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Study of biological detoxification of lignocellulose hydrolysate

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Summary

Second-generation bioethanol is produced from non-food sources, usually from lignocellulosic biomass, which is made up of cellulose, hemicellulose and lignin. In order to produce bioethanol, the cellulase enzymes have to break down the cellulose into glucose, which can then be fermented. However, lignocellulosic biomass has a recalcitrant structure due to the crystallinity of cellulose. Hemicellulose and lignin also act as barriers preventing an easy access to cellulose for the enzymes. In order to facilitate cellulose accessibility, a pretreatment is applied to the biomass. Although there are multiple different pretreatments available, this thesis focuses on steam explosion pretreatment. By subjecting the biomass to a sudden change in pressure, cellulose surface area available for enzymes to convert increases. However, a number of different inhibitors is also produced during this process, which hinder the efficiency of the following enzymatic hydrolysis and fermentation steps. These inhibitors have to be removed, or at least their concentrations have to be lowered, in order to achieve an economically viable yield of bioethanol. The goal of this thesis was to evaluate the changes in the concentration of inhibitors, as well as sugars, present in lignocellulosic biomass that had undergone a steam explosion pretreatment.

Three different experiments were carried out in order to determine the effect of the addition of no enzyme, only cellulase, only laccase or both cellulase and laccase to the liquid fraction, the solid fraction and both fractions together of the biomass left over after the steam explosion. Samples were taken daily over a period of five consecutive days and concentrations of glucose, xylose, formic acid, acetic acid, 5-hydroxymethylfurfural, furfural, 3,4-dihydroxybenzaldehyde, 4-hydrobenzoic acid, vanillic acid, syringic acid, vanillin and syringaldehyde were measured by HPLC.

The highest sugar concentrations were achieved when only the cellulase was present, as the enzyme aided the breakdown of cellulose and hemicellulose. Lower sugar concentrations yielded when cellulase was combined with laccase, point to laccase having an inhibitory effect on cellulase activity. As expected, neither the addition of only laccase or the absence of enzymes resulted in an increase in sugar concentration. In addition, the lowest inhibitor concentrations were achieved when only the solid fraction of pretreated biomass was present. Finally, the detoxifying effect of laccase was more significant when only the liquid fraction of pretreated biomass was present.

Samenvatting

Tweede generatie bio-ethanol wordt geproduceerd uit non-food bronnen, meestal uit lignocellulosische biomassa, die bestaat uit cellulose, hemicellulose en lignine. Om bio-ethanol te produceren, moeten de cellulase-enzymen de cellulose afbreken tot glucose, die vervolgens kan worden gefermenteerd. Lignocellulosische biomassa heeft echter een recalcitrante structuur vanwege de kristalliniteit van cellulose. Hemicellulose en lignine fungeren ook als barrières die een gemakkelijke toegang tot cellulose voor de enzymen verhinderen. Om de toegankelijkheid van cellulose te vergemakkelijken, wordt een voorbehandeling toegepast op de biomassa. Hoewel er meerdere verschillende voorbehandelingen beschikbaar zijn, richt dit proefschrift zich op stoomexplosievoorbehandeling. Door de biomassa te onderwerpen aan een plotselinge drukverandering, neemt het celluloseoppervlak dat beschikbaar is voor enzymen om te converteren toe. Tijdens dit proces worden echter ook een aantal verschillende remmers geproduceerd, die de efficiëntie van de volgende enzymatische hydrolyse- en fermentatiestappen belemmeren. Deze remmers moeten worden verwijderd, of in ieder geval hun concentraties moeten worden verlaagd, om een economisch haalbare opbrengst van bio-ethanol te bereiken. Het doel van dit proefschrift was om de veranderingen in de concentratie van remmers, evenals suikers, aanwezig in lignocellulosische biomassa die een stoomexplosie voorbehandeling hadden ondergaan, te evalueren.

Drie verschillende experimenten werden uitgevoerd om het effect te bepalen van de toevoeging van geen enzym, alleen cellulase, alleen laccase of zowel cellulase als laccase tot de vloeibare fractie, de vaste fractie en beide fracties samen van de biomassa die overblijft na de stoomexplosie. Gedurende een periode van vijf opeenvolgende dagen werden dagelijks monsters genomen en concentraties glucose, xylose, mierenzuur, azijnzuur, 5-hydroxymethylfurfural, furfural, 3,4-dihydroxybenzaldehyde, 4-hydrobenzoëzuur, vanillinezuur, syringinezuur, vanillinezuur, vanilline en syringaldehyde werden gemeten met HPLC.

De hoogste suikerconcentraties werden bereikt wanneer alleen de cellulase aanwezig was, omdat het enzym hielp bij de afbraak van cellulose en hemicellulose. Lagere suikerconcentraties die ontstonden wanneer cellulase werd gecombineerd met laccase, wijzen erop dat laccase een remmend effect heeft op de cellulase-activiteit. Zoals verwacht resulteerde noch de toevoeging van alleen laccase, noch de afwezigheid van enzymen in een toename van de suikerconcentratie. Bovendien werden de laagste inhibitorconcentraties bereikt wanneer alleen de vaste fractie van voorbehandelde biomassa aanwezig was. Ten slotte was het ontgiftende effect van laccase belangrijker wanneer alleen de vloeibare fractie van voorbehandelde biomassa aanwezig was.

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Abbreviations

LB	Lignocellulosic biomass
XRF	Xylose Rich Fraction
SF	Solid Fraction
MM	Mineral Medium
5-HMF	5-Hydroxymethylfurfural

Glossary

Enzymes: Biological catalysts that speed up chemical reactions without getting used up in the process.

List of figures and tables

Chapter 1: Introduction

Despite the steady increase in the use of renewable energy resources around the world such as hydroelectric or solar energy, 84% of world energy consumption in 2019 was supplied by fossil fuels [1]. This does not present a sustainable situation, as fossil fuels are due to run out in the not so distant future. Due to the fact that economic development heavily depends on having a reliable source of energy available, alternative renewable energy sources are necessary [2], making biofuels the logical alternative to turn to. Biofuels are produced from plants or crops which are often recycled. They offer a reliable source of energy at a much lower environmental impact.

The most common biofuel in use is bioethanol. Some of the advantages of bioethanol include reduced smog formation, reduced toxicity for humans and 80% less carbon emission [2]. Bioethanol is produced from the fermentation of sugars. If these sugars come from an edible biomass source, as is more common, then the bioethanol produced is known as first-generation bioethanol. However, the ethical debate surrounding the use of potential food for fuel, puts the focus on the alternative second-generation bioethanol, which is produced from lignocellulosic biomass such as wood.

The recalcitrant structure of lignocellulosic biomass hinders the production of second-generation bioethanol, and forces the need of having a pretreatment to enable a more efficient breakdown of the cellulose found in the cell wall of plants into sugars. These sugars can then be used in the fermentation for bioethanol production [3]. However, inhibitors formed during the pretreatment step lower the efficiency of the whole process and therefore, have to be either eliminated or reduced to a minimum in order for second-generation bioethanol to be an economically feasible fuel.

Chapter 2: Literature

2.1 Bio-ethanol production process

2.1.1 Lignocellulosic biomass

Lignocellulosic biomass (LB) consists of cellulose, hemicellulose and lignin in different ratios. Specifically, for poplar wood, LB is made up of approximately 50% cellulose, 30% hemicellulose and 20% lignin [4].

Cellulose is the most abundant polymer present in lignocellulosic biomass. It is a polysaccharide which consists of linear chains of glucose subunits linked together by β-1,4-glycosidic bonds and has cellobiose as its repeating unit, as shown in Figure 2.1. Polymer chains are linked to each other with strong hydrogen bonds. The longer the chain, the more hydrogen bonds present and, in consequence, the more difficult it is to hydrolyse [5]. These intramolecular bonds account for cellulose being insoluble in water as well as in dilute acidic and dilute alkali solutions at room temperature [6]. Cellulose molecules also have a tendency to form intermolecular hydrogen bonds. This encourages the formation of microfibrils which in turn form aggregates, creating a crystalline structure responsible for the strength that characterises cellulose. This strength is necessary, as the main function of cellulose in plant cells is to provide mechanical support to the plant by keeping the cell wall rigid.

