To do so, firstly, the chemical hydrolysis of the wastes was performed using different concentrations of hydrochloridric acid (HCl) with the aim of optimizing the conversion of glucose and, consequently, the production of ethanol.

The process was then scaled-up to a 2 L bioreactor according to the shake flasks results previously obtained.

Afterwards, the achieved productivity was compared with the use of commercial enzymes instead of the chemical hydrolysis.

With the goal to achieve a cost effective process, the last part of the project was to produce enzymes from a mold (*Phanerochaete chrysosporium*) and use them to compare the bioethanol production in the bioreactor.

2 MATERIALS AND METHODS

This section presents the materials used in the different experiments conducted, as well as the brands and model of all chemicals and instruments. The experimental setup and methods used during the experimental work are also described.

2.1 RAW MATERIALS

The substrates used to perform this experiments were rapeseed, coffee and bread. The rapeseed was provided by an oil producing facility, corporation ADM, located in Olomouc, Czech Republic. The coffee was a single origin Arabica from Ethiopia, Sidamo Nefas region. This coffee was used in an expresso machine for coffee brewing in the Faculty of Chemistry, in Brno University of Technology, and dried before being used in these experiments. The commercial breadcrumbs from Castello were used as bread waste.

The wastes' composition is presented in Table 2.1, in percentage of content.

Table 2.1 Waste composition in % of content for rapeseed, coffee and bread waste.

Composition (%)	Rapeseed	Coffee	Bread
Cellulose	17.8	9.6	-
Hemicellulose	14.8	34.8	-
Total soluble sugars	-	8.3	6.3
Proteins	34.1	14.1	12.3
Lipids	7.8	9.3	1.5
Starch	12.5	-	63.8

2.2 *SACCHAROMYCES CEREVISIAE* STRAIN

The yeast used in this experimental study was *Saccharomyces cerevisiae* 6646 and was obtained from The Czech National Collection of Type Cultures, Czech Republic. The stock culture was conserved in Petri dishes at 3ºC in the same medium described in Table 2.2.

2.3 CULTURE MEDIUM

The culture medium for the *S. cerevisiae* was the growth medium for yeast described in Table 2.2. The medium was sterilized in Systec V-95 autoclave at standard conditions (1 atmosphere relative pressure and 115ºC) for 15 minutes.

Table 2.2 Composition of the medium used for the yeast *S. cerevisiae* culture.

	Concentration (g/L)	Manufacturer
Yeast extract	4	Roth, Germany
KH_2PO_4	1	Lach:ner, Czech Republic
$MgSO_4.7H_2O$	0.5	Lach:ner, Czech Republic
$(NH_4)_2SO_4$	1	Lach:ner, Czech Republic
Peptone	3	Roth, Germany
Glucose	30	Lach:ner, Czech Republic
Deionized water	Adjust to 1 L	-

For solid medium 20 g/L of agar (HiMedia Laboratiories, Czech Republic) were added.

2.4 AGAR PLATES AND SAMPLE PLATING

Agar plates were prepared using wastes hydrolysates to cultivate *S. cerevisiae*. The waste hydrolysates were obtained by substrates hydrolysis with HCl (Lach:Ner, Czech Republic) in final different concentrations, 0.1, 0.2, 0.3, 0.6 M. For every 30 gr of substrate, 180 mL of HCl solution was used.

The hydrolysis was performed in a water bath at 100ºC during 1.5 hours with the substrate in solution. After cooling down, 20 g L^{-1} of agar was added to the hydrolysate and the pH was set to 5.5-6.0 with NaOH 1.2 M (Lach:Ner Czech Republic), and the solution was sterilized using standard conditions.

After sterilization, the solution was poured into Petri dishes, in sterile conditions varying from the traditional flame method and laminar flux chamber (ESCO class II BSC), then let to solidify in the chamber. After solidification, 500 µL of inoculum diluted to 1-10^5 was plated. The inoculum was prepared as described in 2.5.1.

The agar plates were incubated in a Heidolph inkubator 1000 incubator at 29ºC and photographed every 24h to analyse the growth of yeast in the waste hydrolysates.

A plate containing the waste material with distilled water was used as a control to observe the difference of using the waste material without being hydrolysate, along with a control plate with *S. cerevisiae* inoculated with medium described in 2.3.

2.5 BIOETHANOL PRODUCTION ASSESSMENT IN SHAKE FLASKS

2.5.1 INOCULUM

Every inoculum was prepared in Erlenmeyer flasks without air, in a Heidolph inkubator 1000 incubator, at 29ºC, with an agitation of 144 rpm. *S. cerevisiae* was grown during, approximately, 12 hours. Figure 2.1 shows a type of inoculum used.

Figure 2.1 Inoculum growing in the incubator.

2.5.2 EXPERIMENTAL CONDITIONS

The first experiments had the goal of determining the most efficient way to perform the cellulose hydrolysis of the waste material. For this effect, various concentrations of HCl were tested.

To conduct the bioethanol fermentation it was necessary to hydrolyse the waste material in order to break down the lignocellulosic material. 30 gr of the waste were weighted for a total volume of 180 mL (16.7% w/v) in Erlenmeyer flasks and each flask was brought to the desired HCl concentration, namely 0.1, 0.2, 0.3 and 0.6 M.

The Erlenmeyer flasks were sealed with a stopper and the content was boiled in a water bath for 1.5 h. After cooling, the pH of the content was set to 5.5-6.0 using a burette to control the volume of NaOH (1.2 M) added so the final volume would be known.

Finally, the content was sterilized using standard conditions and inoculated (see section 2.5.1) with 10% of the total volume.

The fermentation took place in a Heidolph inkubator 1000 incubator, at 29ºC, with an agitation of 144 rpm for, at least, 48 h. For determination of the concentration of reducing sugars, glucose, polyphenols, flavonoids and ethanol, samples were collected in time intervals of 0, 4, 8, 12, 24, 28, 32, 36, 48 hours. If glucose was still available, the fermentation was carried out a little longer and additional samples were collected.

This protocol was followed for the three studied waste materials and repeated at different times. Before performing the scale-up to a 2 L bioreactor, one last fermentation was considered for the 3 waste materials at the same time using the 3 concentrations of HCl that showed the best results so the more efficient one could be distinguished.

2.6 BIOETHANOL PRODUCTION – LABORATORY SCALE

After selection of the HCl concentration that led to better results in the acid hydrolyses, a bioreactor (New Brunswick® BioFlo/CelliGen 115) with 2 L of volume was used to reproduce the results. Fermentations with commercial and produced enzymes were also performed, so that the productivity achieved under different experimental conditions could be compared.

2.6.1 EXPERIMENTAL CONDITIONS

Each substrate was used at a concentration of 16.7% (w/v) and placed into 1.5 L of solution. The bioreactor with the content was then autoclaved at standard conditions and operated in batch mode without air supply.

The fermentation started with the addition of inoculum. The volume of inoculum needed was 10% of the initial solution volume, prepared as described in section 2.5.1.

The set-up of the bioreactor is shown in Figure 2.2, in particular the water jacket, the pH meter with the buffers' flasks, the agitation and the monitor.

Samples were collected at 0, 4, 8, 12, 24, 28, 32, 36, 48 hours. After the beginning of fermentation when glucose was still available, the fermentation was carried out a little longer and additional samples were collected.

Figure 2.2 Experimental set-up for the 2 L bioreactor experiments.

2.6.2 CHEMICAL HYDROLYSIS

After analysing the production of bioethanol in shake flasks, the 0.6 M HCl concentration was determined as optimal for the chemical hydrolysis in the 1.5 L volume.

To perform the chemical hydrolysis, the waste material and the acid solution were adding into the bioreactor vessel, after this it was autoclaved at standard conditions.

The fermentation was performed with a temperature of 25ºC, 200 rpm agitation and the pH was kept at 6, being regulated by 1 M HCl and 1 M of NaOH.

2.6.3 COMMERCIAL ENZYMES

In this part of the studies, the waste substrates weren't pre-treated. The pH and temperature used in this work were based on the experiments obtained by Eng. Hlaváček (Hlaváček, 2013) where he studied the variation of pH and temperature using paper waste adding commercial enzymes from the biomass kit Novozymes analysing which condition led to higher bioethanol concentration. For the bread waste fermentation it was used an α-amylase from Sigma-Aldrich as commercial enzymes.

2.6.3.1 RAPESEED AND COFFEE WASTE FERMENTATION

For the fermentation of the waste substrates rapeseed and coffee, each of these materials were placed into a solution of 1.5 L phosphate citrate buffer at pH 4.8. The fermentation started with the addition of the inoculum and the commercial enzymes.

The commercial enzymes used were the biomass kit Novozymes provided by Novozymes A/S (Denmark). The enzyme loading was 500 µL/15 g biomass for the NS50013 (cellulase complex) and 50 µL/15 g biomass for NS50010 (β-glucosidase). Table 2.3 lists the activity and optimal conditions of these enzymes.

Table 2.3 Enzyme activity of the biomass kit, pH, temperature and dosing considering the weight of total solids (TS) (Novozymes, 2016).

Enzymes	Activity	Density (g/mL)	pH	Temperature (ºC)	Dosing (% w/w TS)
NS50013 (cellulose complex)	700 EGU/g (~ 70 FPU/g)	1.2	4.5 - 6.5	45 - 50	2 – 6%
NS50010 (β-glucosidade)	250 CBU/g	1.2	2.5 – 6.5	45 - 70	0.2 – 0.6%

The pH was kept at 4.8 by the use of 0.2 M dibasic sodium phosphate (Lach:Ner Czech Republic) and 0.1 M citric acid (Lach:Ner Czech Republic), the temperature at 40ºC and agitation of 200 rpm.

2.6.3.2 BREAD WASTE FERMENTATION

The bread waste was placed in a phosphate citrate buffer with a pH of 5.5. This pH was regulated during the fermentation with 0.2 M dibasic sodium phosphate and 0.1 M citric acid, as with the other two waste materials.

In this case, 200 µL of α-Amylase from *Bacillus amyloliquefaciens* (Sigma-Aldrich, Denmark) was used. The activity of this enzyme is shown in Table 2.4.

Table 2.4 Enzyme activity and specifications of the α-Amylase from *Bacillus amyloliquefaciens* (Sigma-Aldrich, 2016b).

Enzymes	Activity	Density (g/mL)
α-Amylase	250 KNU/g	1.10 - 1.30

This fermentation started with the loading of the enzyme, the temperature was set to 65ºC and the agitation speed was 300 rpm. These conditions were kept during 2 h, after this time, the temperature was change to 40ºC and the inoculum was added.

2.6.4 In-house produced enzymes

Fermentations of waste products were also carried out using in-house produced enzymes during this work. In this case, after inoculating the bioreactor, 30 mL of the in-house produced enzymes were added. The pH was kept at 5.5 through the use of 0.2 M dibasic sodium phosphate and 0.1 M citric acid, the temperature at 40ºC and the agitation at 200 rpm.

2.6.4.1 *Phanerochaete chrysosporium* cultivation and enzyme preparation

The enzymes were produced from a mold. The mold used was *Phanerochaete chrysosporium* CCM 8074, kept at 39ºC in a medium with 10 g of glucose, 100 mL of Basal III medium and 900 mL distilled water. The Basal III medium is composed of 0.147 M KH_2PO_4, 0.042 M $MgSO_4$ and 0.009 M $CaCl_2$ (Lach:Ner Czech Republic), in 1 L of distilled water. The medium was sterilized in an autoclave Systec V-95 at standard conditions (1 atmosphere and 115ºC) for 15 minutes.

To use the mold, 10 mL of a spore suspension were taken into an Erlenmeyer flask with 150mL of the medium described and added 12 mg/mL of ampicillin sodium salt (Sigma-Aldrich, Denmark), in sterile conditions in the laminar flux chamber (ESCO class II BSC). Then, the mold was cultivated during 7 days inside a Heidolph inkubator 1000 incubator at 39ºC and 144 rpm of agitation.

After the incubation period, the liquid was transferred to an Erlenmeyer flask and 1% (w/v) of carboxilmethyl cellulose was added. Broth from *P. chrysosporium* cultures carried out for 7 days were filtered and the resulting filtrate was used as the in-house enzyme preparation. Part of the content was frozen until needed.

2.6.4.2 Protein analysis

The protein content of the in-house enzyme preparation was determined using the procedure of Hartree-Lowry Assay, which allows to determine the protein concentration present within the sample, based on a spectrophotometric method.

For this procedure it was necessary to prepare 3 reagents. The reagent A consisted of 7 mM NaK tartrate, 0.81 M sodium carbonate and 1 M NaOH. The reagent B was made with 70 mM NaK tartrate, 40 mM copper sulfate, 10 mL of 1 M NaOH to 90mL H_2O. The last reagent, C, was Folin-Ciocalteau reagent (PENTA, Czech Republic) diluted to 1:15.

1 mL of the produced enzymes (proper diluted) was added to a test tube with 0.9 mL reagent A and incubated 10 min in a bath at 50ºC. Then, it was allowed to cool to room temperature and 0.1 mL of reagent B was added, mixed and incubated 10 min at room temperature. After this, 3 mL of reagent C was rapidly added, mixed and the test tube was placed into the 50ºC bath for 10min. When the test tube was cooled, the absorbance was measured at OD_{650} (1cm light path cuvette) with Helios ε (UV-VIS) spectrophotometer. The calibration curve was prepared using serial dilutions of a bovine serum albumin (BSA; Sigma-Aldrich, Denmark) solution with known concentration (see Appendix A).

2.6.4.3 SDS-PAGE

The molecular weight of the proteins present in the in-house enzyme preparation was estimated by SDS-Page. All the solutions needed to prepare the separation and concentration gel are described in Appendix E. The separation gel used was 12% (w/v).

After adding the separation gel mixture to the electrophorese apparatus (BIO-RAD), 1 mm of isopropanol (enough to cover the separation gel) was added and polymerisation reaction was allowed to occur. After polymerisation, the excess water was removed and the concentration gel was added to the support of the apparatus.

Then, the electrode buffer was poured into the lower part of the apparatus and the sample buffer in the upper part of the tank (buffers' composition describe in Appendix E), the sample was added to the wells. The molecular mass standard mix used was Precision Plus Protein™ Dual Color Standards (BIORAD, Czech Republic).

The gel was submitted to electrophoresis during 1 hour at 120 V.

After this the gel was immersed in a staining solution and cooked during 30 s in the microwave. The gel was then transferred to a destaining solution and microwaved for 30 s and further washed (the preparation of these solutions are described in Appendix E).

2.7 Analytical methods

2.7.1 Growth curves

Yeast growth in shake flasks was determined by measuring the cultures OD_{600} (1cm light path cuvette) with a Helios ε (UV-VIS) spectrophotometer. Proper dilutions were made in order to keep absorbance values below 1.0.

2.7.2 Determination of reducing sugars

The determination of the concentration of reducing sugars was done following the Somogyi-Nelson analysis (Somogyi, 1952).

3 solutions were prepared, the first one containing 0.283 M Na_2CO_3 anhydrous, 0.238 M $NaHCO_3$, 1.27 M Na_2SO_4 anhydrous and 0.053 M $KNaC_4H_4O_6 \cdot 4H_2O$, for 800 mL H_2O. The solution 2 was composed of 0.080 M $CuSO_4.5H_2O$ and 0.845 M Na_2SO_4 anhydrous, for 200 mL H_2O. Solution 3 contained 0.283 M $(NH_4)_2MoO_4$ dissolved in 450 mL H_2O, 0.091M $Na_2HAsO_4 \cdot 7H_2O$ to 25 mL of H_2O and 21 mL of H_2SO_4 concentrated was added. This last solution needed to be left in the dark 48h prior to use.

To determine the sugars present in each sample of the fermentation and the waste material hydrolysate, 1 mL of the sample, 0.5 mL of solution 1 plus 0.5 mL of solution 2 were pipetted into a test tube. Solutions were well mixed in a vortex and boiled for 10 min in a water bath. After letting it cool to room temperature, 0.5 mL of solution 3 was added and topped with distilled water to 10 mL. Afterwards, the content of the test tube was mixed and the OD_{720} was measured (1cm light path cuvette) with Heλios ε (UV-VIS) spectrophotometer. Proper dilutions were made in order to keep absorbance value below 1. A correlation between glucose concentration and OD_{720} was made to estimate the reducing sugars content in the sample, in mg glucose per mL. The calibration curve is shown in Appendix B.

2.7.3 Determination of flavonoids

In order to determine the concentration of flavonoids in each sample, 0.5 mL of sample was pipetted to a test tube and 1.5 mL of H_2O and 0.2 mL of 5% $NaNO_2$ were added. This was mixed and incubated for 5 min. Afterwards 0.2 mL of 10% $AlCl_3$ was added and the sample rested for 5min. To finalize, a solution of 1.5 mL 1 M NaOH plus 1 mL H_2O was mixed with the rest of the test tube content and incubated for 15 min.

The OD_{510} was then measured (1 cm light path cuvette) with a Heλios ε (UV-VIS) spectrophotometer. Proper dilutions were made in order to keep absorbance value below 1. A correlation between catechin concentration and OD_{510} was made in order to estimate the catechin mg per mL. The calibration curve was prepared with catechin solutions (0.05 – 0.3 mg/mL) in ethanol, as presented in Appendix C.

