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

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Abstract: The promise of biofuels as a sustainable energy source required to meet the world’s increasing energetic demand is leading to new discoveries 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 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 production 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 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.

1 INTRODUCTION

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 [1], [2].
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 [3], [4].
Among the dominant renewable biofuels: bioethanol, biodiesel and biogas, the most common is ethanol from corn grain (starch) and sugar cane (sucrose) [5]. Nevertheless, these conventional crops are unable to meet the global demand of bioethanol production due to their primary value as food and feed [6]. 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

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 [2].
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.
When using lignocellulosic materials as feedstock it is necessary to convert lignocellulosic biomass into intermediates (cellulose, hemicellulose, lignin) before conversion into the final products [3].
Regarding the potential of plant biomass to produce liquid biofuels, most of its composition consists of cell walls, where 75% of it are polysaccharides [7]. This makes it necessary to disrupt the cell wall before further processing.

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 [8], [9].
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 (celllobiose) [2], [8], [9].
In order to be used, these tightly packed nanofibers must be broken down through chemical or enzymatic hydrolysis to create sugar, being later converted into ethanol or other liquid fuels [7]. Its stability is also due to the presence of hemicellulose and lignin.
Hemicellulose differs from cellulose as they are heteropolymers of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose) and acetylated sugars [8]. 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. 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 [10]. 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 [8], [11]. 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 [2], [9]. 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 its structural integrity [8], [12]. 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-coumary, coniferyl and sinapyl alcohol, linked through different types of ether and ester bonds, and minor phenolic compounds [12]. 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.

### 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 it was studied the use of rapeseed, coffee and breadcrumbs waste as substrates for the bioethanol production.

#### Rapeseed

Rapeseed (Brassica napus) straw is a lignocellulosic agricultural residue with high sugar content (approximately 60%) [13], namely 44±0.2% cellulose and 20±0.2% hemicellulose [14]. 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 [15], [16].

#### Coffee

Coffee is one of the most popular beverages around the world and has grown in commercial importance during the last years [17]–[19]. 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 [19], [20]. Besides carbohydrates, coffee bean is composed of cellulose, minerals, sugars, lipids, tannin and polyphenols [20]. Spent coffee constitutes almost 50% of the worldwide production of soluble coffee preparation, it is rich in sugars containing mannose and galactose.

#### 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 [21]. 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 [22]. 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.

### Biomass conversion for bioethanol production

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

#### 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 [6], [23]. 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 [4], [6], [24]. Additionally, most of hemicellulose should be hydrolysed and lignin released or degraded in a process also called delignification [24], [25]. 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 [4]. 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 [4], [6].

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 [26]. There are four pretreatment techniques used in this process: chemical, physical, physicochemical and biological. These techniques can be used individually or combined.

#### Enzymatic hydrolysis
After the pretreatment, the cellulose from the lignocellulosic material 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 (H₂SO₄ or HCl) is used, or an enzymatic reaction.

Sulphuric and hydrochloridric acids are the most commonly used diluted acids for the chemical hydrolysis of lignocellulose biomass. When diluted acids (concentrations of 1-10%) are employed, high temperatures (in the range of 100-150°C) are required in order to hydrolyse the crystalline cellulose of lignocellulose [4], [24]. 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 [24], [27]. 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 [27]. 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 [28].

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 [29]. 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, cellulose is converted into glucose by β-glucosidases (EC 3.2.1.21) [26]. Hemicellulolytic enzymes are more complex, involving a mixture of eight enzymes [6]. 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 specificity.

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 [24], [26]. *Trichoderma reesei* is a saprobic, known for efficient production of extracellular enzymes, most commercial cellulolytic enzymes are obtained from this aerobic microorganism [4], [28].

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 50°C and pH between 3 and 7, since these conditions lead to less degradation of sugar and higher yields comparing to chemical hydrolysis [6], [26], [28], [30].

### 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. This yeast is capable to withstand high concentrations of ethanol and high temperature, be tolerant to inhibitors present in the hydrolysates and have cellulolytic activity [6]. 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, xyllose.

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 [31].

The aim of this work was to compare the production of bioethanol using three different waste substrates, rapeseed, coffee and bread crumbs, 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.