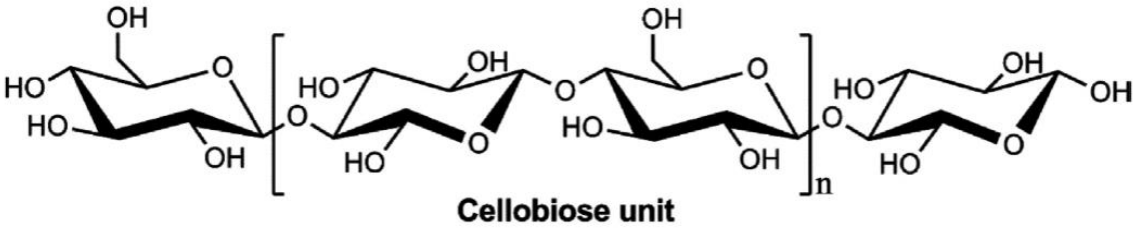

Figure 2.1: The chemical structure of cellobiose [7].

On the other hand, hemicellulose has an amorphous structure which grants it little strength compared to cellulose. Hemicellulose consists of various linear and/or branched polysaccharides made up of pentose and hexose monosaccharide units such as xylose, glucose, mannose, galactose and arabinose [6]. The exact ratio of the different monosaccharides is not fixed and varies depending on the origin of the biomass. Hemicellulose has a lower degree of polymerization than cellulose. In consequence, it is easily dissolved in dilute acidic and dilute alkali solutions [7]. Hemicellulose interacts with cellulose in such a way as to help strengthen the cell wall but it also limits the access of enzymes to the cellulose.

Finally, lignin is the third component of lignocellulosic biomass. It is a polyphenolic substance derived from three phenylpropanoid units named monolingols: p-coumaryl, coniferyl, and sinapyl alcohol. These units link together through ester bonds to produce the monomers syringyl (S), guaiacyl (G), and p-hydroxyphenyl (H). The amount of lignin present in lingocellulosic biomass is directly related to its recalcitrance [8] as it acts like a barrier blocking access to the cellulose for the enzymes. Lignin further discourages cellulose breakdown by absorbing some cellulase during enzymatic hydrolysis because of its ability to form hydrogen bonds. Lignin is also accountable for hydrophobicity of the interior of the

cell wall and influences its strength, as it binds the hemicellulose and cellulose together, providing further rigidity.

The structure of lingocellulosic biomass is shown in Figure 2.2.

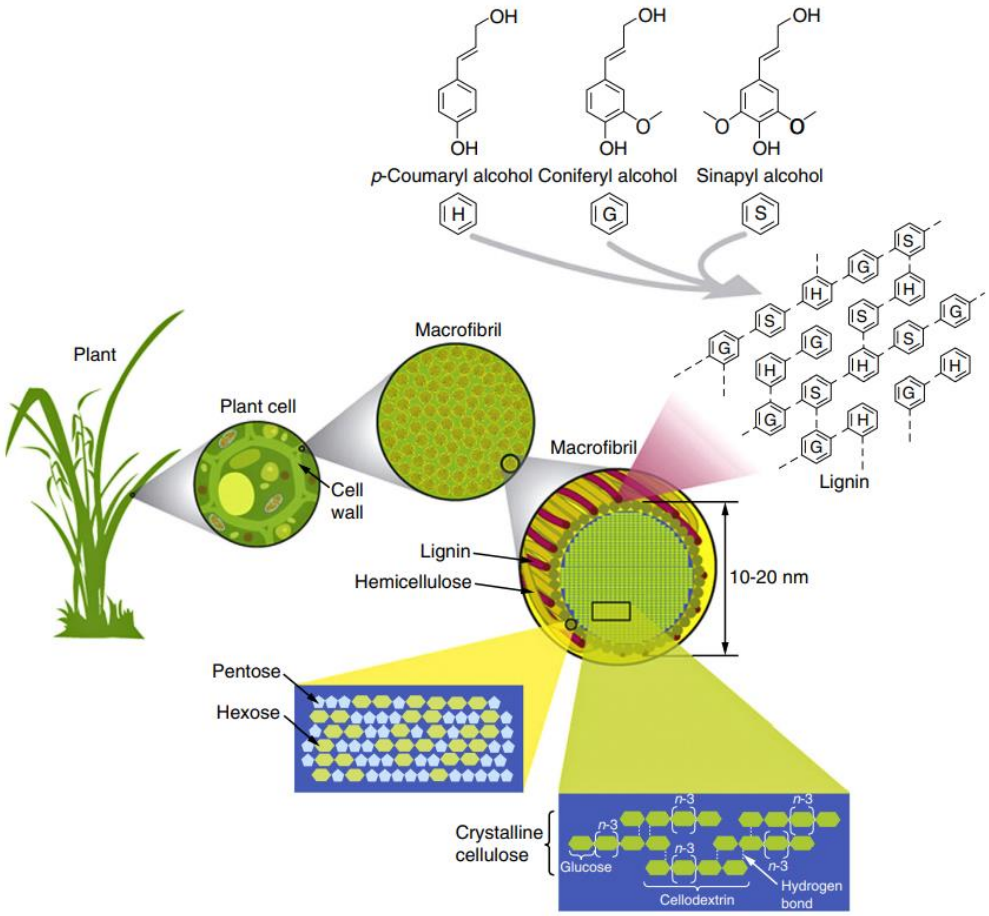

Figure 2.2: Structure of lignocellulosic biomass, lignin, hemicellulose and cellulose [9].

2.1.2 Pretreatment

As mentioned above, the lignin and hemicellulose components in lignocellulosic biomass limit the access of enzymes to the cellulose. Pretreatment is needed to facilitate these enzymes accessing the cellulose as well as the hemicellulose fractions, in order to overcome biomass recalcitrance. This is achieved by lowering the degree of polymerisation and the crystallinity index, increasing the porosity and breaking lignin-carbohydrate linkages [10]. An increase in the amount of cellulose surface area available will increase the concentration of sugars produced in enzymatic hydrolysis, which will in turn increase the overall yield and efficiency of bioethanol produced in fermentation. The effects of pretreatment on the lignocellulosic biomass are represented in Figure 2.3.

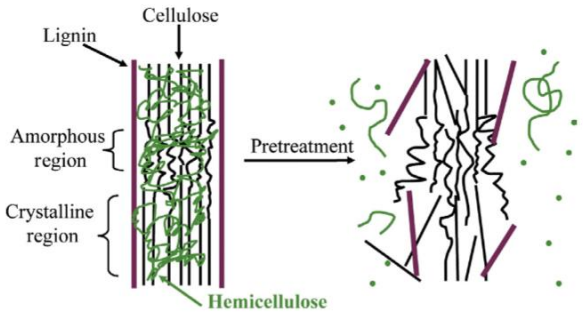

Figure 2.3: The effects of pretreatment on lignocellulosic biomass [11].

However, toxic compounds are also formed during pretreatment due to the harsh conditions the lignocelllosic biomass is subjected to. The presence of these toxic compounds, which will be discussed further in section 2.2, means that a compromise has to be reached during pretreatment between increasing enzyme access to cellulose and hemicellulose and keeping the concentration of inhibitors to a minimum.

There are a number of different pretreatments that have been developed over the last years. These pretreatments are often classified into biological, physical, chemical, and physicochemical pretreatments. The pre-treatment chosen will have significant repercussion on the following enzymatic hydrolysis and fermentation steps, as it will affect the concentration of toxic compounds present [10]. For this thesis, the focus was put on steam explosion pretreatment which falls under the category of physicochemical pretreatment.

Steam explosion pretreatment consists of applying hot steam of up to approximately 240ºC [12] to the biomass under high pressure for a set time and then quickly decompressing the biomass back to atmospheric pressure. This sudden pressure change disrupts the structure of the biomass. In addition, acetyl groups present in hemicellulose undergo autohydrolysis due to the high temperatures, forming acetic acid. The combination of the mechanical forces and chemical effects [10], redistributes and partially removes the lignin and hemicellulose in the biomass, increasing cellulose accessibility for the enzymes.

Steam pretreatment results in biomass being separated into two distinct fractions: a liquid and a solid fraction. The liquid fraction is known as the xylose rich fraction (XRF) and is largely made up of monomeric sugars derived from hemicellulose while the remaining cellulose and lignin make up the solid fraction.

Depending on the particle size and the exact temperature and time, the effects of steam pretreatment can range from small cracks to total defibrillation [12]. The severity factor (R_0) is used to compare the effects between steam pretreatments where different temperatures and times have been used, and it is given by the following expression:

$$\log R_0 = \log(t \cdot e^{(\frac{T-100}{14.75})})$$

Where t is time in minutes and T is temperature in degrees Celsius. A high severity factor means more hemicellulose has been removed and the degree of polymerisation of cellulose has decreased, allowing for easier enzymatic access to the cellulose for degradation. However, it also means a higher concentration of inhibitors is formed from increased sugar degradation. Therefore, a balance between

decreasing the recalcitrance of biomass and keeping the concentration of inhibitors low, has to be taken into account when establishing the conditions for the steam pretreatment.