2.7.4 Determination of polyphenols

The measurement of polyphenols was performed based on the methods of Folin-Ciocalteu. Briefly, 1 mL of Folin-Ciocalteu diluted to 1:9 were added to 1 mL of H_2O and 50 µL of sample into one test tube. After mixing, the tube was left up to 5 min at room temperature, then 1 mL of a saturated Na_2CO_3 solution was added.

After mixing and leaving it to rest for 15 min, the sample OD_{750} was measured (1 cm light path cuvette) in a Heλios ε (UV-VIS) spectrophotometer. Proper dilutions were made in order to keep absorbance value below 1. A correlation between gallic acid concentration and OD_{750} was made

in order to estimate the gallic acid mg per mL. The calibration curve was made with gallic acid (0.1 – 0.5 mg/mL), as shown in Appendix D.

2.7.5 DETERMINATION OF GLUCOSE AND ETHANOL - HPLC

All the samples from the shake flasks and bioreactor were analysed concerning ethanol and glucose contents using high performance liquid chromatography (HPLC). A Dionex UltiMate 3000 system equipped with a Phenomenex column with 8 µm sized ROA-Organic Acid H^+ (8%) particles (Rezex 00H-0138-K0), operated at 60ºC and 43 bar, was used to separate the glucose and the ethanol. The mobile phase consisted of a 5 mM H_2SO_4 solution prepared with MilliQ water, flowing at 0.850 mL/min. The eluted compounds were detected by an ERC Refractomax 520 refractive index (RI) detector.

RI spectra obtained from a typical sample analysis can be seen in Appendix F where glucose and ethanol peaks are identified. The areas under these peaks can be converted into the amount of component using an analytical software (Chromeleon 7.2) and the calibration curve equation of each component (Appendix F).

2.7.5.1 SAMPLE PREPARATION

Raw samples from the fermentation were taken and centrifuged in a Hermle Labortechnik Gmblt Z36HK centrifuge during 10 min at 4500 rpm before being analysed in the HPLC. The centrifugation was performed in order to separate the waste material and the supernatant. The sample was then filtered using 45 µm (Whatman® GD/XP) syringe filters, PTFE membrane prior to HPLC analysis.

2.7.6 CALCULATIONS

In order to calculate the number of colony-forming unit (CFU) per volume it was used the following equation (2.1) was used.

$$\frac{nº\ colonies \times Dilution\ factor}{sample\ volume} \qquad (2.1)$$

For the ethanol production, the following equations (2.2), (2.3) and (2.4) were used for the determination of ethanol yield factor ($Y_{P|S}$, g/g), ethanol volumetric productivity (Q_P, g/L·h) and the efficiency of sugars conversion to ethanol (η, %).

$$Y_{P|S}\ \frac{g}{g} = \frac{[Ethanol\ \frac{g}{L}]_{final}}{[Polysaccharides]} \qquad (2.2)$$

$$Q_P\ \frac{g}{L \cdot h} = \frac{[Ethanol\ \frac{g}{L}]_{final}}{Fermentation\ time\ h} \qquad (2.3)$$

$$\eta\ \% = \frac{[Ethanol\ \frac{g}{L}]_{\ final} \times 1.5\ L\ (solution\ volume)}{Ethanol\ theoretical\ value} \quad (2.4)$$

To obtain the concentration of polysaccharides the composition of each waste was determined, in order to know its cellulose (C_{cont}) and hemicellulose (H_{cont}) content. Then, with the substrate weight used in the fermentation (W, g) and the conversion factor for cellulose, 1.11 g sugar per g of polysaccharide (Wyman *et al.*, 2004), and hemicellulose to monosaccharides it was possible to calculate the maximum sugars content, 1.11 g per g of polysaccharide for hexoses content and 1.36 g per g of polysaccharide for pentose content (Wyman *et al.*, 2004), for a complete hydrolysis. Equation (2.5) is applicable for the conversion of starch into glucose, replacing C_{cont} with the content in starch.

The hemicellulose is hydrolysed to pentoses and hexoses so the composition in hexoses (Hex_{cont}) and pentose ($Pent_{cont}$), according to each waste content, is also known.

$$[Polysaccharides] = W(g) \times C_{cont} \times 1.11 \quad (2.5)$$

$$[Polysaccharides] = W(g) \times H_{cont} \times Hex_{cont} \times 1.11 \quad (2.6)$$

$$[Polysaccharides] = W(g) \times H_{cont} \times Pent_{cont} \times 1.36 \quad (2.7)$$

To obtain the theoretical ethanol production for each substrate content, the polysaccharides concentration times the ethanol theoretical value, 0.51 g/g (Mussatto *et al.*, 2012) was calculated.

2.7.7 STATISTICAL ANALYSIS

Experimental errors are expressed as standard deviations displayed as vertical error lines when presenting the experimental data.

3 RESULTS AND DISCUSSION

In this section the results obtained for the experiments conducted are shown. These results are divided into three parts, the first part consisting in the evaluation of the *S. cerevisiae* growth ability in the waste materials used in this work, followed by the presentation of the results obtained for the shake flasks experiment regarding the chemical hydrolysis. In the final part it is reported the performance of the 2 L bioreactor for each waste substrate, in three sub-sections associated to chemical hydrolysis, the use of commercial enzymes and the in-house produced enzymes.

3.1 YEAST GROWTH IN THE WASTE MATERIALS HYDROLYSATES. EVALUATION IN AGAR PLATES

To determine if the waste materials chosen for this work were appropriated for the growth of *S. cerevisiae*, a chemical hydrolysis of the waste materials supplemented with 20 g/L of agar was performed as described in section 2.4.

As a control, the media described in 2.3 was used and the inoculum prepared was plated in the Petri dish. It was noticed that the yeast had grown after 48 h of incubation (Figure 3.1).

Different concentrations of HCl were tested for the hydrolysis of the rapeseed waste, namely, 0.2, 0.3 and 0.6 M. One plate with waste material was prepared without HCl, only deionised water. The pictures represented in Figure 3.1 were taken more than 48 h after the incubation and compared to the pictures taken between 24 h and 48 h of incubation. The comparison between these two set of pictures was able to show that the yeast was growing slowly in the waste material, especially when the concentration of HCl was higher. Besides the slower growth, the morphology of the colonies grown in the medium with higher concentration of HCl were larger than the ones grown with lower HCl concentration.

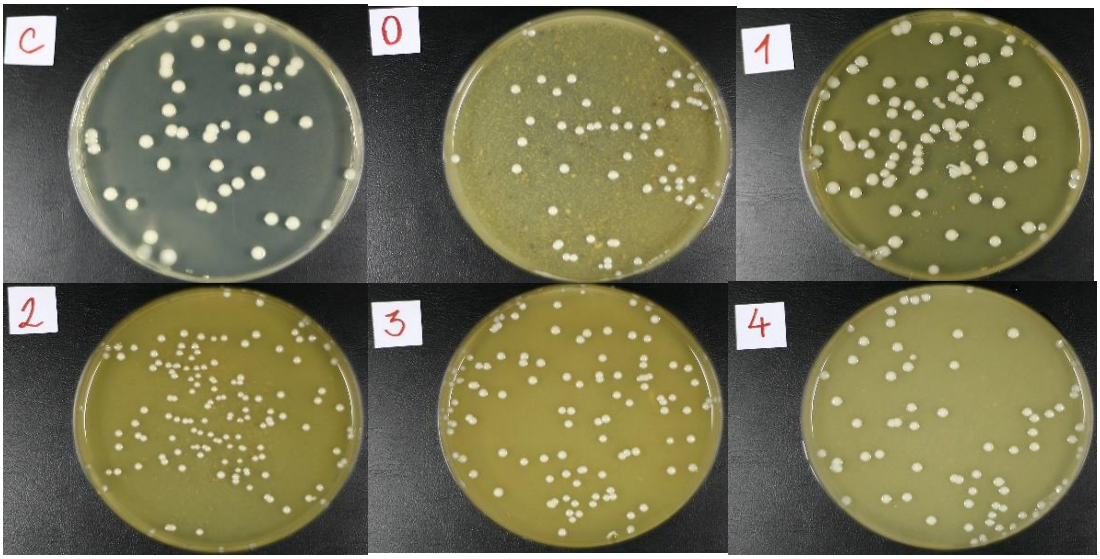

Figure 3.1 Rapeseed hydrolysates with 20 g/L agar inoculated with *S. cerevisiae*. In the up row on the left, C represents the control Petri dish, 0 is the hydrolysate without HCl and the hydrolysis in 1 was conducted

with 0.6 M of HCl. In the bottom row, 2 was grown with 0.3 M HCl, 3 in the presence of 0.2 M and the dish 4 with 0.1 M HCl.

Table 3.1 represents the number of colony-forming units obtained for each plate analysed. The hydrolysate with 0.3 M HCl had higher number of CFU/mL, these data should have more measurements in order to establish a statiscally significant difference between the conditions since they all fall within the same power of ten it is hard to assess the best condition overall. Looking at Figure 3.1, the colonies from plate 1 (the sample with 0.6 M HCl) are larger than the ones in plate 2 (the sample with 0.3 M) which means that some colonies can be an aggregate of more than one colony that was count as only one.

Table 3.1 Colony-forming units obtained for each plate tested in the rapeseed hydrolysis.

	CFU/mL
Control	9.80×10^6
Without HCl	1.54×10^7
0.6 M HCl	1.92×10^7
0.3 M HCl	2.82×10^7
0.2 M HCl	2.22×10^7
0.1 M HCl	1.52×10^7

The plates made with the coffee hydrolysates were grown for one week because after 48 h signs of growth were barely noticed. At this point it was believed that the coffee waste would not show satisfying results in the production of bioethanol with this strain, however the concentration of reducing sugars presented in the coffee waste after chemical hydrolysis was five times higher than in the rapeseed waste, approximately, except for the sample without HCl in its composition. And, later on, the shake flask results showed production of bioethanol (section 3.2.2). For all these reasons, it was decided that despite the slow grow, the production of bioethanol using coffee waste was still a viable choice to continue working on.

Figure 3.2 shows that after one week of incubation, the colonies on plates ϕ, 5 and 15 (low HCl concentrations) weren't as grown as the others, according to their morphology and aspect. The plates 45 and 60 (high HCl concentrations) show how the yeast growth is hindered in this higher amount of acid. Plate 30 showed the desired look and number of colonies.

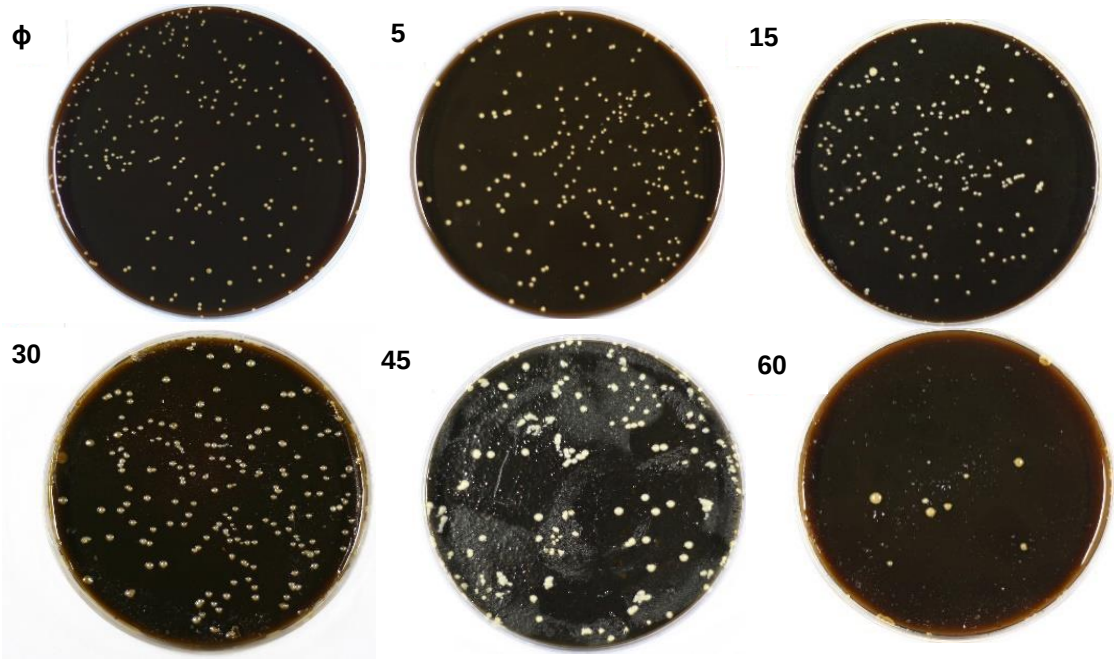

Figure 3.2 Coffee hydrolysates with 20 g/L agar inoculated with *S. cerevisiae*. On the top row on the left, ϕ represents the hydrolysate without HCl, 5 shows the hydrolysis with 0.1 M HCl and the hydrolysis in 15 was conducted with 0.3 M of HCl. In the bottom row, 30 was grown with 0.6 M HCl, 45 presented 0.9 M and the Petri dish 60 has 1.2 M HCl.

From Table 3.2 it is possible to conclude that the use of 0.1 M and 0.6 M of HCl led to the highest CFU/mL after chemical hydrolysis. The sample without HCl show that the *S. cerevisiae* is able to grow in this waste material without needing to hydrolyse the sugars contained in the coffee waste.

Table 3.2 Colony-forming units per volume obtained for each plate tested in the coffee waste hydrolysis.

	CFU/mL
Without HCl	4.04×10^7
0.1 M HCl	3.92×10^7
0.3 M HCl	3.44×10^7
0.6 M HCl	3.48×10^7
0.9 M HCl	3.38×10^7
1.2 M HCl	1.80×10^6

As stated previously (section 1.3.2.3), the bread waste has a high content of sugars, which provide for a higher CFU/mL after 48 h than the coffee waste.

In Figure 3.3 it is noticeable that no growth occurred in plates 45 and 60 (with respectively 0.9 and 1.2 M of HCl used for the hydrolysis of this waste material). This may be due to a highly acidic

media where the yeast is not able to grow, since when there is too much acid, the glucose molecule breaks down to dehydrated form which is the opposite of the hydrolysis reaction.

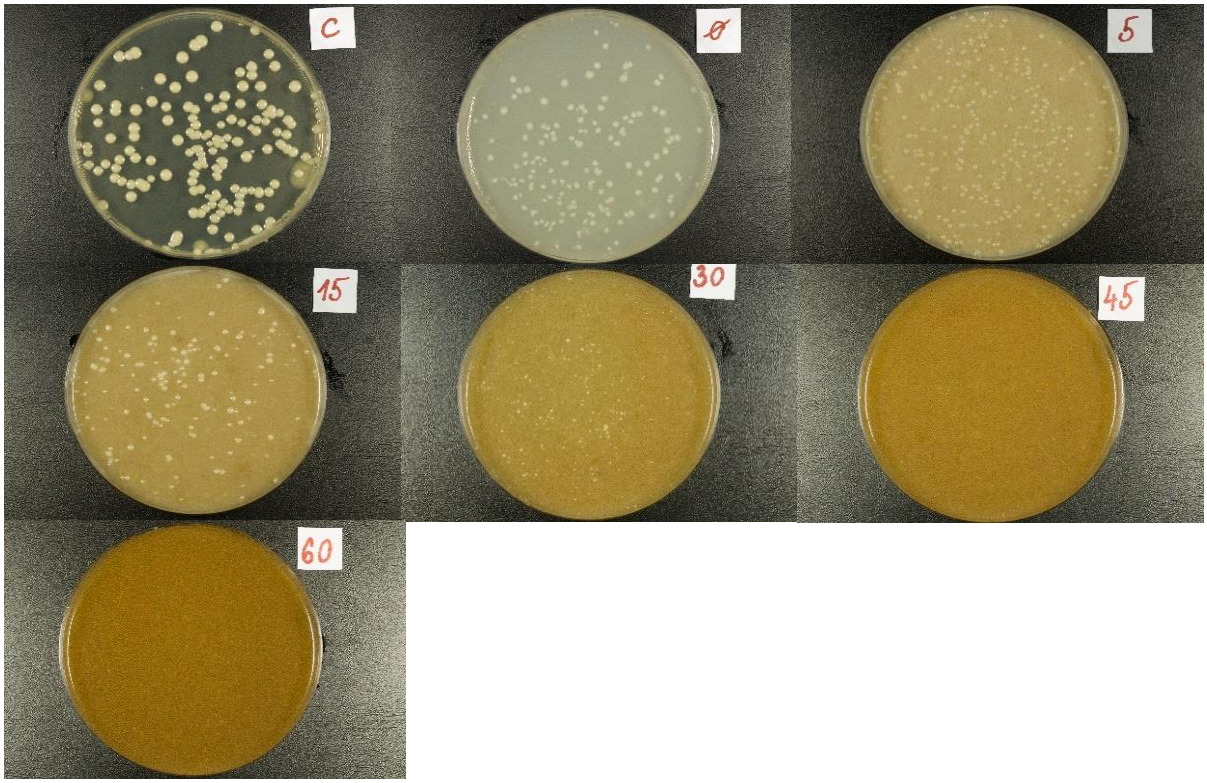

Figure 3.3 Bread hydrolysates with 20 g/L agar inoculated with *S. cerevisiae*. On the top row on the left, C represents the control Petri dish, ϕ represents the hydrolysate without HCl and 5 shows the hydrolysis with 0.1 M HCl. On the middle and bottom rows, 15 shows the hydrolysis with 0.3 M HCl, 30 was grown with 0.6 M HCl, 45 presented with 0.9 M and the Petri dish 60 has 1.2 M HCl.