## 2 MATERIALS AND METHODS

### 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 1, in percentage of content.

<table>
<thead>
<tr>
<th>Composition (%)</th>
<th>Rapeseed</th>
<th>Coffee</th>
<th>Bread</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose</td>
<td>17.8</td>
<td>9.6</td>
<td>-</td>
</tr>
<tr>
<td>Hemicellulose</td>
<td>14.8</td>
<td>34.8</td>
<td>-</td>
</tr>
<tr>
<td>Total soluble sugars</td>
<td>-</td>
<td>8.3</td>
<td>6.3</td>
</tr>
<tr>
<td>Proteins</td>
<td>34.1</td>
<td>14.1</td>
<td>12.3</td>
</tr>
<tr>
<td>Lipids</td>
<td>7.8</td>
<td>9.3</td>
<td>1.5</td>
</tr>
<tr>
<td>Starch</td>
<td>12.5</td>
<td>-</td>
<td>63.8</td>
</tr>
</tbody>
</table>

*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 3°C in medium with 4 g/L yeast extract, 1 g/L KH₂PO₄.
0.5 g/L MgSO₄·7H₂O, 1 g/L (NH₄)₂SO₄, 3 g/L peptone and 30 g/L glucose.

Inoculum
Every inoculum was prepared in Erlenmeyer flasks without air, in a Heidolph inkubator 1000 incubator, at 29°C, with an agitation of 144 rpm. S. cerevisiae was grown during, approximately, 12 hours.

Experimental conditions
A bioreactor (New Brunswick® BioFlo/CelliGen 115) with 2 L of volume was used to reproduce the results. Fermentations with commercial and in-house produced enzymes were also performed. 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. 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.

Chemical hydrolysis
0.6 M HCl concentration was used for the chemical hydrolysis in 1.5 L volume. The waste material and solution were poured into the 2 L bioreactor and autoclaved at standard conditions. The fermentation was performed with a temperature of 25°C, 200 rpm agitation and the pH was kept at 6, being regulated by 1 M HCl and 1 M of NaOH.

Commercial enzymes
The pH and temperature used in this work were based on the experiments obtained by Eng. Hlaváček [32] where the variation of pH and temperature were studied using paper waste adding commercial enzymes from the biomass kit Novozymes analysing which condition led to higher bioethanol concentration.

For the fermentation of rapeseed and coffee waste, 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).

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 40°C and agitation of 200 rpm. 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 amyloliquificaciens (Sigma-Aldrich, Denmark) was used. This fermentation started with the loading of the enzyme, the temperature was set to 65°C and the agitation speed was 300 rpm. These conditions were kept during 2 h, after this time, the temperature was change to 40°C and the inoculum was added.

In-house produced enzymes
Fermentations of waste products were also carried out using enzymes produced during this work. In this case, after inoculating the fermenter, 30 mL of the 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 40°C and the agitation at 200 rpm.

The enzymes were produced from a mold. The mold used was Phanerochaete chrysosporium CCM 8074, kept at 39°C 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 KH₂PO₄, 0.042 M MgSO₄ and 0.009 M CaCl₂ (Lach:Ner Czech Republic), in 1 L of distilled water. The medium was sterilised in an autoclave Systec V-95 at standard conditions (1 atmosphere and 115°C) for 15 minutes.

To use the mold, 10 mL of a spore suspension were taken into an Erlenmeyer flask with 150 mL 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 incubator 1000 incubator at 39°C and 144 rpm of agitation.

After the incubation period, the liquid was transferred to an Erlenmeyer flask and 1% (w/v) of carboximethyl 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.

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.

SDS-PAGE
The molecular weight of the proteins present in the in-house enzyme preparation was estimated by SDS-Page.

Determination of reducing sugars
The determination of the concentration of reducing sugars was done following the Somogyi-Nelson analysis [33].

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 60°C and 43 bar, was used to separate the glucose and the ethanol. The mobile phase consisted of a 5 mM H₂SO₄ 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.

The areas under these peaks can be converted into the amount of component using an analytical software (Chromleven 7.2). Raw samples from the fermentation were taken and centrifuged in a Hermle Labortecnik GmbH 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.

Calculations
For the ethanol production, the following equation 2.1, was used for the determination of the efficiency of sugars conversion to ethanol (η, %).

$$\eta \% = \frac{[\text{Ethanol}]_{\text{final}}}{\text{Ethanol theoretical value}}$$ 2.1
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 of cellulose, 1.11 g cellulose per g of polysaccharide [34], 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 [34], for a complete hydrolysis.