One of the main advantages of steam pretreatment over other pretreatment methods, is that no chemicals are needed, only water. This way, less economic investment is needed in security measures corresponding to chemical hazards, the environmental impact is lower and the equipment needed to carry out the pretreatment is simpler and therefore requires less investment. Heat generated during the pretreatment can also be used to improve overall energy efficiency of the process.

2.1.3 Enzymatic hydrolysis

The second step in the production of bioethanol is an enzymatic hydrolysis where the soluble enzymes have to be absorbed into the surface of an insoluble substrate. A high concentration of sugars produced during enzymatic hydrolysis is the prerequisite to obtaining a high yield of bioethanol in the final fermentation step.

As mentioned above, enzymatic hydrolysis is heavily and negatively influenced by the degree of polymerisation and crystallisation of cellulose and hemicellulose, as those parameters determine the area available for cellulase enzymes to hydrolyse cellulose into monosaccharides (hexoses and pentoses) such as glucose and xylose. This makes enzymatic hydrolysis a generally slow process, as the number of active sites available for the enzymes to bind to are limited [13]. Lignin content also impacts the rate and extent of the hydrolysis, as lignin acts like a barrier which prevents cellulase from reaching the cellulose. The enzymatic hydrolysis is only performed after the pretreatment in order to have a biomass with a lower polymerisation and crystallisation degrees and lower in lignin and hemicellulose content for enzymes to act upon.

2.1.3.1 Cellulase enzymes

Cellulase enzymes can be classified into three major categories, which are endo-1,4-β-glucanases (EG), exo-1,4-β-glucanases/cellobiohydrolases (CBH) and β-glucosidases (BGL). These three types of cellulase enzymes have to work synergistically in order to fully hydrolyse cellulose [14].

EG cuts the cellulose chain at random points, creating new reducing and non-reducing chain ends which are then attacked by the CBH. CBH catalyses the liberation of cello-oligosaccharides and cellobiose by acting upon these new chain ends. Finally, the BGL aids in the breakdown of cellobiose into glucose [14].

In addition to this, hemicellulase activities are also present in the cellulase complex, in order to catalyse the liberation of xylose from hemicellulose. The main components in hemicellulase are endoxylanase, which is involved in the breakdown of xylan in order to produce xylo-oligosaccharides, and β-D xylosidase, which is responsible for the hydrolysis of xylo-oligosaccharides into xylose [15].

2.1.4 Fermentation

Fermentation is the process by which microorganisms break down sugars such as glucose into ethanol and carbon dioxide in the absence of oxygen. This step can be carried out right after the enzymatic hydrolysis takes place or it can be carried out simultaneously to the hydrolysis, which is the preferred method [16] as it reduces the time needed for the whole process as well as the risk of contamination. However, optimal temperature and pH values might differ for hydrolysis and fermentation.

Different microorganisms can be used for fermentation, but *Saccharomyces cerevisiae* is the most common one due to its high tolerance to inhibitors and high ethanol concentration [16].

One of the main problems encountered during fermentation is the presence of inhibitors derived from the pretreatment. These inhibitors can not be degraded into ethanol and therefore may lower the rate of fermentation and economic feasibility of the process.

2.2 Inhibitors formed during pretreatment

As mentioned above, harsh conditions during pretreatment lead to a partial hemicellulose and lignin degradation and generation of toxic compounds. These toxic compounds negatively affect the enzyme hydrolysis and fermentation steps, lowering the yield of bioethanol produced and therefore hindering economic viability of producing second generation bioethanol.

The kind and concentration of inhibitors formed, not only depends on the pretreatment method and its conditions, but it is also significantly affected by the composition of the lignocellulosic biomass. This is because different types of biomass have different ratios of cellulose, hemicellulose and lignin. As most organic inhibitors derive from hemicellulose and lignin, [3] the concentration of these two substances in the biomass used is particularly important when determining the amount of inhibitor that will be formed.

2.2.1 Classification of inhibitors

Inhibitors formed during pretreatment can be classified into three main categories: short-chain organic acids and aldehydes, sugar-derived aldehydes, and aromatic compounds [3], as shown in Figure 2.4.

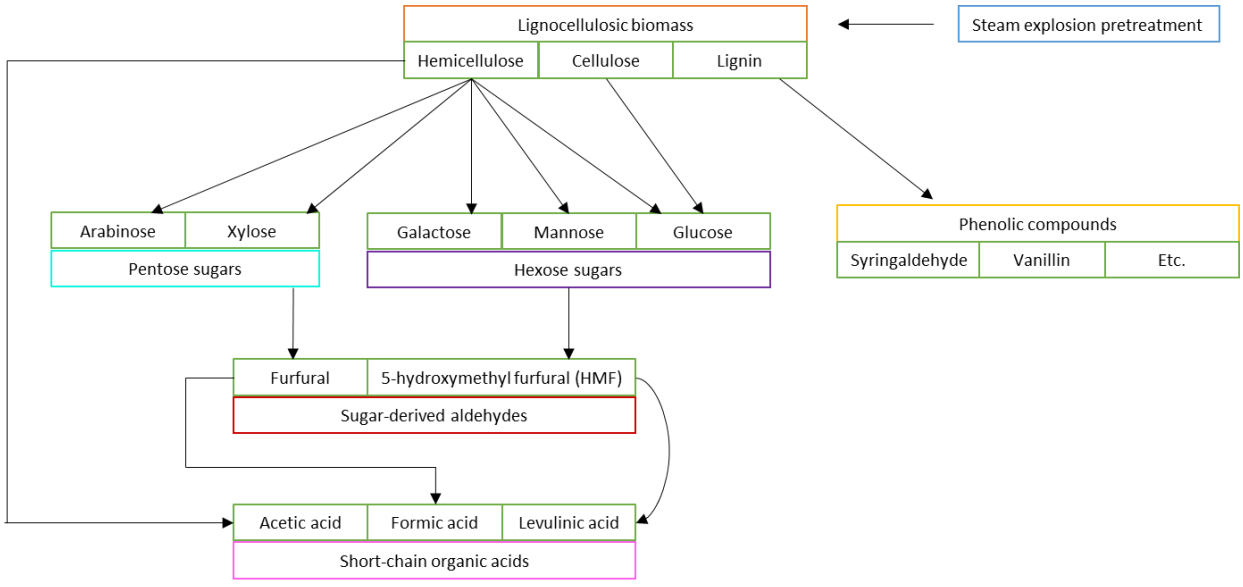

Figure 2.4: Origins and classification of lignocelluosic inhibitors.

2.2.1.1 Sugar-derived aldehydes

Sugar-derived aldehydes come from the degradation of hexose and pentose sugars found in hemicellulose and cellulose.

An example of a sugar-derived aldehyde is furfural. Furfural is formed from the degradation of pentose sugars such as xylose and arabinose which are found in hemicellulose, as shown in Figure 2.5. The formation of this inhibitor is favoured by acidic conditions, high water concentration and a temperature of under 150ºC in the pretreatment, as well as the dehydration reaction [3]. Furfural influences the fermentation step negatively, as it inhibits glycolytic enzymes which are essential for ATP production. A decrease in the yield of ATP, hinders yeast growth and in consequence bioethanol yield decreases. The concentration of reactive oxygen species (ROSes) in yeast is also affected by the presence of furfural, which encourages its build up, damaging cells and hampering yeast survival and reproduction [3].

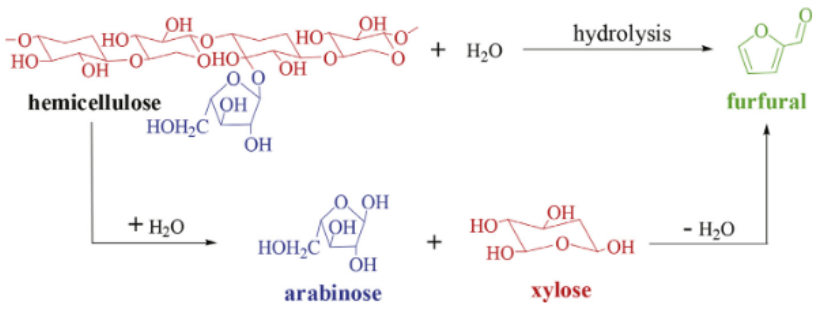

Figure 2.5: Hydrolysis of hemicellulose into furfural [17].