After analysing the colonies that developed in the solid media contained in Petri dishes and calculating the CFU/mL for each waste hydrolysate it was noticed that the conditions with 0.1 and 0.6 M of HCl lead to a higher number of colonies (Table 3.3).

Table 3.3 Colony-forming units per volume obtained for each plate tested in the bread waste hydrolysis.

	CFU/mL
Control	3.10×10^7
Without HCl	3.16×10^7
0.1 M HCl	6.36×10^7
0.3 M HCl	3.10×10^7
0.6 M HCl	5.88×10^7
0.9 M HCl	0

1.2 M HCl	0

In conclusion, the concentrations of HCl that showed higher production of CFU/mL in the hydrolysates in agar were the lowest ones, up to 0.6 M. With higher concentrations, the growing is slower or, in some cases (bread waste), it didn't occur. As such, these higher concentrations of acid were determined to be unsuitable for the production of bioethanol through acid hydrolysis, when *S. cerevisiae* is the selected yeast.

3.2 BIOETHANOL PRODUCTION IN SHAKE FLASKS

Before studying the production of bioethanol in shake flasks, several tests for the chemical hydrolysis were made in order to test which concentration of HCl led to release of more reducing sugars. These tests were consistent with the yeast growth in agar plates, as expected. In lesser acidic media, there was a higher concentration of reducing sugars in comparison with more acidic media.

After this step, three different acid concentrations were chosen to further continue the study of the bioethanol production with chemical hydrolysis in shake flasks, according to the results obtained until this point. They were 0.1, 0.3 and 0.6 M of HCl.

3.2.1 RAPESEED WASTE

Two types of rapeseed waste were available with different lipid's concentration. The first one had 2.55% lipids in its composition and the second one had 9% lipids. The first chemical hydrolysis showed that the concentration of reducing sugars was higher in the sample with less lipids. This was expected due to lower availability of cellulose when the lipid structure is more compact. From this point onwards, only the rapeseed waste sample with 2.55% of lipids was used, since it showed higher concentration of reducing sugars after hydrolysis.

After several chemical hydrolysis experiments, the three HCl concentrations (0.1, 0.3 and 0.6 M) were chosen as appropriated for the bioethanol production in rapeseed waste, according to the concentration of reducing sugars released. A shake flask with deionised water and no HCl was used for control purposes.

Figures 3.4 and 3.5 show the glucose consumption and ethanol production during the shake flask fermentation. The sample without acid always presented lower values of sugar and ethanol produced than the samples where acid was used for the hydrolysis, as expected. According to the two figures, it is possible to conclude that the fermentation reaches a maximum of production after 24 h and stabilises on these values until the end of fermentation at 48 h.

The sample with 0.1 M for the chemical hydrolysis by HCl shows lower glucose concentration in its initial composition (after chemical hydrolysis), while the production of ethanol is lower than the

other two samples. The glucose concentration post-hydrolysis for the 0.3 and 0.6 M HCl experiments wasn't that different, but the sample with 0.6 M showed higher glucose availability after 24 h of fermentation and had a maximum of ethanol production of 7.5 g/L whereas for 0.3 M the maximum production was 6.98 g/L.

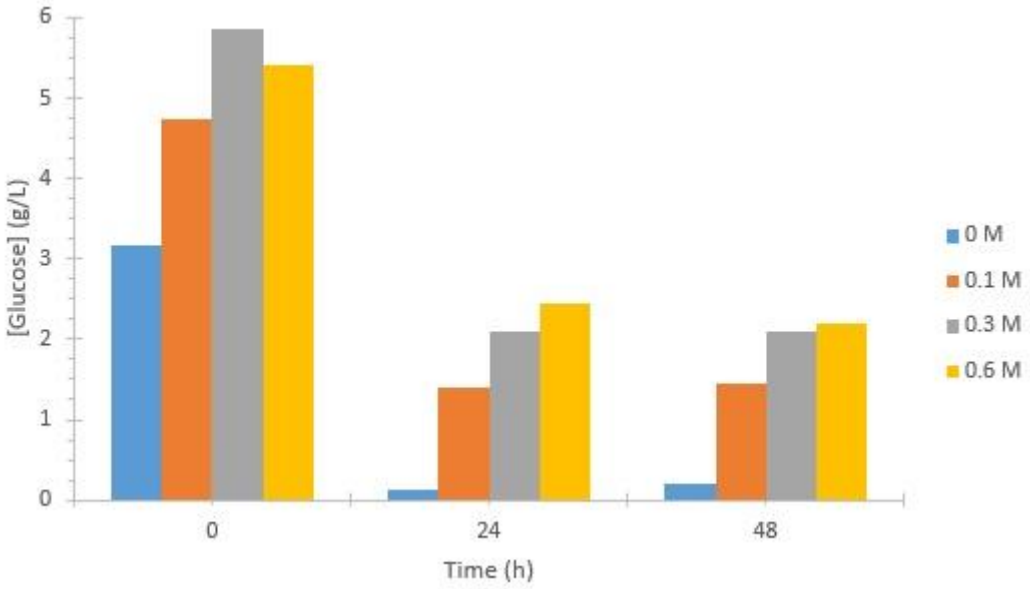

Figure 3.4 Glucose consumed (g/L) during the fermentation in shake flasks of rapeseed waste hydrolysates obtained using the indicated HCl concentrations.

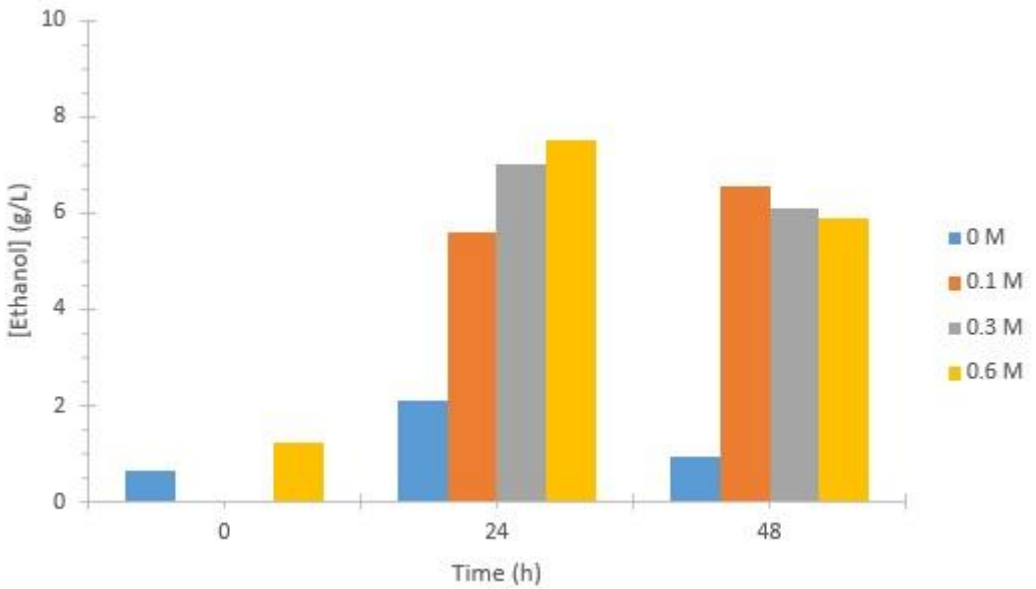

Figure 3.5 Ethanol produced (g/L) during the fermentation in shake flasks of rapeseed waste hydrolysates obtained using the indicated HCl concentrations.

3.2.2 COFFEE WASTE

The use of coffee waste showed more potential when compared to rapeseed, as the concentration of glucose was almost four times higher than the concentration of glucose released from the hydrolysis of rapeseed waste on one condition (Figure 3.6 compared to Figure 3.4).

The initial concentration of glucose was almost the same in the samples hydrolysed with 0.1, 0.3 M HCl and without it. However, when 0.6 M of acid were used, the concentration of glucose after the hydrolysis was 23.2 g/L.

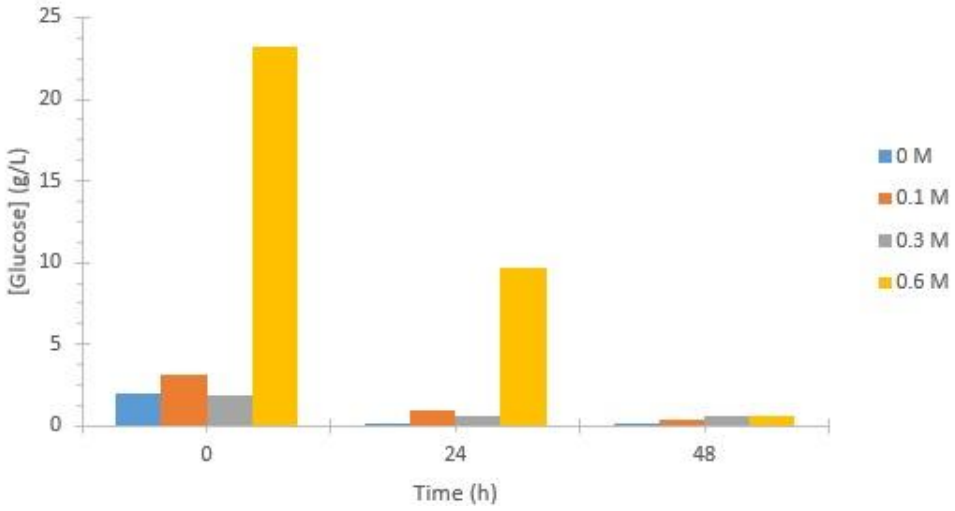

Figure 3.6 Glucose consumed (g/L) during the fermentation in shake flasks of coffee waste hydrolysates obtained using the indicated HCl concentrations.

As with the rapeseed waste, the samples with 0.1, 0.3 M and without HCl had a maximum production of ethanol at 24 h, subsequently stagnating its production. For the sample where 0.6 M HCl was used for the chemical hydrolysis, there is a continuing decrease of glucose and increase of ethanol concentration, reaching a maximum at 48 h, obtaining 9.97 g/L (Figure 3.7).

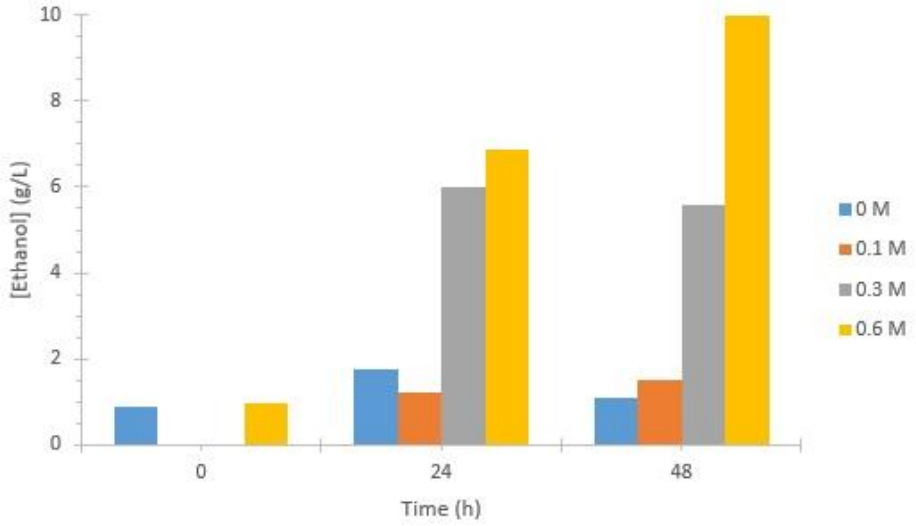

Figure 3.7 Ethanol produced (g/L) during the fermentation in shake flasks of coffee waste hydrolysates obtained using the indicated HCl concentrations.

3.2.3 BREAD WASTE

A closer examination of Figure 3.8 and Figure 3.9 shows a similar behaviour of the glucose consumption between coffee waste and bread waste. The amount of initial glucose for the 0.6 M acid concentration after hydrolysis is twice as high as for 0.3 M (60.8 g/L and 30.2 g/L, respectively) and lower for the other two samples. Again, at 24 h there is a maximum peak of production that is stable until the 48 h fermentation. The 0.6 M HCl sample is the exception, where the increase of ethanol production is gradual during the 48 h obtaining 34.8 g/L at the end of the fermentation.

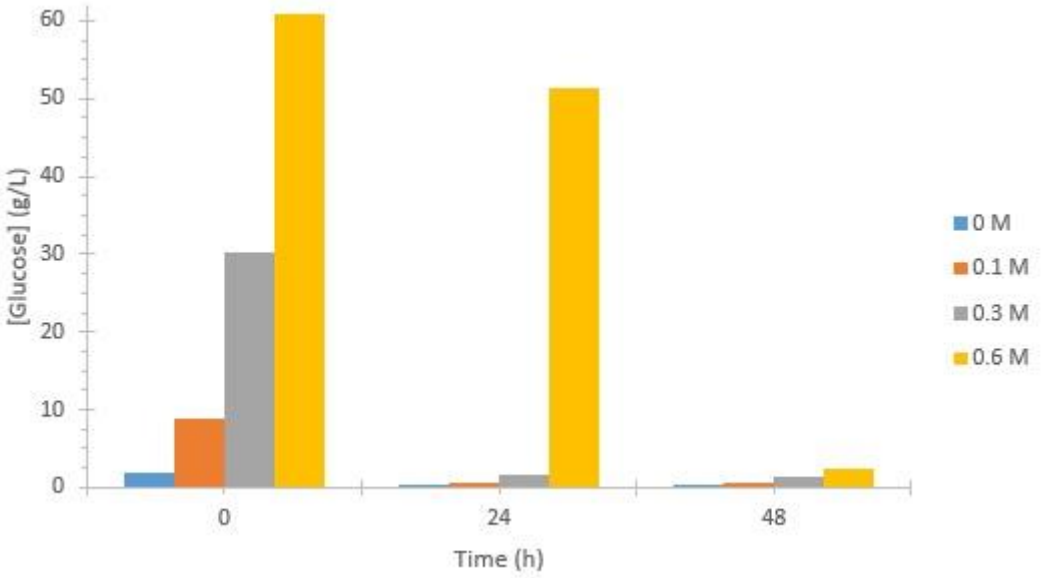

Figure 3.8 Glucose consumed (g/L) during the fermentation in shake flasks of bread waste hydrolysates obtained using the indicated HCl concentrations.

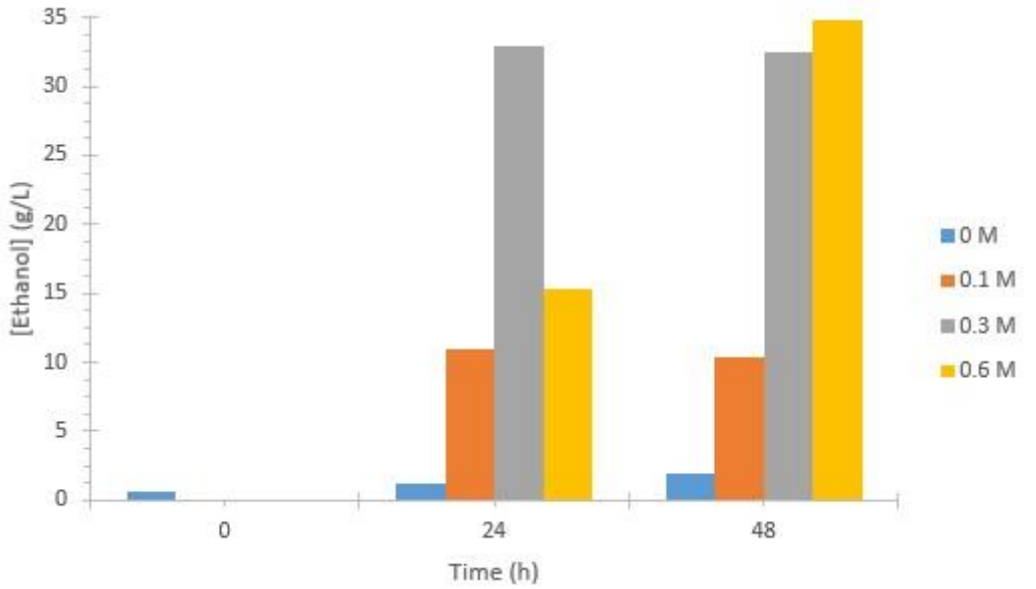

Figure 3.9 Ethanol produced (g/L) during the fermentation in shake flasks of bread waste hydrolysates obtained using the indicated HCl concentrations.