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.

\[ [\text{Polysaccharides}] = W(g) \times C_{cont} \times 1.1 \]  
\[ [\text{Polysaccharides}] = W(g) \times H_{cont} \times Hex_{cont} \times 1.1 \]  
\[ [\text{Polysaccharides}] = W(g) \times H_{cont} \times Pent_{cont} \times 1.36 \]

To obtain the theoretical ethanol production for each substrate content, the polysaccharides concentration times the ethanol theoretical value, 0.51 g/g [35] was calculated.

3 RESULTS AND DISCUSSION

Bioethanol production with chemical hydrolysis of raw wastes

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 1. 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.

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 maximum production of this fermentation 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) [15].

Coffee waste

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 [20], the digestion time of conventional coffee varies from 48 to 72 h, depending on temperature.

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 2 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.

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.

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 was very low, so it is possible to assume that further production of ethanol was not possible. 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.

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 0 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.

**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 4).

**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 5 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.

**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 6 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.

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.

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 [36]. 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 produced enzyme sample. Two bands of 41.7 and 106.6 kDa were detected.

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.

Rapeseed waste

Figure 7 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 24 h, when a residual concentration of glucose was reached. This behaviour was matched by the increase of ethanol concentration until 24 h of fermentation.

Table 2 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 [37]–[39]. 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.

**Coffee waste**

Figure 8 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.

According to the results obtained (Table 2), 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.

**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 9.
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 2). 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.

Comparison of the results obtained for the three hydrolysis used for the production of bioethanol

Table 2 summarises the results obtained with the three different hydrolysis tested for rapeseed, coffee and bread waste. The best efficiency obtained in the ethanol production was achieved when using chemical hydrolysis, with 21, 16.1 and 50.9% for the rapeseed, coffee and bread waste, respectively.

![Figure 9 Glucose consumption and ethanol formation during the 48 h fermentation using bread as substrate and hydrolysis with in-house produced enzymes.](image)

Table 2 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.

<table>
<thead>
<tr>
<th>Waste substrates</th>
<th>Chemical hydrolysis</th>
<th>Hydrolysis with commercial enzymes</th>
<th>Hydrolysis with in-house produced enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[Ethanol] (g/L)</td>
<td>η (%)</td>
<td>[Ethanol] (g/L)</td>
</tr>
<tr>
<td>Rapeseed</td>
<td>6.86</td>
<td>21.0</td>
<td>4.28</td>
</tr>
<tr>
<td>Coffee</td>
<td>7.42</td>
<td>16.1</td>
<td>3.04</td>
</tr>
<tr>
<td>Bread</td>
<td>30.6</td>
<td>50.9</td>
<td>26.8</td>
</tr>
</tbody>
</table>

Moreover, 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-hydroxymethylfurfural (HMF) or furfural can be formed from the dehydration of pentoses [41]. 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).

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 2). 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) [13].
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).

4 Conclusions

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 bio-refinery, 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. 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, where a noticeable shift in the sugar production rate was evident when the acid concentration increased from 0.3 M to 0.6 M HCl. Later on, this study was scaled-up to a 2 L bioreactor using 0.6 M HCl for the chemical hydrolysis.

ATTENDING TO THE THREE CONDITIONS STUDIED, CHEMICAL HYDROLYSIS, SIMULTANEOUS SACCHRIFICATION AND FERMENTATION USING A “COCKTAIL” OF CELLULOSE ENZYMES FROM NOVOZYMES, AMYLASE FROM SIGMA OR USING ENZYMES PRODUCED IN-HOUSE FROM THE MOLD P. CHRYSOSPORIUM, THE BEST RESULTS WERE OBTAINED FOR THE CHEMICAL HYDROLYSIS (HIGHER ETHANOL PRODUCTION). 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.

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. 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).

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 NOMENCLATURE

- **C**<sub>cont</sub> Cellulose content
- **H**<sub>cont</sub> Hemicellulose content
- **Hex**<sub>cont</sub> Hexose content
- **HMF** 5-hydroxymethylfurfural
- **HPLC** High performance liquid chromatography
- **η (%)** Efficiency of sugars conversion to ethanol
- **Pent**<sub>cont</sub> Pentose content
- **PTFE** Polytetrafluoroethylene

6 REFERENCES