The formation of 5-hydroxymethylfurfural (5-HMF) is favoured under the same conditions as those described for furfural and its effects on the fermentation are similar too. The difference is that 5-HMF is derived from the degradation of hexoses such as galactose, mannose and glucose, as shown below in Figure 2.6.

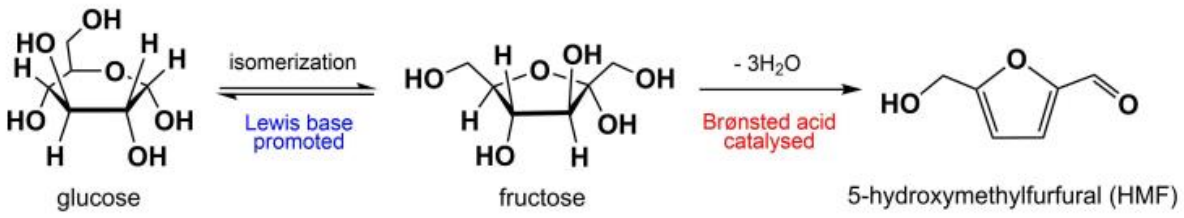

Figure 2.6: Hydrolysis of glucose to 5-hydroxymethylfurfural [18].

2.2.1.2 Aromatic compounds

Inhibitors classified under this group contain a benzene ring in their structure. They can be further classified into three subgroups: phenolic compounds, non-phenolic compounds and benzoquinones [3]. In this thesis we will focus on phenolic inhibitors such as syringaldehyde, syringic acid, vanillin,

vanillic acid, 4-hydroxybenzoic acid, 3,4-hydroxybenzaldehyde and 4-hydroxybenzaldehyde which originate from the lignin fraction of lignocellulosic biomass.

Aromatic compounds inhibit both the enzymes in the enzymatic hydrolysis and the microorganisms in the fermentation step [3]. They form an aromatic compounds' complex around cellulase, which causes cellulase to precipitate and deactivate, no longer being able to hydrolyse the pretreated biomass. Furthermore, aromatic compounds have hydrophobic properties which allow them to destabilise the integrity of the cell walls in microorganisms, inhibiting their role in fermentation [3].

2.2.1.3 Short-chain organic acids

Short-chain organic acids mainly inhibit microorganisms during the fermenting stage [3]. They derive mostly from the hemicellulose and lignin in lignocellulosic biomass. Examples of short-chain organic acids are formic, acetic and levulinic acids.

Acetic acid mostly comes from the degradation of acetyl-groups in the hemicellulose and lignin fraction. Therefore, the concentration of inhibitor depends on the type of biomass. A lignocellulosic biomass that is more acetylated, will result in a higher concentration of acetic acid. Acetic acid concentration also increases with an increase in the temperature and duration of the pretreatment applied to lignocellulosic biomass but is independent of the chemicals used. While acetic acid does not affect cellulase activity during enzymatic hydrolysis, it does inhibit nutrient uptake of *Saccharomyces cerevisiae* [3], affecting the fermentation stage.

In addition, the concentration of formic acid increases with increased temperature and time of the pretreatment in a similar way to acetic acid. This inhibitor is derived from degradation of furfural and 5-HMF. The degradation of 5-HMF also gives rise to levulinic acid, whose formation is incentivised by acidic conditions during pretreatment.

All short-chain organic acids mentioned above are weak acids with low values of the equilibrium constant pKa. A low pKa means the acids can donate their protons more easily. As mentioned above, short-chain organic acids mainly inhibit microorganisms. This is due to the fact that they can diffuse through cell membranes in their undissociated form and start to dissociate once inside, where the pH is approximately 7. As the hydrogen ions accumulate inside de cells, microorganisms use up energy to pump them out of the cells with the aim of keeping a neutral pH. The energy used up in this process is not being used for growth and, therefore, the bioethanol production will not be as efficient [3].

2.3 Detoxification

As discussed in the sections above, the presence of inhibitors hinders the efficiency of bioethanol production. In order to make second generation bioethanol competitive against first generation bioethanol, the lignocellulosic hydrolysate has to be detoxified. There are multiple detoxification methods such as filtration of inhibitors, absorption of inhibitors, chemical degradation or extraction to name a few [3]. However, in this thesis the focus is going to be put on biological detoxification.

Biological detoxification can be carried out using either enzymes or microbes. Enzymatic biological detoxification is the chosen method of detoxification for this thesis. Nevertheless, it is worth mentioning the importance of the role of *Rhodococcus opacus* in detoxification and lignin valorisation due to its multiple catabolic pathways for aromatic compounds and sugars.

The extent to which the selected detoxification method manages to remove inhibitors, is determined by the properties of the inhibitor such as its size, polarity, chemical reactivity and concentration [3].

2.3.1 Laccase in biological detoxification

Biological detoxification, also known as bioabatement, consists in using enzymes such as laccases to detoxify the lignocellulosic hydrolysate. Laccase is a copper-containing oxidase which polymerises phenolic inhibitors into harmless oligomers/polymers, thus detoxifying the hydrolysate. On the other hand, the use of laccase to detoxify also results in a decrease in the yield of bioethanol as some glucose is consumed during incubation, leading to lower sugar production [19]. Therefore, detoxification via laccase should be carried out after the enzymatic hydrolysis [3].

Chapter 3: Material and methods

3.1 Measurement of cellulase activity

In order to determine the activity of the cellulase enzyme, a calibration curve was needed. 3,5-Dinitrosalicylic acid (DNS) reagent was used as an indicator. DNS reagent reacts in the presence of reducing sugars (sugars with a free aldehyde or ketone group) and heat. The nitro groups in DNS molecules are reduced to amine groups, forming 3-amino-5-nitrosalicylic acid. The colour of the solution changes from yellow to orange. The intensity of this change in colour depends on the concentration of reducing sugars present. Therefore, absorbance was measured to determine the concentration of reducing sugars and subsequently, enzyme activity was calculated. A higher enzyme activity would mean an increase in the degradation of cellulose present in plant cells and in consequence, a higher concentration of reducing sugars, which would lead to a higher colour intensity and higher absorbance values.

3.1.1 Citrate buffer

The citrate buffer was prepared by dissolving 210.0503 g of citric acid monohydrate in 750 ml of demineralised water. The 1M solution was then made up to 1 L in a volumetric flask. The pH was measured to be aproximately 1 and therefore, pellets of NaOH were added until the pH reached 4.5. Then, the buffer was diluted until the desired value of 0.05M and the pH was measured to be 4.8.

3.1.2 DNS reagent

For the preparation of the DNS reagent, 1.87 g of 3,5-Dinitrosalicylic acid were dissolved in 75 ml water, with the help of a magnetic stirring bar and a heating plate at a temperature of 50°C in order to speed up the rate at which the acid dissolved. This solution was bright yellow in colour.

Separately, 3.5 g NaOH were dissolved in 75 ml demineralised water. When all the NaOH dissolved, 53.9 g of sodium potassium tartrate tetrahydrate 1.46 g of sodium disulfite and 1.45 g of phenol where added to the solution. This was all mixed under a fume hood in order to minimise the phenol vapours produced, as phenol is a toxic substance. Thicker gloves were also used in order to minimise the risk of any phenol coming in contact with the skin.

Finally, both solutions were mixed together and brought up to 250mL in a volumetric flask. The resulting solution turned bright orange in colour. The volumetric flask was covered in an aluminium foil due to its light sensitivity and stored in the fridge until further use.

The exact amounts of each component used are listed in Table 3.1.

Table 3.1: Mass of components used in the preparation of DNS reagent.

	Mass (g)
3,5-Dinitrosalicylic acid	1.8700
NaOH	3.5790
Sodium potassium tartrate tetrahydrate	53.9086
Sodium disulfite	1.4611
Phenol	1.4550

3.1.3 Filter paper assay for cellulose saccharification

3.1.3.1 Enzyme assay tubes

The cellulase activity was determined in accordance to the National Renewable Energy Laboratory method [20]. Firstly, 6 Whatman N0.1 filter paper strips each weighing 50 mg were placed inside six different test tubes. 0.1 mL of the 0.05M citrate buffer was added to each test tube and the test tubes were warmed up to 50ºC.