3.3 BIOETHANOL PRODUCTION IN 2 L BIOREACTOR

3.3.1 BIOETHANOL PRODUCTION WITH CHEMICAL HYDROLYSIS OF RAW WASTES

After the shake flasks tests the HCl concentration of 0.6 M was chosen as the most appropriate for the chemical hydrolysis of the waste materials under study, and therefore it was selected as the only acid concentration used for the chemical hydrolysis of wastes for the bioethanol production in the 2 L bioreactor.

3.3.1.1 RAPESEED WASTE

As mentioned before, the majority of rapeseed's sugar composition is made up of hexoses, in the form of cellulose. Therefore it is not surprising that the majority of the sugar yield derived from acid hydrolysis is comprised of glucose. The glucose concentration during the fermentation is showed in Figure 3.10.

As noticed before, there is a decrease in the glucose's concentration, followed by a stabilisation after 24 h.

The ethanol concentration grows until, approximately, 30 h of fermentation. After this point there was a decrease which may be due to ethanol evaporation or the sampling was not well performed, if the sample was not taken from an agitated place, it may be diluted or saturated. Because of this unexpected decrease and the results obtained in the shake flask test, the maximum ethanol concentration was taken into account for the fermentation calculus.

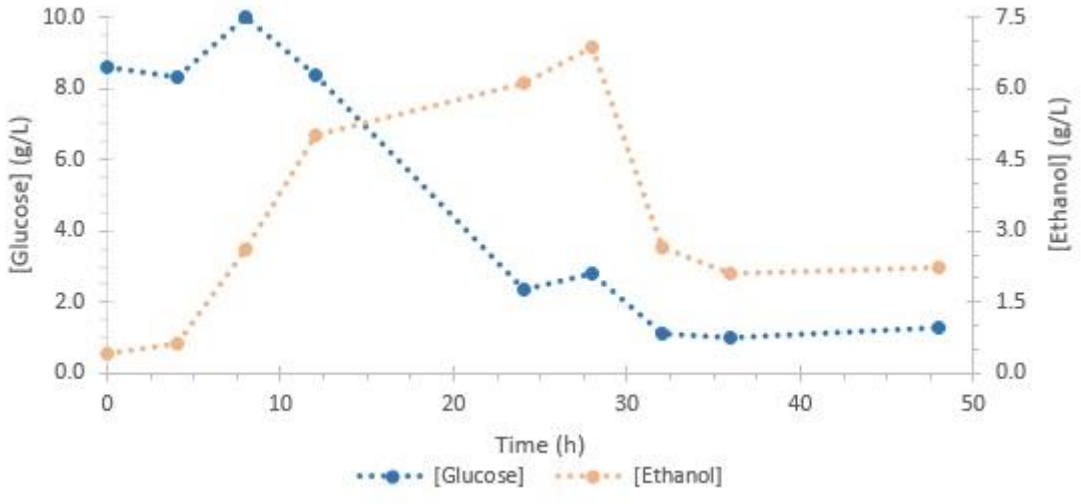

Figure 3.10 Glucose consumption and ethanol formation during the 48 h fermentation using rapeseed as substrate for the chemical hydrolysis.

The sugar concentration increases, in the first hours of fermentation, before reaching a maximum level of 10 g/L of glucose. This level is demised by the degradation of sugars as only 1.0 g/L of this sugar remains at the end of the reaction.

It is important to notice that the initial composition of rapeseed accounted for 17.8% of cellulose and 14.8% hemicellulose (content). Cellulose is hydrolysed into glucose but hemicellulose hydrolyses to pentose and hexoses. The glucose concentration was only determined after the hydrolysis, obtaining 11.1 g/L. If the hydrolysis was complete the monosaccharides content should be around 63.9 g/L. These values are not being compared, because the concentration of all the monosaccharides and disaccharides wasn't determined, but it is possible to say that the hydrolysis was not complete.

According to these results, it was possible to determine the yield and productivity of this fermentation (Table 3.4) obtaining 6.86 g/L of ethanol at 28 h (maximum value) and 21% efficiency comparing to the theoretical value of ethanol possible to obtain for a complete hydrolysis of the initial content of the rapeseed in use.

This low value is indicative of an inefficient hydrolysis and it can also be due to the fact that *S. cerevisiae* is not able to ferment pentose (C-5 sugars) to ethanol, when around 84% of rapeseed's hemicellulose composition is xylose (and a few percentage of mannose) (Díaz *et al.*, 2010).

Table 3.4 Results obtained for the maximum production of ethanol (g/L), ethanol factor yield ($Y_{P|S}$, g/g), ethanol volumetric productivity (Q_P, g/L·h), fermentation time (h) and efficiency of sugars conversion to ethanol (η, %), using rapeseed waste and chemical hydrolysis.

[Ethanol] (g/L)	$Y_{P\|S}$ (g/g)	Q_P (g/L·h)	Fermentation time (h)	η (%)
6.86	0.072	0.245	28	21.0

The concentration of reducing sugars during the fermentation was also studied. Figure 3.11 shows that the mass of reducing sugars generated per gram of biomass follows the same trend as the glucose concentration, as the lignocellulosic structure is rapidly broken down into its components and further broken down into glucose.

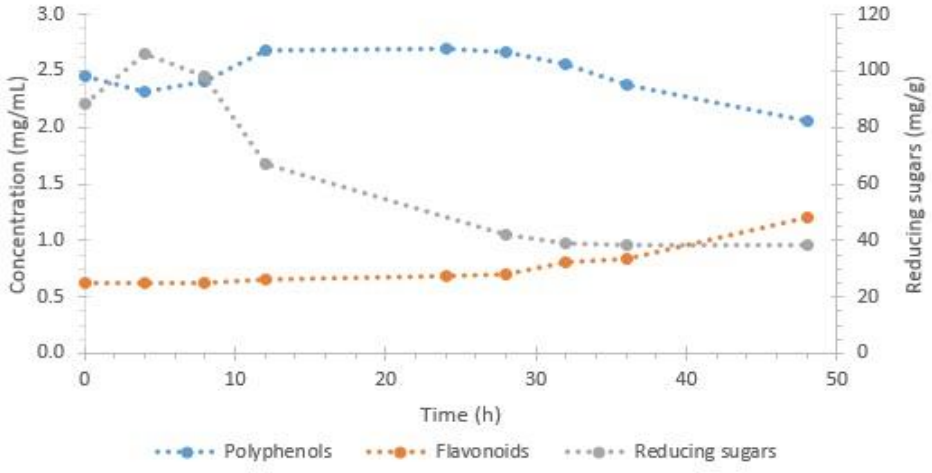

Figure 3.11 Representation of reducing sugars generated by gram of biomass (mg/g) during the fermentation time and concentration of polyphenols and flavonoids obtained (mg/mL), in rapeseed waste using chemical hydrolysis.

The concentration of polyphenols and flavonoids released in the fermentation was also studied for the rapeseed and coffee waste. The concentration of polyphenols decreased slightly after 24 h of fermentation which may be related with the destabilisation of lignin due to its content in phenolic compounds (Hage *et al.*, 2012), while the concentration of flavonoids increased during the same time-frame. This change in the concentration of these antioxidants occurred at the same time as the bioethanol produced was changing and the concentration of reducing sugars and glucose stabilised.

3.3.1.2 COFFEE WASTE

As with the shake flask experiments, the chemical hydrolysis in coffee waste released almost twice as much glucose as using rapeseed waste. This fermentation was designed to run for a long period because according to the literature (Murthy and Madhava Naidu, 2012), the digestion time of conventional coffee varies from 48 to 72 h, depending on temperature.

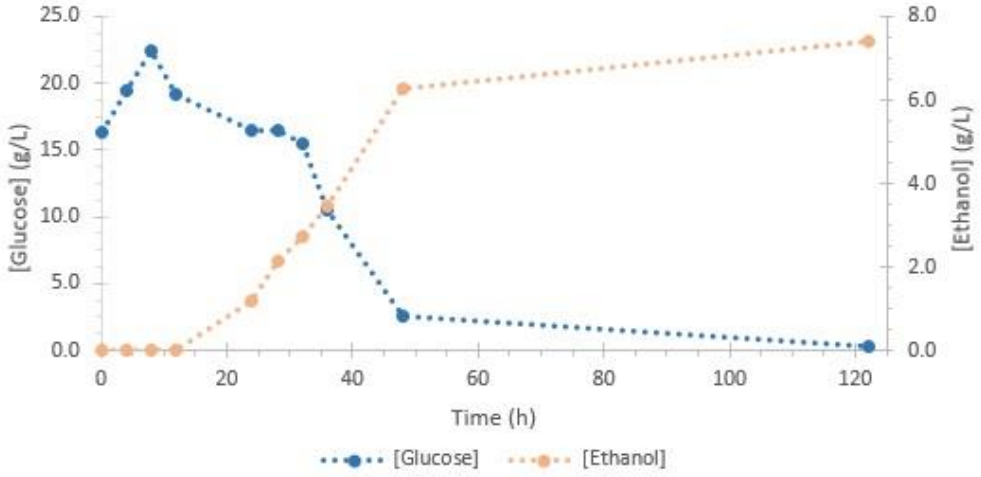

Figure 3.12 Glucose consumption and ethanol formation during the 122 h fermentation using coffee as substrate for the chemical hydrolysis.

Again, an initial release of glucose was observed until 8 h of fermentation, followed by its consumption until the stationary phase is reached, leading to a residual glucose concentration at the end of fermentation. The formation of bioethanol follows the same trend as the glucose consumption, with a stationary phase being reached between 48 and 122 h, where only residual amounts of ethanol were formed. Table 3.5 reports the results obtained for this fermentation.

Comparing the production of ethanol from rapeseed waste with coffee waste, while there is one more gram per litre of ethanol using coffee waste as substrate, the yield is lower. The global concentration of cellulose and hemicellulose (% content) is much higher in coffee waste than in rapeseed waste but the coffee waste has a lower composition in cellulose (9.6%) and 34.8% of its composition is hemicellulose formed of mannose only, because spent coffee grounds is not composed of xylose. But mannose is difficult to hydrolyse, it requires a strong alkaline environment, the opposite of the HCl based hydrolysis in study.

For a complete hydrolysis, there should had been 136 g of sugars and glucose measured after this process, however, only 17 g were available, which indicates that this type of chemical hydrolysis is not ideal when using spent coffee grounds as a substrate.

Table 3.5 Results obtained for the maximum production of ethanol (g/L), ethanol factor yield ($Y_{P|S}$, g/g), ethanol volumetric productivity (Q_P, g/L·h), fermentation time (h) and efficiency of sugars conversion to ethanol (η, %), using coffee waste and a chemical hydrolysis.

[Ethanol] (g/L)	$Y_{P\|S}$ (g/g)	Q_P (g/L·h)	Fermentation time (h)	η (%)
7.42	0.055	0.061	122	16.1

Figure 3.13 represents the consumption of reducing sugars per gram of biomass. As expected, these decrease during the fermentation time.

The coffee waste contains high phenolic antioxidants, around 4 g/L of polyphenols and 2.5 g/L flavonoids. During this fermentation the concentration of the antioxidants didn't oscillate much and as such, the bioethanol production was not inhibited by them.

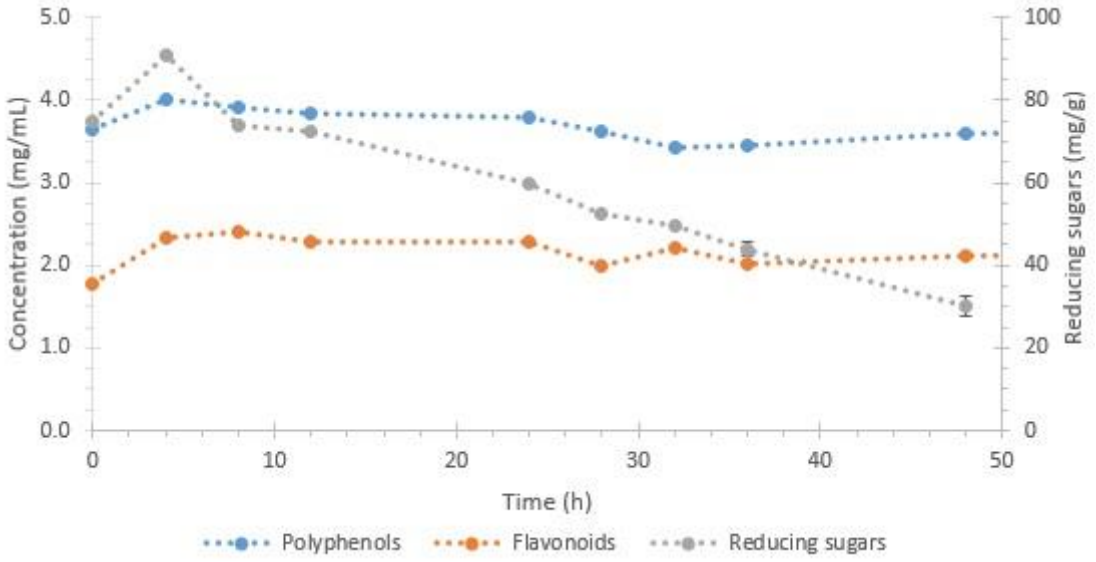

Figure 3.13 Representation of reducing sugars generated by gram of biomass (mg/g) during the fermentation time, and concentration of polyphenols and flavonoids obtained (mg/mL), with coffee waste using chemical hydrolysis.

3.3.1.3 BREAD WASTE

As with the fermentation using rapeseed and coffee as substrates for the production of bioethanol, bread waste also showed a production of glucose in the first hours of fermentation. This production of glucose can also indicate that the yeast is producing its own enzymes to break down the starch into sugars. After this point, around 4 h of fermentation, the production of glucose starts to decrease and the concentration of ethanol steadily increases until 48 h of fermentation.

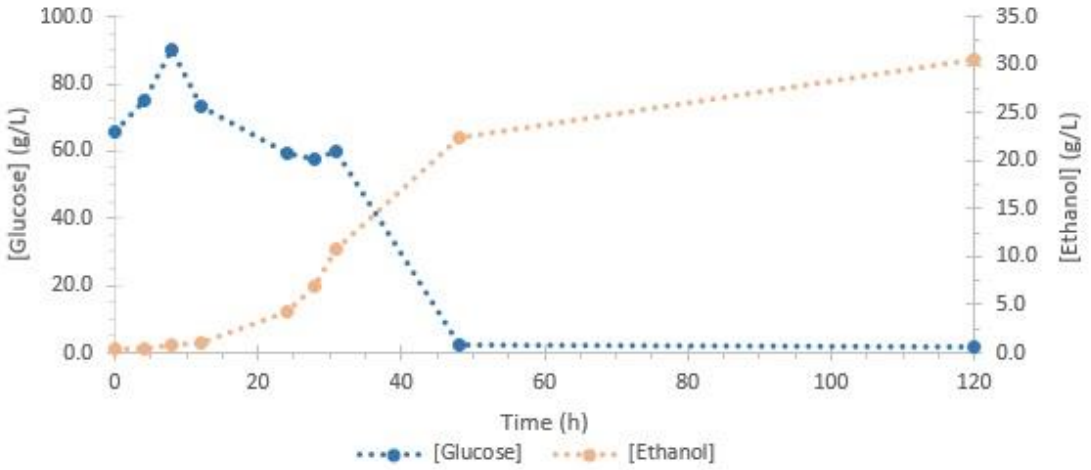

Figure 3.14 Glucose consumption and ethanol formation during the 120 h fermentation using bread as substrate for the chemical hydrolysis.

The highest concentration of ethanol was observed at 120 h, when the fermentation was stopped, with 30.6 g/L of ethanol. At this point the concentration of glucose was almost zero and the concentration of residual sugars (Figure 3.15) was very low, so it is possible to assume that further production of ethanol was not possible. These results were compatible with the shake flasks test.

The bread waste is composed of high content in starch (63.8%), and with full hydrolysis of this content it would be possible to obtain 177 g of polysaccharides and a maximum production of 90 g of ethanol. In this case, 30.6 g/L of ethanol were obtained with 50.9% of efficiency, where 100% efficiency would describe the so far unreported optimum case of complete hydrolysis and complete conversion to bioethanol.

Table 3.6 Results obtained for the maximum production of ethanol (g/L), ethanol factor yield ($Y_{P|S}$, g/g), ethanol volumetric productivity (Q_P, g/L·h), fermentation time (h) and efficiency of sugars conversion to ethanol (η, %), using bread waste and a chemical hydrolysis.

[Ethanol] (g/L)	**$Y_{P\|S}$ (g/g)**	**Q_P (g/L·h)**	**Fermentation time (h)**	**η (%)**
30.6	0.173	0.255	120	50.9

In Figure 3.15 the large amount of reducing sugars released in the chemical hydrolysis of bread waste (beginning of fermentation) are shown, along with how they are consumed to produce ethanol during the fermentation. The last sample taken for this analysis is not shown, but it exhibited a residual concentration of the reducing sugars per gram of biomass (at 120 h of fermentation).