0.5mL of diluted enzyme solution was added to each test tube. The diluted enzyme solution was prepared by firstly diluting the enzyme solution by a factor of 50. In order to achieve this, 0.2mL of enzyme solution were added to a 10mL volumetric flask and bought up with demineralised water. Then, and a second dilution was carried out following the dilution factors shown in Table 3.2.

Table 3.2: Enzyme dilutions.

Dilution from original enzyme solution	**Dilution factor from 1:50 diluted solution**	**1:50 diluted enzyme solution (ml)**	**0.05M citrate buffer (ml)**
100	2	0.30	0.30
400	8	0.10	0.70
800	16	0.10	1.50
1200	24	0.05	1.15
1500	30	0.05	1.14

3.1.3.2 Blank and control

A reagent blank was also made using only 1.5 ml of citrate buffer. In addition, a substrate control was made using 1.5 ml of citrate buffer and the 50 mg filter paper strip but no enzyme solution. This is used to confirm that any glucose present when measuring the dilutions containing enzymes is due to the enzyme solution and not due to the filter paper.

3.1.3.3 Glucose standards

Glucose standards were made by diluting a 10 mg/ml solution of anhydrous glucose, which was made up by dissolving 1 g of glucose in 100 ml of demineralised water. Glucose standard dilutions are shown in Table 3.3. 0.5 ml of each glucose dilution were added to 1ml of citrate buffer in a test tube.

Table 3.3: Dilution of glucose standards for the construction of the standard curve.

Glucose solution (ml)	Citrate buffer (ml)	Dilution	Dilution concentration (mg/0.5ml)
1	0.5	1:1.5	3.35
1	1.0	1:2	2.50
1	2.0	1:3	1.65
1	4.0	1:5	1.00

All test tubes containing glucose standards, blanks, control and enzymes were left for 60 minutes in a water bath at 50ºC. Once the one hour was over, 3 ml of DNS reagent were added to the test tubes in order to stop the reaction.

3.1.3.4 Colour development

All test tubes were boiled for 5 minutes and then transferred to an ice-water bath. After the pulp had settled, all 11 test tubes (one blank, one substrate control, 5 diluted enzyme solution and 4 glucose standards for calibration curve) were diluted by adding 0.2 ml of test tube content and 2.5 ml of demineralised water in a spectrophotometer cuvette.

The absorbance of every test tube was measured with a spectrophotometer against the reagent blank at 540 nm.

3.2 Measurement laccase activity

Laccase activity was determined using absorbance values and the Beer-Lambert law:

$$A = \varepsilon \cdot l \cdot c$$

Where A is the absorbance, c is the concentration, l is the optical path length in cm (1 cm) and ε is the molar attenuation coefficient.

The substrates used were syringaldazine and ABTS. Absorbance for syringaldazine was measured at 530 nm and for ABTS at 420 nm. The ε coefficients were 50000 1/(M·cm) for Syringaldazine and 36000 1/(M·cm) for ABTS.

The specific enzyme used was Laccase from *Trametes versicolor*. An enzyme solution of 10mg/100mL was prepared.

3.2.1 Laccase activity with ABTS as a substrate

A 0.25 mM solution of ABTS was made up by dissolving 137.2 mg of ABTS in a 1 L volumetric flask.

2 cuvettes were prepared, one containing the blank and the other containing the sample. Both had 2.2 ml of buffer solution but 0.3 ml of ABTS solution were then added to the sample and not to the blank.

Once the cuvettes were placed inside of the spectrophotometer, 0.5 ml of enzyme solution was added to both cuvettes (so that the total volume in each cuvette was 3 ml) and the spectrophotometer was immediately run for 10 minutes. This was done in order to start measuring absorbance as soon as the reaction started. The procedure was repeated twice more.

3.2.2 Laccase activity with syringaldazine as a substrate

A 0.25 mM solution was made up by dissolving 9.009 mg in a 1L volumetric flask of Methanol. However, in this case, Methanol and not demineralised water was used to bring the volume up to 1L.

Just as with ABTS, 2 cuvettes were prepared, one blank and one sample. Both had 2.2 ml of buffer solution but 0.3 ml of syringaldazine solution were then added to the sample and not to the blank. The blank contained 0.3 ml of methanol.

Once the cuvettes were placed inside of the spectrophotometer, 0.5 ml of enzyme solution were added to both cuvettes and the spectrophotometer was immediately run for 10 minutes at 530 nm.

3.3 Detoxification of the liquid fraction

The goal in this first experiment was to detoxify the liquid xylose rich fraction (XRF) leftover from the steam explosion and to observe the effects two different enzymes (cellulase and laccase) have on this fraction. For this, 12 Erlenmeyers were set up. In the first three, only mineral medium (MM) was added to the Erlenmeyer containing the XRF. Three other Erlenmeyers contained MM and cellulase, three had MM and laccase and the final three Erlenmeyer flasks contained XRF, MM, and both cellulase and laccase.

3.3.1 Mineral medium

The mineral medium was made up by dissolving the exact amount of each component indicated in Table 3.4 in demineralised water in a 1 L volumetric flask with 2 ml of Hoagland solution.

Table 3.4: Composition of the mineral medium used in experiment 1.

Component	Mass (g)
$NaH_2PO_4 \cdot 2H_2O$	4.4748
KH_2PO_4	1.5031
$CaCl_2 \cdot 2H_2O$	0.0350
$MgSO_4 \cdot 7H_2O$	0.2002
$FeNH_4 - citrate$	0.0016
NH_4Cl	1.0003
$NaHCO_3$	0.5034

The pH was measured and adjusted to 4.5 using NaOH pellets.

3.3.2 Xylose rich fraction (XRF)

The Xylose rich fraction (XRF) used in this experiment corresponds to the reference numbers 1703, 1705, 1710, 1712.

The pH of the liquid XRF was adjusted to 7.3 using NaOH pellets. This pH is not optimum for cellulase activity but it corresponds to the ideal pH for the microorganisms used in the original experiment and therefore, it was kept the same. The actual pH reached was 7.28.

Sterilization of the XRF was done through filtration, as doing so with heat or UV light may have provided the molecules with enough energy to reach their activation energy, changing the molecular structure of hemicellulose. The filter used was a 0.45 µm membrane. The solution was left to filter overnight but as barely any solution was filtered, it was decided to filter first through a 47 µm, 30 µm, 11 µm, 2 µm filter papers and then through the 0.45 µm membrane. However, the filtering through the last membrane was still quite slow, which indicates that many of the particles present in the XRF solution had a particle size between 2 and 0.45 µm.

3.3.3 Laccase solution

0.0104 g of Laccase from *Trametes versicolour* were added to the 0.5M citrate buffer previously prepared as explained in title 2.1.1. This solution was then filtered through a 0.22 µm membrane using a syringe into a sterile Falcon tube and under the biosafety cabinet.

3.3.4 Cellulase solution

Cellulase (Genencor, Viscamyl flow) was filter sterilised through a 0.22 µm membrane in the same way as the Laccase solution.

3.3.5 Setup for experiment 1

The total volume of the 12 Erlenmeyers in experiment 1 was 50 ml. This corresponds to half the volume of the Erlenmeyers in the original experiment. The volumes of all components were halved due to the limited amount of XRF available.

20.205 ml of XRF were added to the 12 Erlenmeyers under the biosafety cabinet. This corresponds to the optimal amount (40.41%) of XRF as found in the master thesis of Jeroen Van Cleef [21].

Then, the volumes specified in Table 3.5 were added. In total, there were 4 different setups and 3 Erlenmeyers with the same proportion of components for each setup.

Table 3.5: Content of Erlenmeyers in experiment 1.

	Component	Volume (mL)
Erlenmeyers 1-3	XRF	20.205
	Mineral medium (MM)	29.795
Erlenmeyers 4-6	XRF	20.205
	MM	28.660
	Cellulase	1.135
Erlenmeyers 7-9	XRF	20.205
	MM	25.250
	Laccase	4.545
Erlenmeyers 10-12	XRF	20.205
	MM	24.115
	Cellulase	1.135
	Laccase	4.545

Samples were taken of every Erlenmeyer and placed in an Eppendorf immediately after mixing all corresponding components. Samples continued to be taken daily for the following 5 days.

3.4 Detoxification of the solid fraction

In this second experiment, the aim was to detoxify the solid fraction left over after the steam explosion pretreatment, and to observe the effects of cellulase and laccase in the same way as in the detoxification of the liquid fraction. The solid used had reference number 1729 and its severity factor for the steam explosion was 3.838.