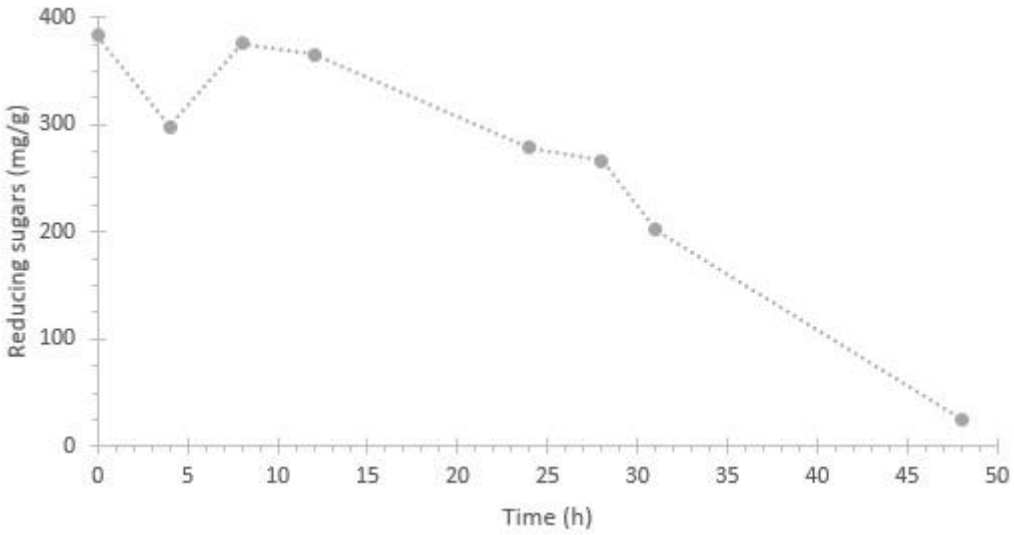

Figure 3.15 Representation of reducing sugars generated by gram of biomass (mg/g) during the fermentation time, when using bread waste and chemical hydrolysis.

3.3.2 BIOETHANOL PRODUCTION USING COMMERCIAL ENZYMES

In addition to experiments using chemical hydrolysis, ethanol production was also studied with no chemical hydrolysis but with the addition of commercial enzymes. The “cocktail” from Novozymes described in section 2.6.3 used a cellulase enzyme to break down cellulose to cellobiose and a β-glucosidase to hydrolyse the saccharides. These enzymes were added to the rapeseed and coffee waste fermentation. For the bread waste fermentation experiments, a commercial preparation of α-Amylase from *Bacillus amyloliquefaciens* was used.

3.3.2.1 RAPESEED WASTE

The “cocktail” of enzymes was added to the bioreactor two hours before to the addition of inoculum. As expected, it is possible to observe a release of glucose in the first hours of reaction. After the inoculum was added at the 2 h time point, the bioethanol production started.

Due to impossibility to access the laboratory between the 12 and 72 h of the fermentation, no samples were taken during this period of time. The fermentation was stopped at 72 h (70 h of fermentation), with a maximum of 4.28 g/L of ethanol (Figure 3.16).

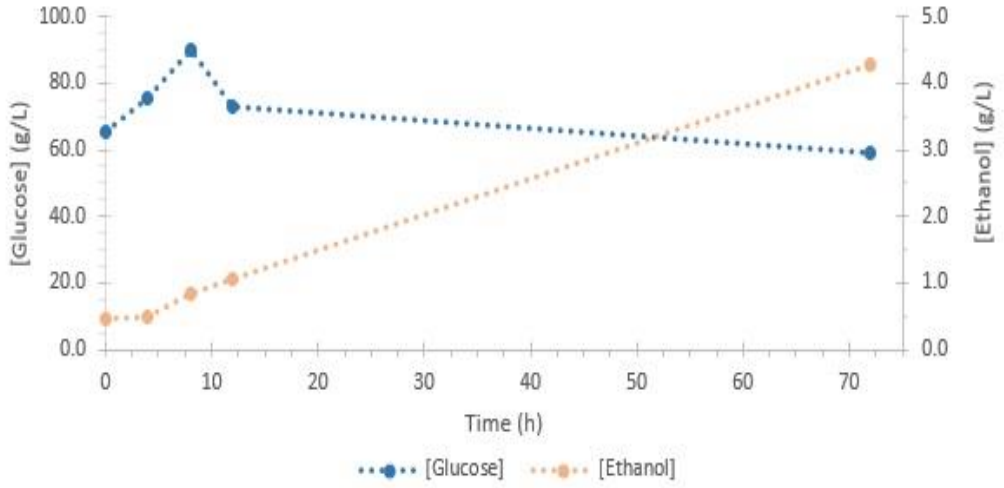

Figure 3.16 Glucose consumption and ethanol formation during the 72 h fermentation using rapeseed as substrate for the enzymatic hydrolysis using commercial enzymes.

Table 3.7 presents the results obtained in this fermentation with 13.1% of the theoretical yield of ethanol. The concentration of bioethanol obtained for this fermentation is really low compared to the value obtained for the chemical hydrolysis (separate hydrolysis and fermentation). A higher yield was expected when simultaneous saccharification and fermentation was employed, but looking at Figure 3.16, the glucose was consumed in a low rate during the fermentation which lead to a low production of bioethanol in the 72 h of fermentation.

Table 3.7 Results obtained for the maximum production of ethanol (g/L), ethanol factor yield ($Y_{P|S}$, g/g), ethanol volumetric productivity (Q_P, g/L·h), fermentation time (h) and efficiency of sugars conversion to ethanol (η, %), using rapeseed waste and enzymatic hydrolysis using commercial enzymes.

[Ethanol] (g/L)	**$Y_{P\|S}$ (g/g)**	**Q_P (g/L·h)**	**Fermentation time (h)**	**η (%)**
4.28	0.045	0.059	72	13.1

Again, the reducing sugars generated per gram of biomass, Figure 3.17, show the same trend as the glucose concentration in Figure 3.16. An increase in the reducing sugars occurred in the first hours. This was related to the use the “cocktail” of enzymes which hydrolysed the cellulose and the saccharides, a feature of the simultaneous saccharification and fermentation process. But, again, the concentration of reducing sugars didn’t decrease that much from the initial value at the beginning of the fermentation.

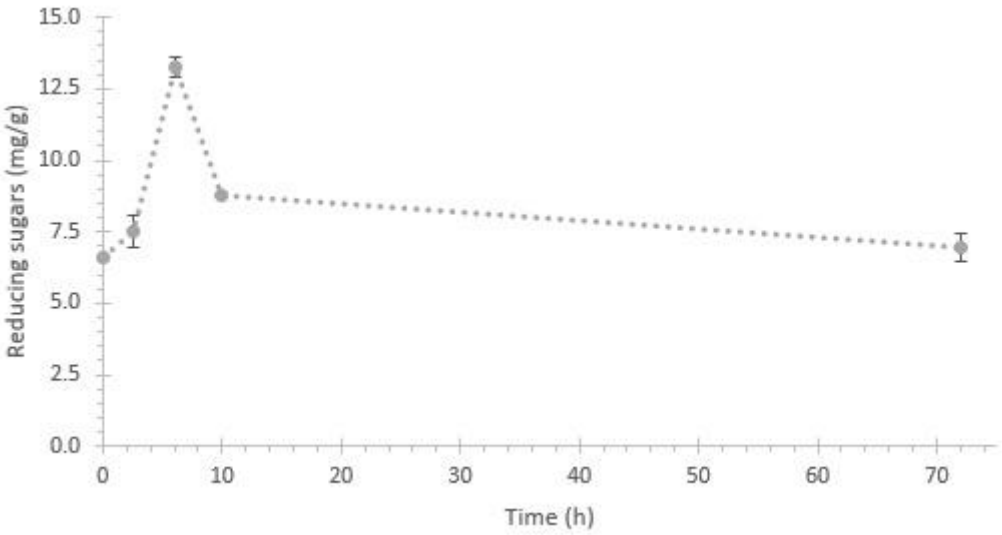

Figure 3.17 Representation of reducing sugars generated by gram of biomass (mg/g) during the fermentation time, with rapeseed waste using enzymatic hydrolysis using commercial enzymes.

The concentration of flavonoids and polyphenols behaved in the same way for the two oxidants and was consistently lower than 0.2 g/L, and therefore not considered relevant.

3.3.2.2 COFFEE WASTE

The same enzyme "cocktail" used for rapeseed was used for the fermentation of coffee waste. Again the inoculum was added to the bioreactor only 2 h after the enzymes were added.

Figure 3.18 shows that the glucose concentration slightly decreased in the first 4 h and was kept relatively constant afterwards during the 48 h of fermentation. The initial concentration was not that high when compared to the previous experiments. The ethanol production increased in the first 8 h and after that period was almost constant throughout the total fermentation time.

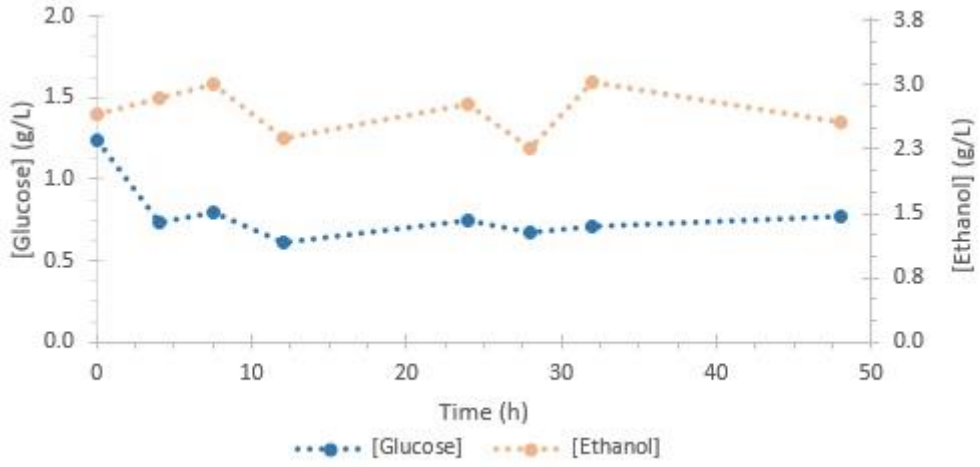

Figure 3.18 Glucose consumption and ethanol formation during the 48 h fermentation using coffee as substrate for the enzymatic hydrolysis using commercial enzymes.

The results obtained for this fermentation (Table 3.8) show a low ethanol production, 3.04 g/L and 6.57% efficiency paired with a really low glucose concentration, according to Figure 3.18. This can be explained by the fact that most of the coffee waste content was hemicellulose, and since *S. cerevisiae* is not able to metabolise pentose and prefers glucose, the fermentation of bioethanol was expected to be difficult to occur in such low glucose concentration.

Table 3.8 Results obtained for the maximum production of ethanol (g/L), ethanol factor yield ($Y_{P|S}$, g/g), ethanol volumetric productivity (Q_P, g/L·h), fermentation time (h) and efficiency of sugars conversion to ethanol (η, %), using coffee waste and enzymatic hydrolysis with commercial enzymes.

[Ethanol] (g/L)	$Y_{P\|S}$ (g/g)	Q_P (g/L·h)	Fermentation time (h)	η (%)
3.04	0.022	0.095	32	6.57

Figure 3.19 shows that the concentration of reducing sugars generated by gram of biomass had some oscillations and is really low for what was predicted for coffee waste. These results are in agreement with the glucose concentration determined by HPLC.

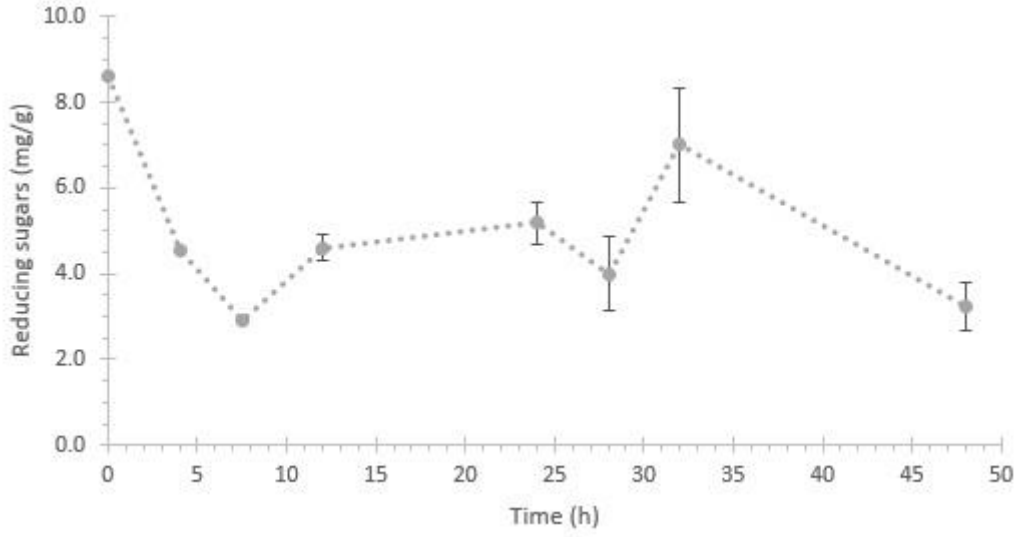

Figure 3.19 Representation of reducing sugars generated by gram of biomass (mg/g) during the fermentation time, with coffee waste using enzymatic hydrolysis using commercial enzymes.

The concentration of polyphenols and flavonoids determined during the 48 h of fermentation show that the concentrations of these components weren't influenced by the ethanol fermentation.

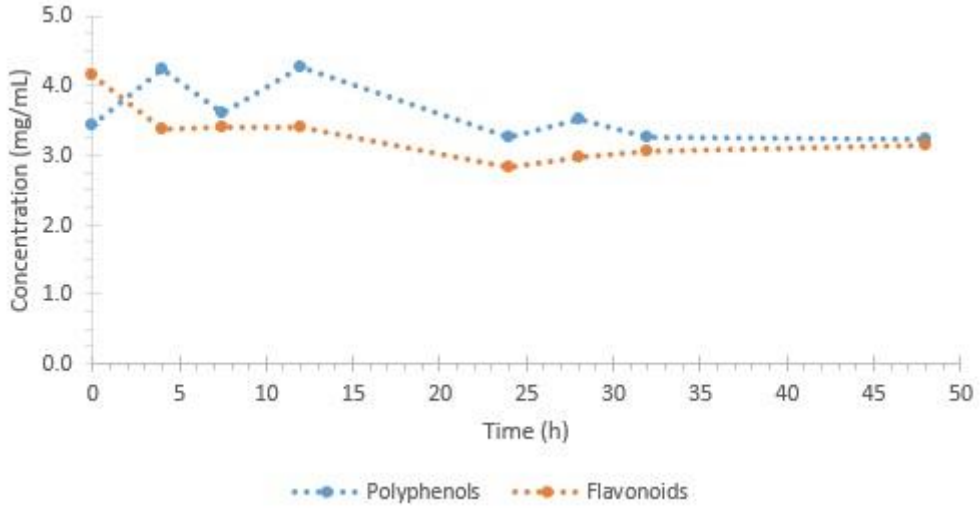

Figure 3.20 Representation of polyphenols and flavonoids concentration obtained (mg/mL), with coffee waste using enzymatic hydrolysis using commercial enzymes.

3.3.2.3 BREAD WASTE

For the bread waste as substrate for the bioethanol fermentation, the commercial enzyme preparation α-Amylase from *Bacillus amyloliquefaciens* was used. These enzymes randomly cleave internal glycosidic linkages in starch molecules to hydrolyse them and yield dextrins and oligosaccharides.

Figure 3.21 shows that after the first 2 h of the fermentation, the glucose concentration was three times higher than in time zero (moment when the enzymes were added). It is possible to suggest that the starch was hydrolysed to monosaccharides. After this point, the glucose concentration decreased and the ethanol production started.

The fermentation was stopped at 52 h, in this moment the ethanol concentration was 26.8 g/L and the glucose concentration was around 5 g/L.

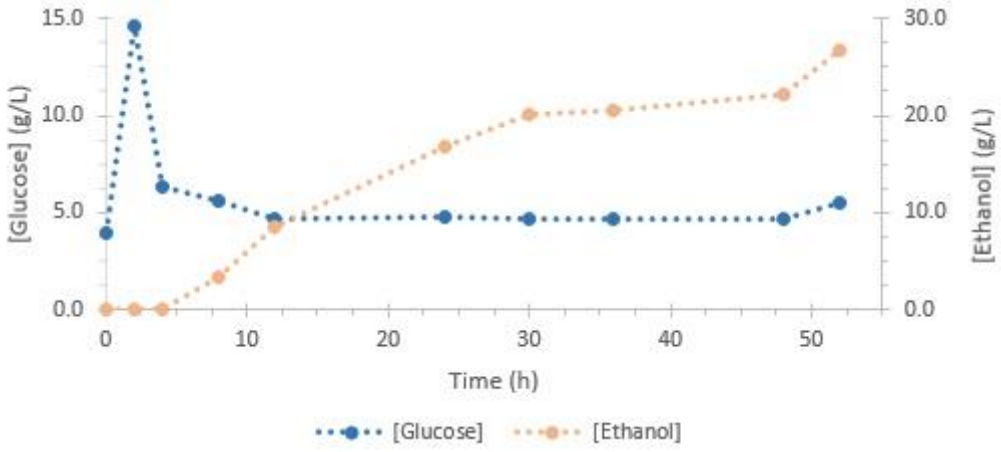

Figure 3.21 Glucose consumption and ethanol formation during the 52 h fermentation using bread waste as substrate for the enzymatic hydrolysis with commercial enzymes.

The ethanol production was 26.8 g/L ethanol and the efficiency was 44.6%, slightly below the values obtained for chemical hydrolysis. It is important to notice that the bread waste wasn't pretreated in both cases, but in the chemical hydrolysis, the acid weakened the glucosidic bonds perhaps making it easier to hydrolyse the starch.

Table 3.9 Results obtained for the maximum production of ethanol (g/L), ethanol factor yield ($Y_{P|S}$, g/g), ethanol volumetric productivity (Q_P, g/L·h), fermentation time (h) and efficiency of sugars conversion to ethanol (η, %), using bread waste and enzymatic hydrolysis with commercial enzymes.

[Ethanol] (g/L)	$Y_{P\|S}$ (g/g)	Q_P (g/L·h)	Fermentation time (h)	η (%)
26.8	0.151	0.515	52	44.6

In Figure 3.22, the reducing sugars produced by gram of biomass had an increase in the first 2 h, when the inoculum wasn't yet added to the media. After the inoculum addition, the concentration of the reducing sugars decreased until 12 h and stabilised until the end of fermentation at 52 h.

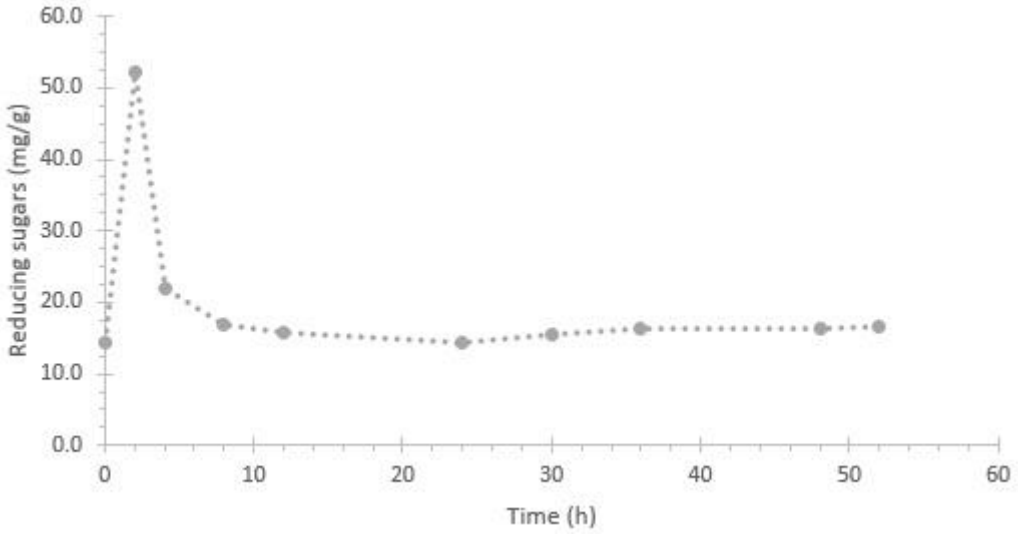

Figure 3.22 Representation of reducing sugars generated per gram of biomass (mg/g) during the fermentation time, using bread waste and enzymatic hydrolysis (commercial enzymes).

3.3.3 BIOETHANOL PRODUCTION USING IN-HOUSE PRODUCED ENZYMES

The last part of this work was to produce enzymes in order to have a more efficient process. A mold, *Phanerochaete chrysosporium*, was chosen to produce the enzymes due to its ability to degrade the aromatic polymer lignin and completely degrading all major components of plant cell walls (Martinez *et al.*, 2004).

After producing the enzymes, the protein concentration was measured, obtaining 6.2±1.4 mg/mL. A SDS-PAGE analysis was performed to determine the molecular weight of the proteins present in the in-house produced enzyme sample. Two bands of 41.7 and 106.6 kDa were detected.

Figure 3.23 shows the gel obtained where lanes 5 and 8 correspond to a duplicate of the enzyme sample analysed and the lane 10 corresponds to the protein standard used to determine the molecular weight.

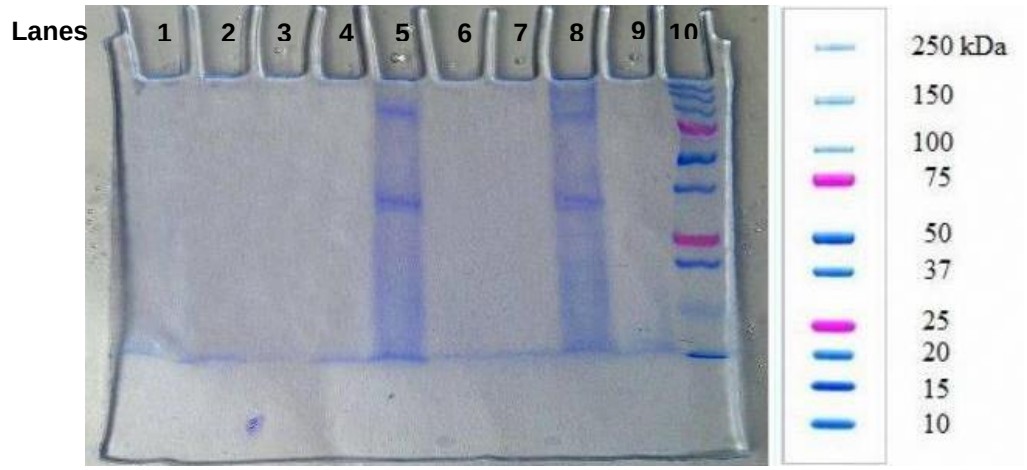

Figure 3.23 SDS-PAGE gel obtained after electrophoresis of in-house produced enzyme. The 5th and 8th wells correspond to a duplicate of the sample under study. The 10th well contains the protein standard.

According to Martinez *et al.* (2004), *P. chrysosporium* has a large repertoire of glycosidases and as for the extracellular oxidases, many of the glycoside hydrolysases appear within large families of closely related genes, consequently the two bands that appear in the SDS-PAGE gel may not correspond to only two proteins but a group of related proteins.

The in-house produced enzymes were added to the bioreactor media with the inoculum, simulating a simultaneous saccharification and fermentation process.

3.3.3.1 RAPESEED WASTE

Figure 3.24 shows that the initial concentration of glucose was lower than both in the fermentations with chemical hydrolysis and using commercial enzymes, not reaching 1.0 g/L glucose. The glucose concentration decreased rapidly until 24h, when a residual concentration of glucose was reached. This behaviour was matched by the increase of ethanol concentration until 24 h of fermentation.

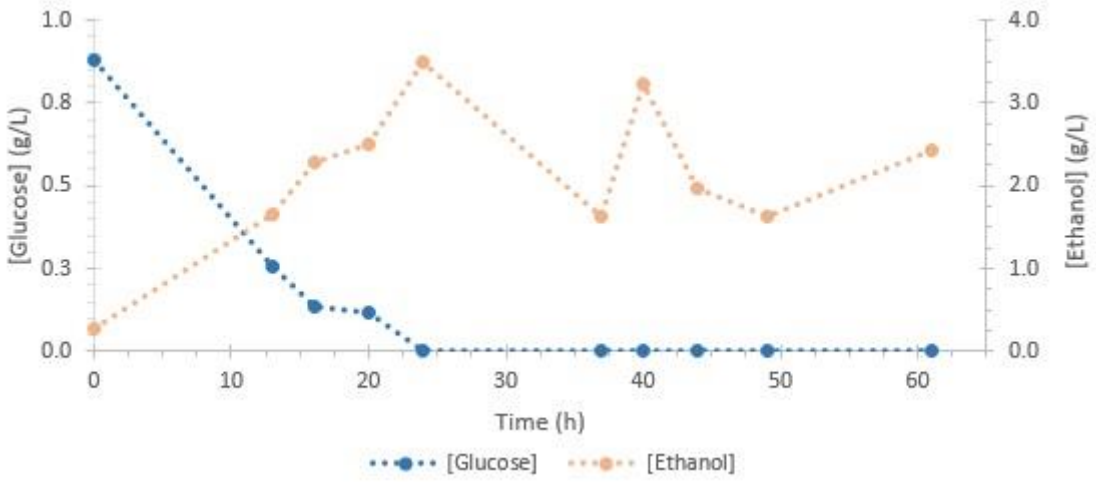

Figure 3.24 Glucose consumption and ethanol formation during the 61 h fermentation using rapeseed as substrate and hydrolysis using in-house produced enzymes.

Table 3.10 shows the ethanol production obtained by this method, 2.44 g/L with 7.5% efficiency. Some studies have reported that the fermentation time when *P. chrysosporium* is used should be longer, above 96 h, and that the glucose concentration decreases abruptly in the first 24 h (Paschos *et al.*, 2015; J. Wang *et al.*, 2016; K. Wang *et al.*, 2013). As such, the fermentation should have been carried on for a longer time, to analyse if there would be any difference in the ethanol production.

Table 3.10 Results obtained using rapeseed waste and hydrolysis using in-house produced enzymes for the maximum production of ethanol (g/L), ethanol factor yield ($Y_{P|S}$, g/g), ethanol volumetric productivity (Q_P, g/L·h), fermentation time (h) and efficiency of sugars conversion to ethanol (η, %).

[Ethanol] (g/L)	$Y_{P\|S}$ (g/g)	Q_P (g/L·h)	Fermentation time (h)	η (%)
2.44	0.025	0.040	61	7.5

As with the glucose concentration, the reducing sugars generated per gram of biomass, also decreased until 16 h of fermentation, stabilising after this period of time until the end of fermentation (Figure 3.25).

Regarding the polyphenols concentration, a slightly increase in its concentration is observed, while may be related to stress conditions. When the glucose concentration was residual, the media was composed of 3.5 g/L of ethanol and enzymes produced from *Phanerochaete chrysosporium*. These elements could lead to the increase in phenolic components (Gomaa, 2012). Flavonoids concentration was residual and constant throughout the fermentation time.

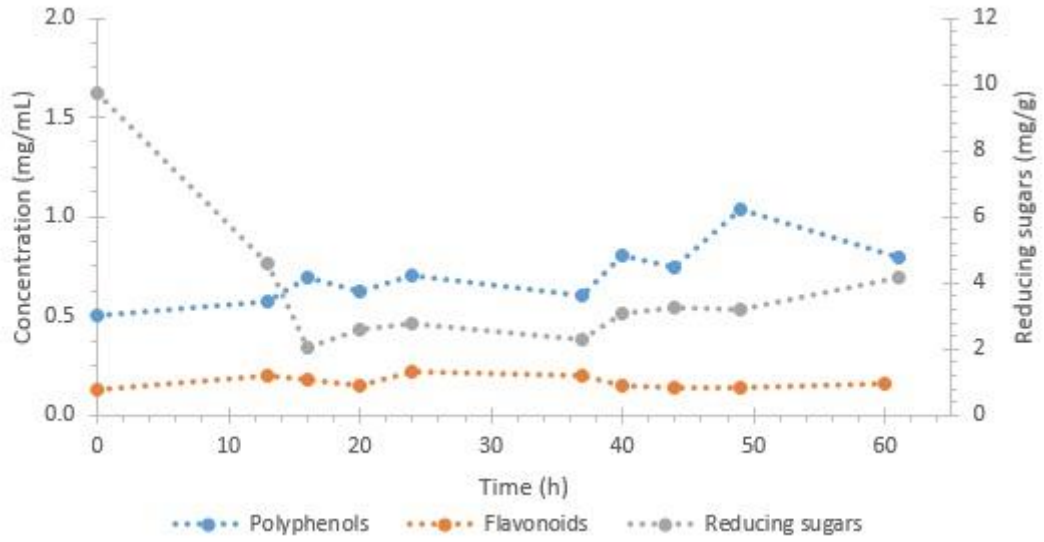

Figure 3.25 Concentration of reducing sugars generated per gram of biomass (mg/g) during the fermentation time, and concentration of polyphenols and flavonoids obtained (mg/mL), when using rapeseed waste and hydrolysis using in-house produced enzymes.

3.3.3.2 COFFEE WASTE

Figure 3.26 shows that while the fermentation stopped at 48 h, the glucose concentration was increasing which may point to the fact that the enzymes were slowly acting in the cellulose of the waste, and this hydrolysis process was slow.

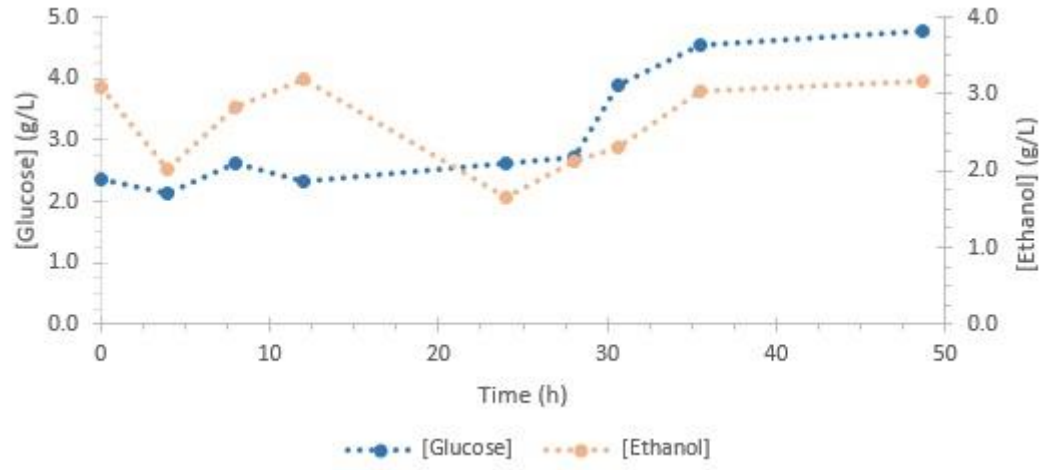

Figure 3.26 Glucose consumption and ethanol formation during the 48 h fermentation using coffee as substrate for the hydrolysis using produced enzymes.

According to the results obtained (Table 3.11), the production of ethanol was really low (6.84%) obtaining 3.16 g/L, a value similar to that observed for the rapeseed waste and with a similar trend in glucose consumption and ethanol production.

Table 3.11 Results obtained for the maximum production of ethanol (g/L), ethanol factor yield ($Y_{P|S}$, g/g), ethanol volumetric productivity (Q_P, g/L·h), fermentation time (h) and efficiency of sugars conversion to ethanol (η, %), using coffee waste and hydrolysis with in-house produced enzymes.

[Ethanol] (g/L)	$Y_{P\|S}$ (g/g)	Q_P (g/L·h)	Fermentation time (h)	η (%)
3.16	0.023	0.065	48	6.84

Figure 3.27 represents the reducing sugars generated per gram of biomass when using coffee waste as substrate. It is noticeable that reducing sugars concentration is almost constant during the fermentation time, with a slight increase in the first hours followed by the stabilisation of its generation during the rest of the time. It shows the same trend as for the glucose consumption in Figure 3.26.

Figure 3.28 shows that the concentration of flavonoids and polyphenols are similar during the fermentation time, only the flavonoids concentration slightly decreases in the first 12 h of fermentation, increases until 28 h and finally stays constant until the end. This period of time is coincident with an increase in the reducing sugars generated by biomass.

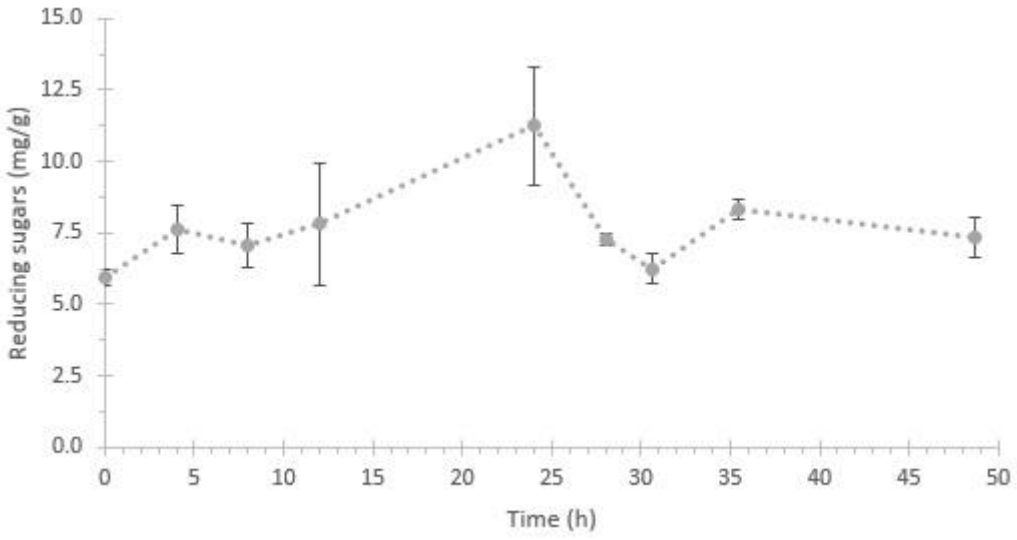

Figure 3.27 Representation of reducing sugars generated per gram of biomass (mg/g) during the fermentation time, with coffee waste using hydrolysis with in-house produced enzymes.