3.4.1 Washing of the solid fraction for experiment 2

As mentioned in chapter 2, the steam explosion pretreatment is applied to the wood with the aim of facilitating the access of enzymes to the cellulose in its cells. It does this by creating pores and therefore increasing the surface area. Some of the liquid fraction (XRF) was still trapped in the pores of the solid sample, so the solid had to be washed in order to get rid of this XRF because in this experiment, there must be no XRF present. The washing of the solid fraction was carried out as described in the original experiment.

5 g of solid fraction were placed into 12 Falcon tubes. The exact amount in each is shown below in Table 3.6. When weighing in the balance, measurements were very unstable, as they kept decreasing slowly. This was due to the fact that the solid fraction had been unfrozen not long ago and some water was possibly still evaporating. Therefore, the weights shown below are the weights at which the measurement started to decrease.

Table 3.6: Weight of the solid fraction present in each falcon tube in experiment 2.

	Weight (g)		Weight (g)
Falcon 1	5.0010	Falcon 7	5.0037
Falcon 2	5.0012	Falcon 8	5.0044
Falcon 3	5.0058	Falcon 9	5.0038
Falcon 4	5.0047	Falcon 10	5.0071
Falcon 5	5.0071	Falcon 11	5.0068
Falcon 6	5.0081	Falcon 12	5.0005

Next, 40 ml of Mineral Medium were added to each falcon tube. The tubes were then shaken and centrifuged at 4500 g (acceleration 9, break 5) for 15 minutes. The supernatant was decanted, and this washing steps with MM were repeated twice more to ensure all XRF had been washed out of the pores of the solid.

Then, the falcon tubes were filed up to 30 ml with MM, shaken and added to the corresponding Erlenmeyer. 10 ml more of MM were used to make sure all solid from the falcon tubes was added to the Erlenmeyer by rinsing any solid stuck in the borders of the tube. As the 10 ml were not enough to also rinse off all the solids from the walls of the Erlenmeyer, so that the cotton stopper could be placed in preparation to autoclave without it touching any solid, 20 ml more of MM were used to rinse off the remaining solids stuck to the walls of the Erlenmeyers. Even then, not all solids were removed from the walls of every Erlenmeyer flask. Each flask had different amounts of solid stuck to its walls. The Erlenmeyers were then autoclaved.

3.4.2 Setup for experiment 2

Just like with the XRF in section 2.3.5, 12 Erlenmeyers were set up, each containing either no enzyme, cellulase, laccase or cellulase and laccase. The laccase and cellulase solutions were the same as the ones described in title 2.3.3 and 2.3.4 respectively.

Table 3.7 shows the exact volume of each component added to the different Erlenmeyer flasks. Each different setup was replicated three times. The total volume of the Erlenmeyers was 100 ml.

Table 3.7: Content of Erlenmeyers in experiment 2.

	Component	Volume (mL)
Erlenmeyers 1-3	Solid fraction	5 g in 55.5 mL MM
	Mineral medium (MM)	45.00
Erlenmeyers 4-6	Solid fraction	5 g in 55.5 mL MM
	MM	42.73
	Cellulase	2.27
Erlenmeyers 7-9	Solid fraction	5 g in 55.5 mL MM
	MM	35.91
	Laccase	9.09
Erlenmeyers 10-12	Solid fraction	5 g in 55.5 mL MM
	MM	33.64
	Cellulase	2.27
	Laccase	9.09

As soon as the enzyme solutions were added, a 2 ml sample was taken from each Erlenmeyer flask. New samples were collected each day for the next 5 days.

3.5 Detoxification of the liquid and solid fractions

In this third experiment, 12 Erlenmeyer flasks were set up in a similar manner to the one described previously but now, the Erlenmeyers contained the XRF as well as the solid fraction.

3.5.1 Washing of the solid fraction for experiment 3

This time, 2.5 g of solid were added to the Falcon tubes due to the limited amount of XRF available. The exact mass of the solid is shown in Table 3.8.

Table 3.8: Weight of the solid fraction present in each falcon tube in experiment 3.

	Weight (g)		Weight (g)
Falcon 1	2.5063	Falcon 7	2.5068
Falcon 2	2.5048	Falcon 8	2.5071
Falcon 3	2.5061	Falcon 9	2.5070
Falcon 4	2.5023	Falcon 10	2.5064
Falcon 5	2.5088	Falcon 11	2.5015
Falcon 6	2.5063	Falcon 12	2.5079

The solid fraction was washed in the same way as described previously in title 2.4.1. Once the solid had been washed, 20 ml were used to transfer the solid in the falcon tube to the corresponding Erlenmeyer. As some solid was still stuck to the sides of the falcon tubes, an additional 10 ml were used in an attempt to transfer all remaining solids into the Erlenmeyer. The Erlenmeyers containing the solid fraction were then autoclaved.

3.5.2 Setup for experiment 3

Erlenmeyers in experiment 3 had a total volume of 50 ml. However, Erlenmeyers 7-12 had a higher volume due to the fact that the quantity of solid fraction, XRF, cellulase and laccase could not be modified. Therefore, no MM was added but the total volume was still over 50 ml.

Table 3.9: Content of Erlenmeyers in experiment 3.

	Component	Volume (mL)
Erlenmeyers 1-3	Solid fraction	2.5 g in 27.5 ml MM
	XRF	20.205
	Mineral medium (MM)	2.295
Erlenmeyers 4-6	Solid fraction	2.5 g in 27.5 ml MM
	XRF	20.205
	MM	1.160
	Cellulase	1.135
Erlenmeyers 7-9	Solid fraction	2.5 g in 27.5 ml MM
	XRF	20.205
	MM	0
	Laccase	4.545

Erlenmeyers 10-12	Solid fraction	2.5 g in 27.5 ml MM
	XRF	20.205
	MM	0
	Cellulase	1.135
	Laccase	4.545

As previously, 2 ml samples were taken as soon as the enzymes were added to the Erlenmeyers and samples continued to be taken for the next five days.

3.6 Follow up of sugar and phenolic compound content

HPLC is used to determine the concentration of sugar and phenolic compound in each sample collected. To prepare samples for HPLC, 1 ml from each Eppendorf was transferred into a plastic vial with a screw cap and heated during 15 minutes in a boiling water bath. This was done in order to denature the enzymes. Then, the samples were cooled down in an ice bath for 5 minutes and filtered through a 0.22 µm membrane using a syringe into a glass vial.

Xylose and glucose concentrations were measured by HPLC using an iron-exclusion column (Coregel ORH 801, Agilent) at 75ºC and a refractive index (RI) detector. The mobile phase used was 8mM of sulfuric acid in demineralised water with a flow rate of 0.6 ml/min. Acetic acid and formic acid concentration was also measured using this column. The calibration curves in 'Chapter 8: Attachments' were used to convert the area value given by the HPLC to concentrations.

The concentration of phenolic and furanic compounds was measured with a C18 column (Agilent, Phenomenex) and Diode array detector (DAD). The mobile phase consisted of a gradient of 2% acetic acid and methanol which is shown in Table 3.10 and a 0.4 ml/min flow rate.

Table 3.10: Mobile phase gradient for phenolic compound detection by HPLC.

Time (min)	2% Acetic acid (%)	Methanol (%)	Flow (ml/min)
0	90.0	10.0	0.4
3	80.7	19.3	0.4
8	67.0	33.0	0.4
40	65.6	34.4	0.4
55	0.0	100.0	0.4
55.1	90.0	10.0	0.4
70	90.0	10.0	0.4

The phenolic and furanic compounds the detection was focused on were furfural, 5-hydroxymethylfurfural, syringaldehyde, syringic acid, vanillin, vanillic acid, 4-hydroxybenzoic acid, 3,4-hydroxybenzaldehyde and 4-hydroxybenzaldehyde.

Chapter 4: Results

4.1 Cellulase activity

4.1.1 Calibration curve

The results for the absorbance of each glucose standard are shown in Table 4.1.

Table 4.1: Absorbance of the glucose standards.

Glucose Concentration (mg/0.5 mL)	Absorbance (A)
3.35	0.631
2.50	0.541
1.65	0.337
1.00	0.191

The National Renewable Energy Laboratory method [20] mentions that glucose standards should be between 0.1 to 1 A. The fact that the absorbance results obtained are within this range, adds to their validity.

The calibration curve is represented in Figure 4.1.

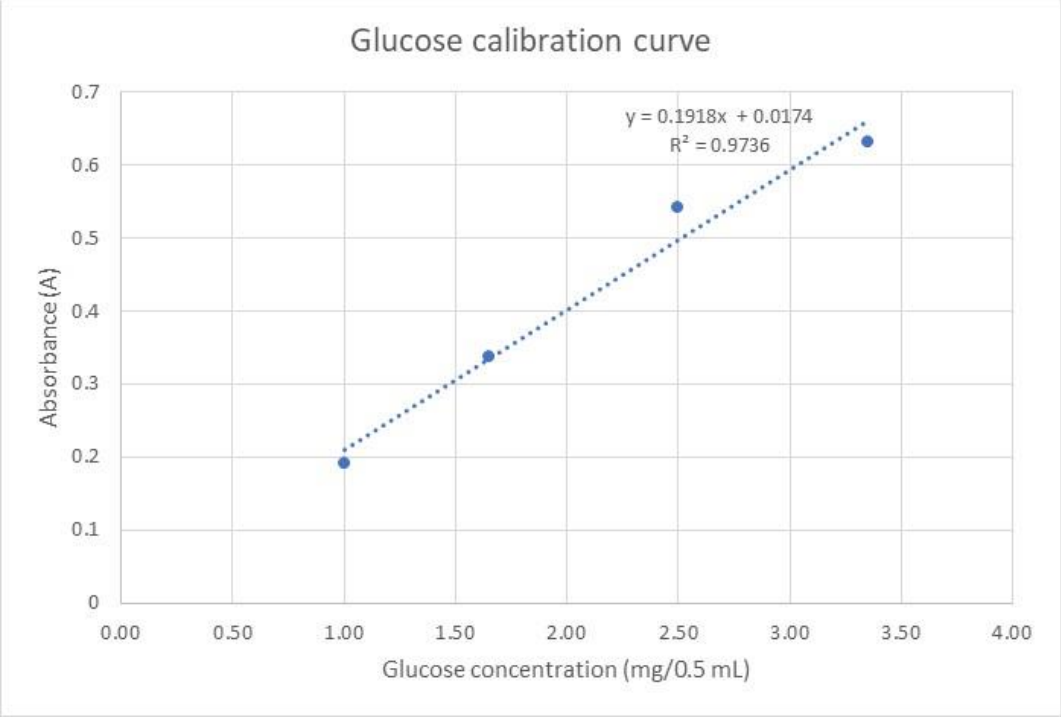

Figure 4.1: Glucose standard calibration curve.

The calibration curve and the absorption results from the enzyme dilutions shown in Table 4.2 are used to determine the concentration of glucose released for each sample. The concentration of enzyme

from the original solution present in each enzyme dilution was calculated by the reciprocal of the dilution factor.

Table 4.2: Absorbance and glucose concentration in enzyme test tubes.

Dilution factor	1/Dilution factor	Absorbance	Glucose Concentration (mg/0.5 mL)
100	0.01000	1.818	9.388
400	0.00250	0.831	4.242
800	0.00125	0.404	2.016
1200	0.00083	0.276	1.348
1500	0.00067	0.275	1.343

In order to determine the concentration of enzyme which would have released 2 mg of glucose, the two data points that are closest to 2 mg, are plotted in Figure 4.2.

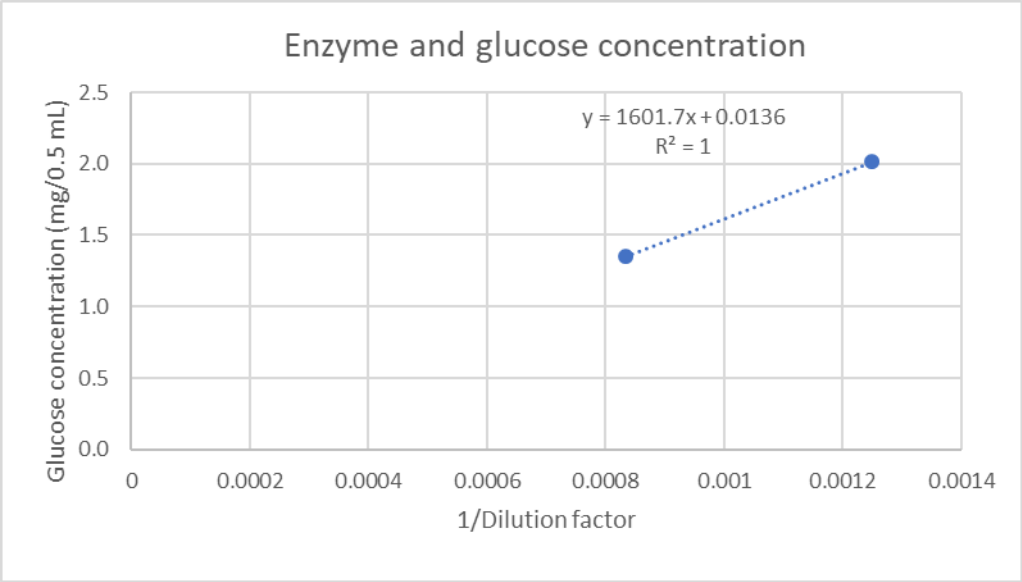

Figure 4.2: Glucose concentration vs enzyme concentration.

By substituting a glucose concentration of 2 mg/0.5 ml, we get a 1/dilution factor of 0.00124. Using the equation [20]:

$$Filter\ Paper\ Activity = \frac{0.37}{[enzyme]\ releasing\ 2mg\ of\ glucose} units/mL$$

$$FPU = 298.34\ units/mL$$

The enzyme blank gave an absorbance of 0.004 A but this value was not subtracted to the absorbance results for the dilutions, as indicated in the procedure, because when done so, the two points in which the 2 mg concentration was found, changed. However, the 2 mg was very close to one of the extremes of the range (1.99mg/0.5mL) therefore the resulting value of FPU deferred more from what was expected than the FPU value without subtracting the blank.

4.2 Laccase activity

4.2.1 Average activity and standard deviation with ABTS as a substrate

Table 4.3 shows the absorbance values for the first run of laccase enzyme with ABTS as substrate. Figure 4.3 represents these data.

Table 4.3: Absorption for ABTS 1.

Time (min)	Abs (A)	Delta	Time (min)	Abs (A)	Delta
0.00	0.035	-	5.17	0.441	0.01
0.17	0.048	0.013	5.33	0.452	0.011
0.33	0.063	0.015	5.50	0.462	0.01
0.50	0.078	0.015	5.67	0.472	0.01
0.67	0.09	0.015	5.83	0.482	0.01
0.83	0.107	0.014	6.00	0.492	0.01
1.00	0.122	0.015	6.17	0.502	0.01
1.17	0.136	0.014	6.33	0.511	0.009
1.33	0.151	0.015	6.50	0.520	0.009
1.50	0.165	0.014	6.67	0.529	0.009
1.67	0.179	0.014	6.83	0.537	0.008
1.83	0.194	0.015	7.00	0.546	0.009
2.00	0.208	0.014	7.17	0.554	0.008
2.17	0.221	0.013	7.33	0.562	0.008
2.33	0.235	0.014	7.50	0.570	0.008
2.50	0.249	0.014	7.67	0.577	0.007
2.67	0.262	0.013	7.83	0.585	0.008
2.83	0.275	0.013	8.00	0.592	0.007
3.00	0.288	0.013	8.17	0.599	0.007
3.17	0.301	0.013	8.33	0.605	0.006
3.33	0.314	0.013	8.50	0.612	0.007
3.50	0.326	0.012	8.67	0.618	0.006
3.67	0.339	0.013	8.83	0.625	0.007
3.83	0.351	0.012	9.00	0.631	0.006
4.00	0.363	0.012	9.17	0.636	0.005
4.17	0.375	0.012	9.33	0.642	0.006
4.33	0.386	0.011	9.50	0.647	0.005
4.50	0.398	0.012	9.67	0.653	0.006
4.67	0.409	0.011	9.83	0.658	0.005
4.83	0.420	0.011	10.00	0.663	0.005
5.00	0.431	0.011			