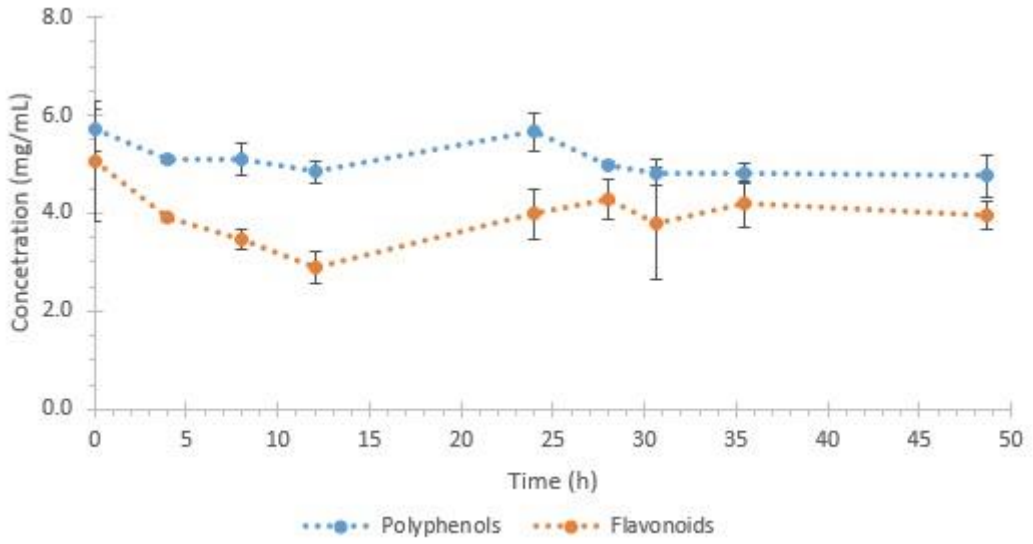

Figure 3.28 Representation of polyphenols and flavonoids concentration obtained (mg/mL), with coffee waste using hydrolysis with in-house produced enzymes.

3.3.3.3 BREAD WASTE

During the first 8 h of fermentation, there was an abrupt consumption of glucose, as expected from *P. chrysosporium*. A slight consumption was followed until the end of the fermentation time. The ethanol concentration gradually increased until the end of fermentation, as shown in Figure 3.29.

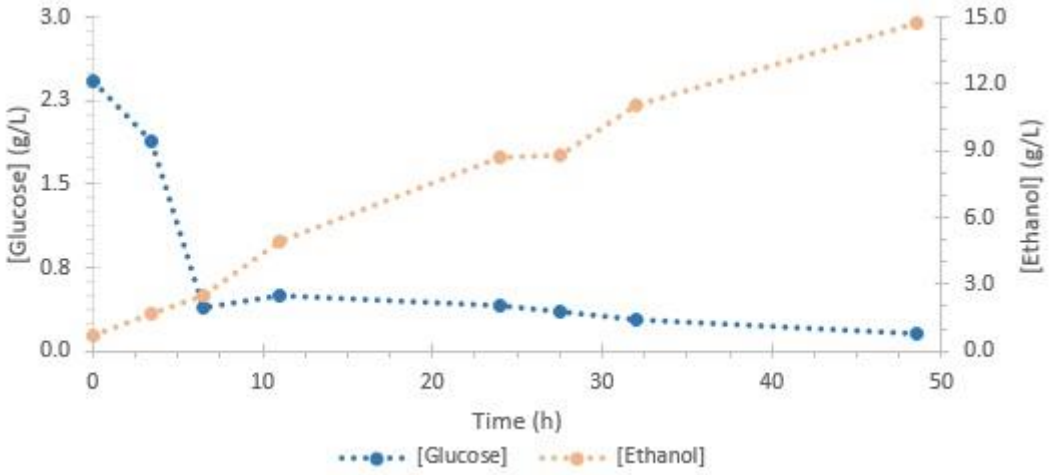

Figure 3.29 Glucose consumption and ethanol formation during the 48 h fermentation using bread as substrate and hydrolysis with in-house produced enzymes.

The ethanol production using bread waste as substrates for the hydrolysis with in-house produced enzymes was the lowest of the three processes studied, reaching only 14.8 g/L with 24.6% efficiency (Table 3.12). Looking at the previous figure, if the fermentation wasn't stopped perhaps the ethanol concentration would have increased, since as commented before, the fermentation process using the mold in study should be conducted during a longer period of time.

Table 3.12 Results obtained for the maximum production of ethanol (g/L), ethanol factor yield ($Y_{P|S}$, g/g), ethanol volumetric productivity (Q_P, g/L·h), fermentation time (h) and efficiency of sugars conversion to ethanol (η, %), using bread waste and hydrolysis with in-house produced enzymes.

[Ethanol] (g/L)	$Y_{P\|S}$ (g/g)	Q_P (g/L·h)	Fermentation time (h)	η (%)
14.8	0.083	0.305	48	24.6

The concentration of reducing sugars generated from biomass decreases in the first hours of fermentation and remained stable until the end, following the same trend as the glucose consumption.

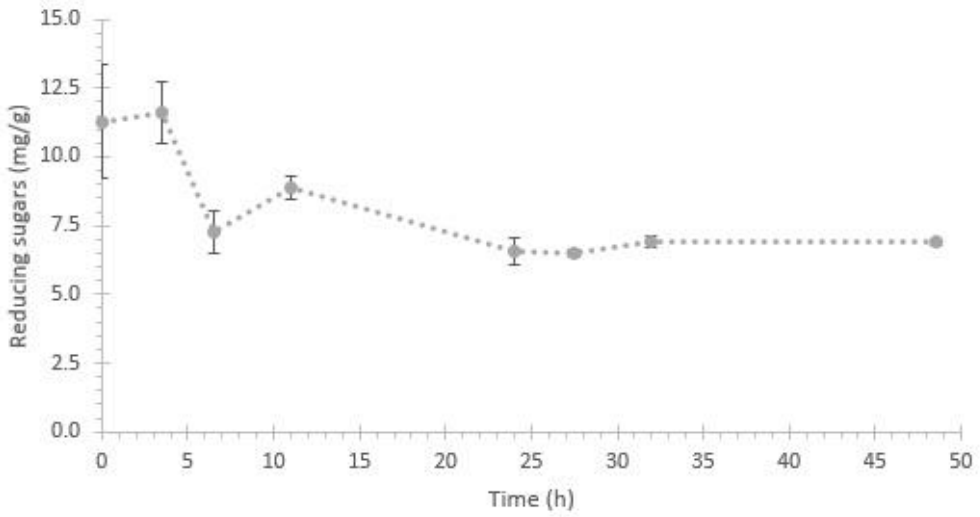

Figure 3.30 Representation of reducing sugars generated per gram of biomass (mg/g) during the fermentation time, using bread waste and hydrolysis with in-house produced enzymes.

4 CONCLUSIONS AND FUTURE PROSPECTS

Considerable progress has been made in order to understand the underlying mechanisms responsible for the hydrolysis process of lignocellulosic materials. The work presented here was performed to investigate the effect of different types of hydrolysis in bioethanol production. Three different feedstocks, with high potential for biorefinery, were subjected to chemical hydrolysis with HCl using different concentrations (0, 0.1, 0.2, 0.3, 0.6, 0.9 and 1.2 M), as well as enzymatic hydrolysis with commercial and in-house produced enzymes. These studies were made in order to understand the contribution of the hydrolysis process in the ethanol production.

The three different feedstocks weren't pretreated; the rapeseed waste was only milled before used, the coffee waste was dried and the bread waste wasn't transformed as it was acquired already as breadcrumbs for the study. It is important to notice that according to the literature the yield of cellulose hydrolysis is less than 20% of theoretical value when there is no pretreatment method employed (Cardona and Sánchez, 2007).

Fermentations for the different hydrolysis studied were performed, using the feedstocks as substrates for the conversion to bioethanol. These studies were conducted firstly in shake flasks and later on scaled-up to a 2 L bioreactor. The composition of the wastes used was known in order to know the amount of cellulose or starch that could be converted in polysaccharides.

The agar plate tests were used in order to guarantee that *S. cerevisiae* was able to grow on the substrates. *S. cerevisiae* grows slowly in coffee waste hydrolysate which has a composition of 9.6% cellulose and 34.8% hemicellulose, and a high content in C-5 sugars which this strain is not able to metabolise.

Also, in the agar plates using rapeseed and bread hydrolysates it is possible to observe a darker colour when a higher concentration of HCl was used in the chemical hydrolysis, concluding that higher acidic concentrations lead to the formation of secondary reactions, as for the Maillard reaction where reducing sugars react with amino acids (Klinke *et al.*, 2004).

In the second part of this study was observed that an increase in the acid concentration led to an increase in the reaction rate. At higher acid concentrations, the reaction rates began to increase rapidly. A noticeable shift in the sugar production rate was evident when the acid concentration increased from 0.3 M to 0.6 M HCl.

The best efficiency obtained in the ethanol production was achieved when using chemical hydrolysis, with 21, 16.1 and 50.9% (Table 4.1) obtained for the rapeseed, coffee and bread waste, respectively. Nevertheless, the process should be controlled because hemicellulose sugars may be degraded to weak acids and furan derivatives which can act as inhibitor of the ethanol fermentation. From the dehydration of hexoses 5-hydroxymethlyfurfural (HMF) can be formed or furfural from the dehydration of pentoses (Li *et al.*, 2014). These by-products have an

inhibitory effect on the rate of reaction during the fermentation process, which is required to convert these low value sugars into high value biofuel products.

One problem that may have occurred was the saturation of the reactor with glucose, as the lignocellulosic structure was rapidly broken down into its components and further broken down into glucose. The fast conversion of glucose into the unwanted by-products due to increased collisions of the suspended particles within the reactor brought on by the increased temperature, ultimately retarding the fermentation of hydrolysates. This situation was already mentioned by Lenihan *et al.* (2010).

Table 4.1 Summary of the bioethanol results obtained for the fermentation in a 2 L bioreactor using chemical hydrolysis, hydrolysis with commercial enzymes or in-house produced enzymes, for the use of rapeseed, coffee and bread waste.

	Chemical hydrolysis		**Hydrolysis with commercial enzymes**		**Hydrolysis with in-house produced enzymes**	
Waste substrates	**[Ethanol] (g/L)**	**η (%)**	**[Ethanol] (g/L)**	**η (%)**	**[Ethanol] (g/L)**	**η (%)**
Rapeseed	6.86	21.0	4.28	13.1	2.44	7.50
Coffee	7.42	16.1	3.04	6.57	3.16	6.84
Bread	30.6	50.9	26.8	44.6	14.8	24.6

When the commercial enzymes from Novozymes were used for simultaneous saccharification and fermentation of waste substrates, the bioethanol concentration achieved was of 4.28, 3.04 and 26.8 g/L, using rapeseed, coffee and bread waste, respectively (see Table 4.1). These values represent an efficiency of 13.1, 6.57 and 44.6% of the theoretical ethanol production, indicating that this method was not a good choice for the rapeseed and coffee wastes, leading to lower efficiency when compared to the chemical hydrolysis. As commented before, one possible reason may be due to the high content of pentoses present in these two wastes that are difficult to metabolise by *S. cerevisiae*, while the efficiency was calculated for a complete hydrolysis and fermentation of the waste substrate.

The expected ethanol production for rapeseed waste as substrate was higher, according to literature ethanol concentration obtained was around 30 g/L for SHF and SSF, representing approximately 60% of ethanol yield (as percentage of theoretical ethanol yield) (López-Linares *et al.*, 2014). The results obtained in this work demonstrate that the process employed was not ideal, a study using rapeseed waste milled to a fine powder should be done to evaluate if the conditions of the waste usage were not ideal for fermentation.

The results obtained for coffee waste as substrate were close to the ones in the literature (Gouvea *et al.*, 2009; Murthy and Madhava Naidu, 2012), however the efficiency obtained in this work was

low compared to other results where 50% yield was obtained. It shows that the conditions in this work are not optimal and further experiments should be performed.

Comparing to Ebrahimi *et al.* (2008), it was obtained 0.35 g ethanol per g of initial dry bread when it was performed a liquefaction at 85°C, saccharification at 60°C and fermentation at 30°C. In this work, it was obtained 0.16 g ethanol per g initial dry bread for simultaneous saccharification and fermentation using commercial enzymes, showing that the temperatures used could be improved and also, the stirring should be improved in order to obtain an ideal mixing of the bioreactor and therefore the results could be similar to the ones in the literature. However, it was obtained 44.6% efficiency in this fermentation, which demonstrates that regarding this method the bread waste is a good choice as waste material for ethanol production.

The final fermentation experiment showed the lowest values for ethanol production in this study, with 2.44, 3.16 and 14.8 g/L for rapeseed, coffee and bread waste respectively (see Table 4.1). The coffee waste was the exception, showing a slightly increase in the ethanol production when compared to the use of commercial enzymes. However, these three fermentations were the only trial perfomed using *P. chrysosporium* and the first one using these method, therefore this process still requires further optimisation in order to find the ideal temperature and pH settings. Furthermore, the trial was done in an anaerobic environment, and the process could benefit from an aerobic environment using the same conditions. Finally, some studies with this mold demonstrated that a longer fermentation time is necessary to increase the ethanol production (Bak *et al.*, 2009).

When the fermentation using the in-house produced enzymes was stopped it was seen a white colour around the waste particles attached to the bioreactor helices, it is possible to conclude that the in-house produced enzymes were able to degrade the lignin of the wastes inducing the uncover of cellulose. This situation relating the white colour around the waste particles was also related by Bak *et al.* (2009).

Attending to the three conditions studied, chemical hydrolysis, simultaneous saccharification and fermentation using a “cocktail” of cellulosic enzymes from Novozymes, α-amylase from Sigma-Aldrich or using enzymes produced in-house from the mold *P. chrysosporium*, the best results were obtained for the chemical hydrolysis (higher ethanol production). In addition, when bread waste was used as substrate, there was a higher ethanol production in all the three conditions studied, with the highest concentration obtained of 30.6 g/L of ethanol. It is possible to conclude that bread waste is a promising waste to be used as substrate for ethanol production, with 50.9% of ethanol production achieved in this study showing promising results.

According to the polyphenols analysis it was observed a slight decrease in its concentration as expected in some of the fermentations performed, regarding rapeseed and coffee waste, as the biodegradation of lignin component decrease the detectable phenolic acids and aldehydes (K. Wang *et al.*, 2013).

It would be helpful for a better understanding of these results, as a future prospect, to study the concentration of C-5 and C-6 sugars in the waste substrates, in order to have the whole composition of cellulose and hemicellulose. Also, after the chemical hydrolysis, it should prove helpful to study the concentration of all the sugars, not only the glucose. HPLC could be used to determine these sugars composition after hydrolysis as well as during the fermentation, and in this way, it would be possible to understand how the reactions of sugar conversions are occurring.

A study related to the stirring of the 2 L bioreactor should be held for improvement of these results. The stirring value used in this work was based on the stirring results previously obtained by Brummer, Skryja, *et al.* (2014), for small volumes and using paper waste as substrate which may not be the ideal stirring for the type of wastes used in this work, leading to not ideal mixture of the bioreactor content.

A pretreatment method could be employed before the hydrolysis of all the substrates to determine if it improves the ethanol production previously obtained and to increase the surface area of cellulose available for the hydrolysis, as many authors have considered (Hendriks and Zeeman, 2009; Lu *et al.*, 2009).

In conclusion, *P. chrysosporium* is a suitable mold for the degradation of cellulose and hemicellulose, in addition to lignin, due to its genetic information that includes, glycoside hydrolases, carbohydrate esterases and glycosyltransferases (Martinez *et al.*, 2004). The protein families and domains in *P. chrysosporium* reflects the complexity of metabolizing lignin derivatives and related aromatic compounds, due to the high number of putative glucose methanol choline reductases, which includes extracellular alcohol oxidases and cellobiose dehydrogenases and these domains are more abundant in filamentous fungi (*P. chrysosporium*).

Further investigation needs to be done in order to understand how the hydrolysis by in-house produced enzymes from *P. chrysosporium* could be optimised, relating to fermentation time, pH, temperature, stirring and, specially, enzyme loading.

After optimisation of the fermentation process with in-house produced enzymes, ultrafiltration technique could be used for concentrating the in-house produced enzymes and evaluation of these results to see if the bioethanol production was improved.

The conclusions of this work could help to further understanding on how to improve the hydrolysis of cellulose and starch on waste substrates and, in a near future, contribute to the reduction of costs of these processes in bioethanol production.

5 REFERENCES

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APPENDIX

A. HARTREE-LOWRY CALIBRATION CURVE

In this Appendix the standard curve for the Hartree-Lowry assay is presented. The standard curve was done in order to be able to correlate the measured absorbance at 650 nm to the proteins concentration in solution, in mg BSA/mL (Figure 5.1).

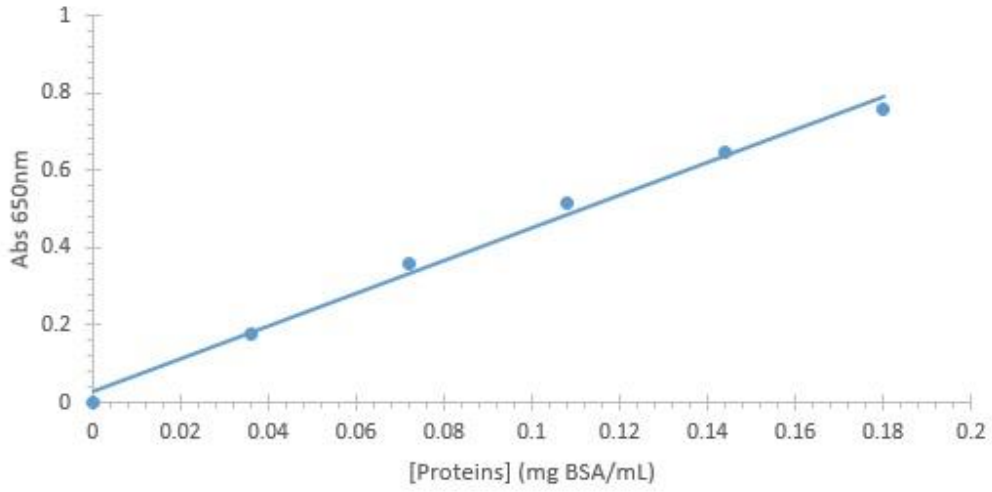

Figure 5.1 Standard curve for the determination of proteins concentration. Abs 650 nm=4.2563·[Proteins (mg BSA/mL)]+0.0254, R^2=0.9921.

B. SOMOGYI-NELSON CALIBRATION CURVE

In this Appendix the standard curve for the Somogyi-Nelson assay is presented. The standard curve was done in order to be able to correlate the measured absorbance at 720 nm to the glucose concentration in solution, in µg/mL (Figure 5.2).

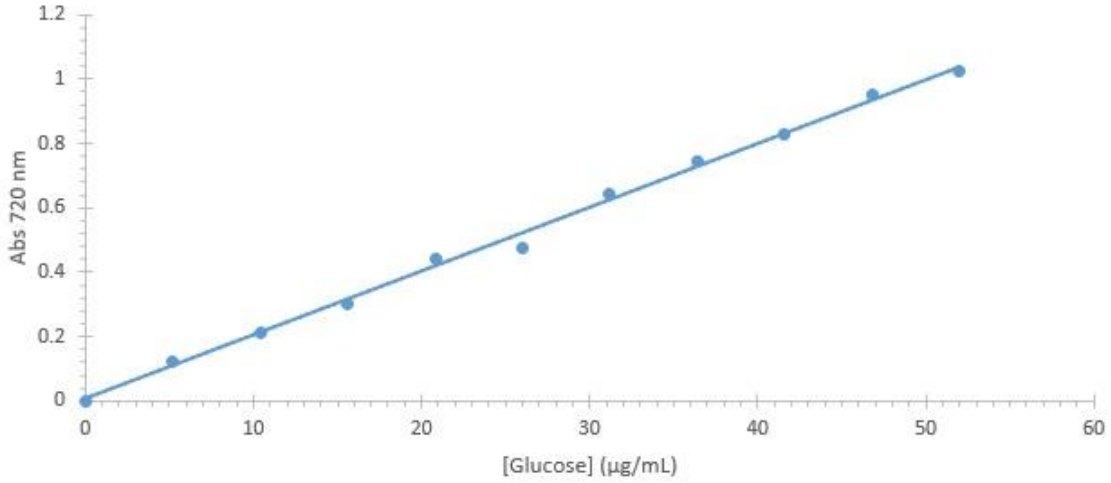

Figure 5.2 Standard curve for the determination of glucose concentration. Abs 720 nm=0.0198·[Glucose (µg/mL)]+0.0068, R^2=0.9967.

C. FLAVONOIDS ANALYSIS CALIBRATION CURVE

In this Appendix the standard curve for the flavonoids determination is presented. The standard curve was done in order to be able to correlate the measured absorbance at 510 nm to the catechin concentration in solution, in mg/mL (Figure 5.3).

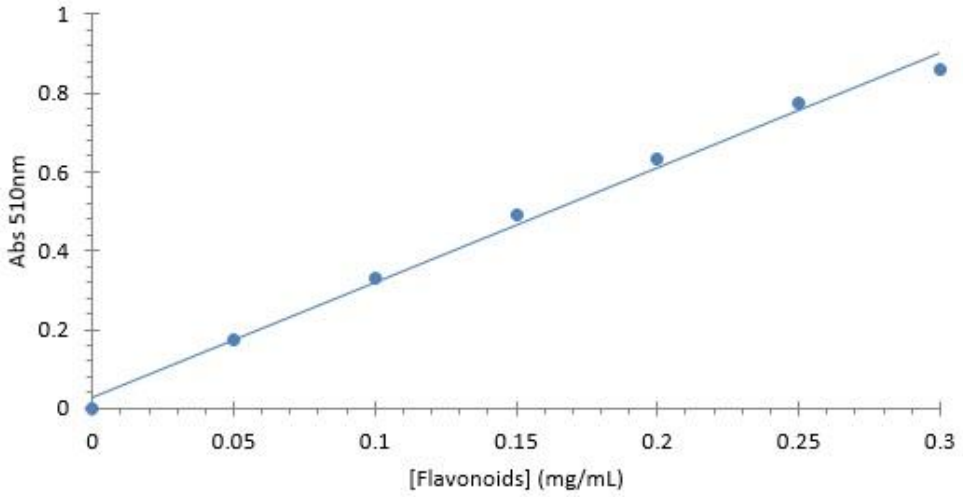

Figure 5.3 Standard curve for the determination of flavonoids concentration. Abs 510 nm=2.9121·[Catechin (mg/mL)]+0.0299, R^2=0.9924.

D. POLYPHENOLS ANALYSIS CALIBRATION CURVE

In this Appendix the standard curve for the Folin-Ciocalts assay is presented. The standard curve was done in order to be able to correlate the measured absorbance at 750 nm to the Gallic acid concentration in solution, in mg/mL (Figure 5.4).

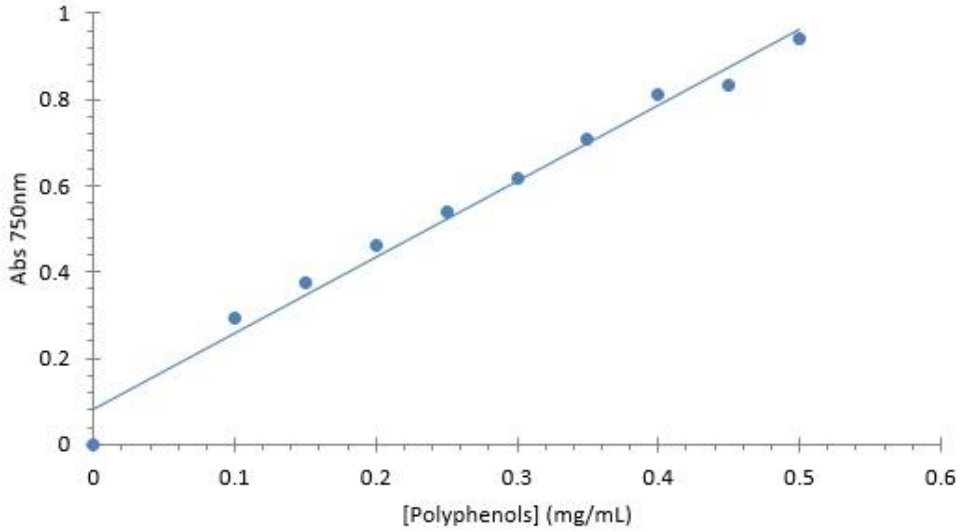

Figure 5.4 Standard curve for the determination of polyphenols concentration. Abs 750 nm=1.7684·[Galic acid (mg/mL)]+0.0802, R^2=0.9823.

E. GELS AND SOLUTIONS COMPOSITIONS FOR SDS-PAGE

For preparation of the separation and concentration gel for SDS-PAGE, the following solutions were necessary (Table 5.1).

Table 5.1 Composition of the solutions needed for the preparation of the gels.

Solutions	Reagents	pH	Volume (mL)	Storage
A	4.22 M acrylamide + 0.52 M bisacrylamide	-	100 mL distilled H_2O	4ºC
B	3 M Tris (in 50 mL H_2O)	Set to 8.8 by 1 M HCl	100 mL distilled H_2O	-
C	3 M Tris (in 50 mL H_2O)	Set to 6.8 by 1 M HCl	100 mL distilled H_2O	-
D	0.35 M SDS	-	100 mL distilled H_2O	In the dark
E	0.44 M APS	-	1 mL distilled H_2O	-
F	0.015 M bromophenol blue	-	1 mL distilled H_2O	-

After preparation of the solutions, the separation and stacking gel were prepared as described in Table 5.2.

Table 5.2 Composition of the 12% (w/v) separation gel and 4% (w/v) concentration gel.

Solutions	Separation gel 12% (w/v)	Concentration gel 4% (w/v)
A	3.30 mL	1.00 mL
B	2.50 mL	-
C	-	380 µL
D	100 µL	30 µL
H_2O	4.00 mL	2.10 mL
TEMED	50 µL	3 µL
E	350 µL	30 µL

The sample buffer was prepared recurring to 4.8 mL of solution C, 4 mL solution D, 0.2 mL F solution, 6 mL glycerol, 4.8 mL H_2O and 1 mL beta-mercapto-ethanol. And, for the running buffer it was prepared 1 L of solution with 50 mM Tris, 250 mM glycine and 6.9 mM SDS.

For the visualisation of the gel, it was prepared three solutions; a stabilisation solution with 400 mL H_2O and 100 mL methanol; the staining solution with 450 mL methanol, 100 mL acetic acid, 450 mL H_2O and 0.5 g Coomassie Brilliant Blue G-250 and, the destaining solution with 250 mL methanol, 100 mL acetic acid and 650 mL H_2O.

F. CHROMATOGRAM EXAMPLE FROM HPLC AND CALIBRATION CURVES FOR ETHANOL AND GLUCOSE

The high performance liquid chromatography (HPLC) system used for analysis of the glucose and ethanol concentration employs a refractive index detector (RI). Figure 5.5 shows an example of RI spectrum.

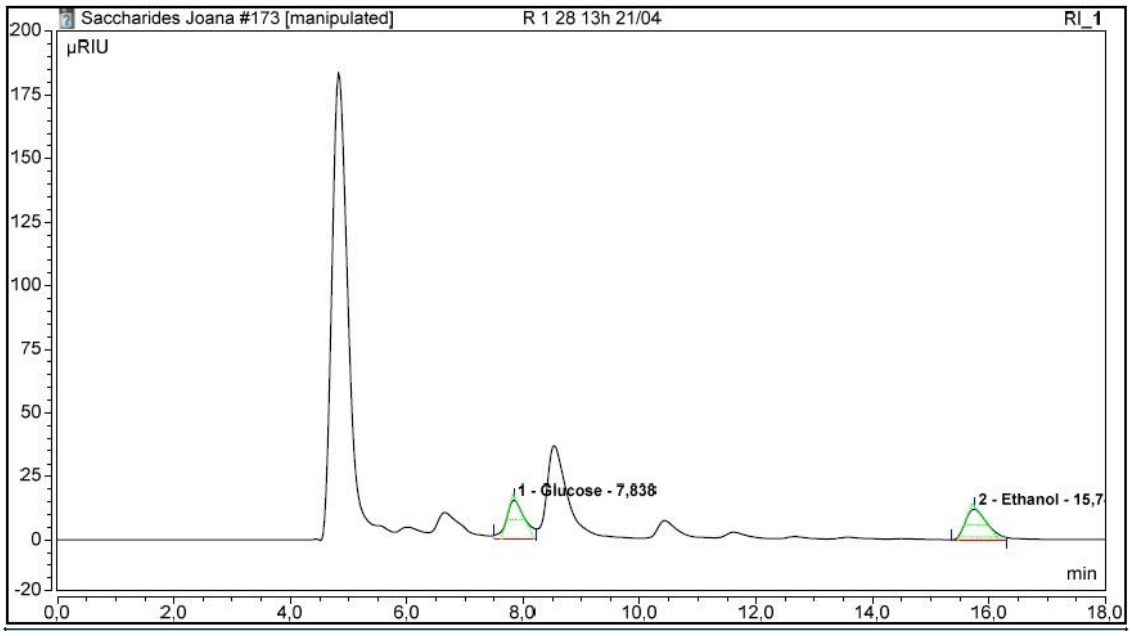

Figure 5.5 Example of RI spectrum of a bioreactor's sample where it is possible to detect glucose and ethanol's presence.

The standard curves were done in order to be able to correlate the measured areas of the absorbance peaks of a compound in the chromatogram to the compound concentration in a solution. The absorbance of glucose and ethanol are measured in terms of refractive index units (RIU), Figure 5.6 and Figure 5.7, respectively.

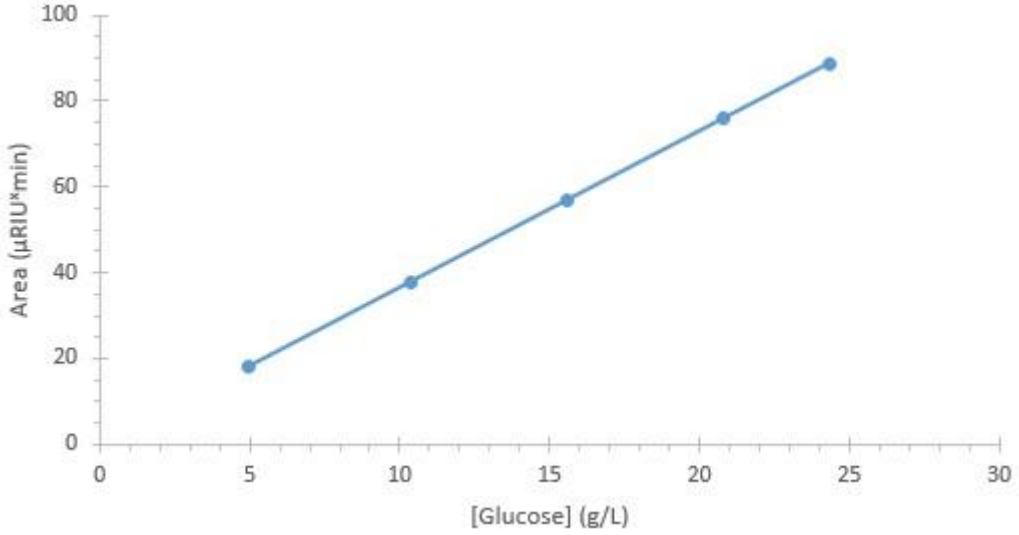

Figure 5.6 Standard curve for the determination of glucose concentration. Area (µRIUxmin)=3.65·[Glucose (g/L)], R^2=1.

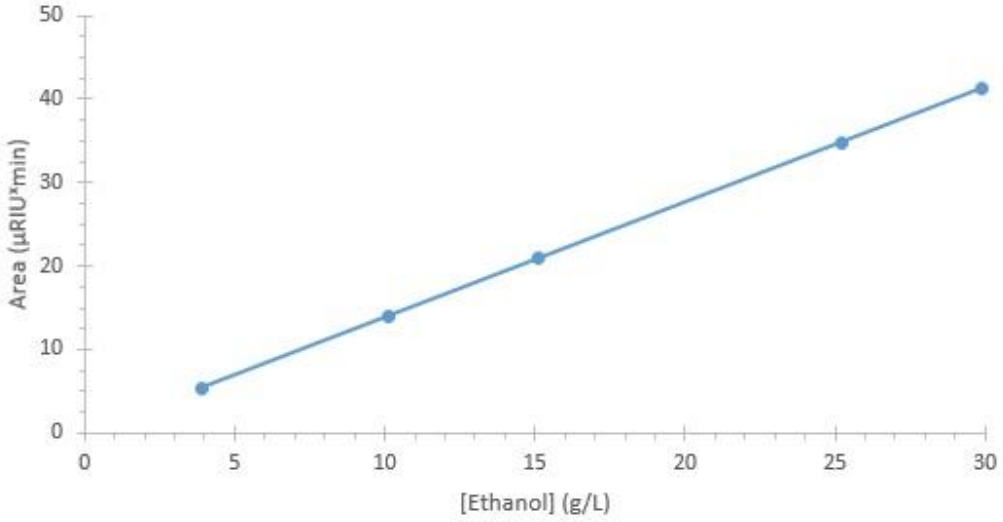

Figure 5.7 Standard curve for the determination of ethanol concentration. Area (µRIUxmin)=1.3821·[Ethanol (g/L)], R^2=1.