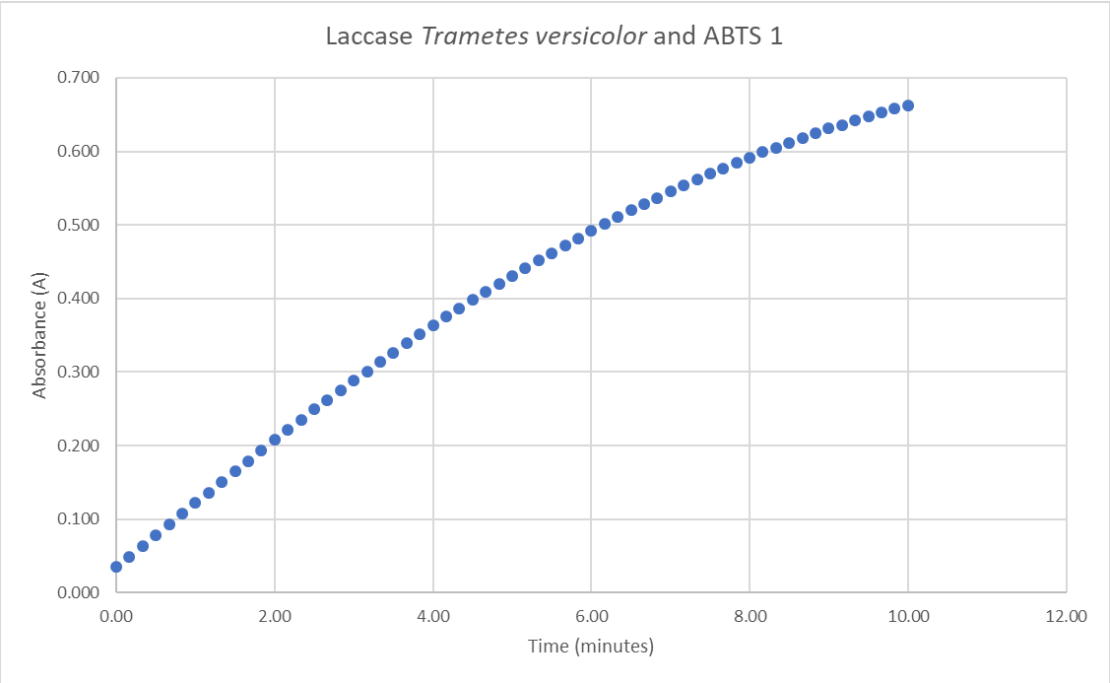

Figure 4.3: Absorbance vs time for ABTS 1.

The measurements were repeated twice more: ABTS 2 data are collected in Table 4.4 and Figure 4.4 and ABTS 3 in Table 4.5 and Figure 4.5.

Table 4.4: Absorption for ABTS 2.

Time (min)	Abs (A)	Delta	Time (min)	Abs (A)	Delta
0	0.068	-	5.17	0.496	0.01
0.17	0.085	0.017	5.33	0.506	0.01
0.33	0.102	0.017	5.50	0.515	0.009
0.50	0.119	0.017	5.67	0.525	0.010
0.67	0.136	0.017	5.83	0.533	0.008
0.83	0.152	0.016	6.00	0.542	0.009
1.00	0.169	0.017	6.17	0.550	0.008
1.17	0.185	0.016	6.33	0.558	0.008
1.33	0.201	0.016	6.50	0.566	0.008
1.50	0.216	0.015	6.67	0.574	0.008
1.67	0.232	0.016	6.83	0.581	0.007
1.83	0.247	0.015	7.00	0.588	0.007
2.00	0.262	0.015	7.17	0.595	0.007
2.17	0.277	0.015	7.33	0.601	0.006
2.33	0.291	0.014	7.50	0.608	0.007
2.50	0.305	0.014	7.67	0.614	0.006
2.67	0.319	0.014	7.83	0.620	0.006
2.83	0.333	0.014	8.00	0.625	0.005
3.00	0.346	0.013	8.17	0.631	0.006
3.17	0.360	0.014	8.33	0.636	0.005
3.33	0.372	0.012	8.50	0.642	0.006
3.50	0.385	0.013	8.67	0.647	0.005
3.67	0.397	0.012	8.83	0.651	0.004
3.83	0.409	0.012	9.00	0.656	0.005

4.00	0.421	0.012	9.17	0.660	0.004
4.17	0.433	0.012	9.33	0.664	0.004
4.33	0.444	0.011	9.50	0.668	0.004
4.50	0.455	0.011	9.67	0.672	0.004
4.67	0.466	0.011	9.83	0.676	0.004
4.83	0.476	0.010	10.00	0.680	0.004
5.00	0.486	0.010			

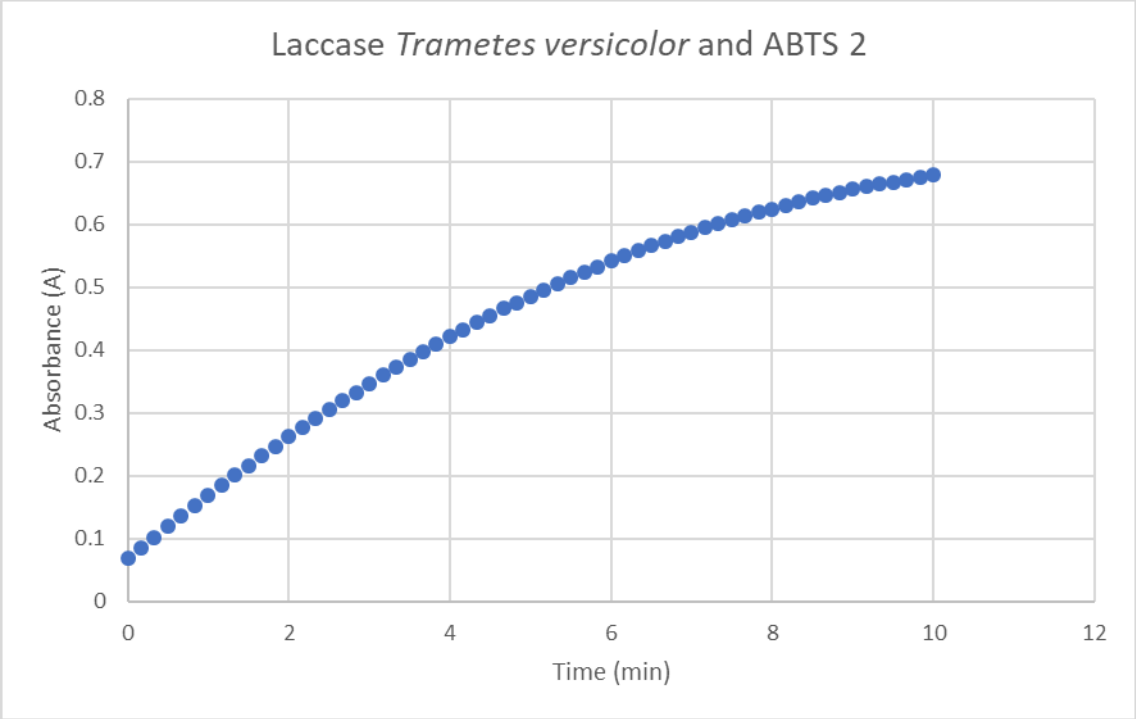

Figure 4.4: Absorbance vs time for ABTS 2.

Table 4.5: Absorption for ABTS 3.

Time (min)	Abs (A)	Delta	Time (min)	Abs (A)	Delta
0	0.098	-	5.17	0.520	0.01
0.17	0.115	0.017	5.33	0.529	0.009
0.33	0.132	0.017	5.50	0.539	0.010
0.50	0.149	0.017	5.67	0.547	0.008
0.67	0.165	0.016	5.83	0.556	0.009
0.83	0.182	0.017	6.00	0.565	0.009
1.00	0.198	0.016	6.17	0.573	0.008
1.17	0.214	0.016	6.33	0.581	0.008
1.33	0.230	0.016	6.50	0.589	0.008
1.50	0.246	0.016	6.67	0.596	0.007
1.67	0.261	0.015	6.83	0.603	0.007
1.83	0.276	0.015	7.00	0.609	0.006
2.00	0.291	0.015	7.17	0.616	0.007
2.17	0.305	0.014	7.33	0.622	0.006
2.33	0.319	0.014	7.50	0.629	0.007
2.50	0.333	0.014	7.67	0.634	0.005
2.67	0.347	0.014	7.83	0.640	0.006
2.83	0.360	0.013	8.00	0.646	0.006

3.00	0.373	0.013	8.17	0.651	0.005
3.17	0.386	0.013	8.33	0.656	0.005
3.33	0.399	0.013	8.50	0.661	0.005
3.50	0.411	0.012	8.67	0.666	0.005
3.67	0.423	0.012	8.83	0.670	0.004
3.83	0.435	0.012	9.00	0.674	0.004
4.00	0.447	0.012	9.17	0.679	0.005
4.17	0.458	0.011	9.33	0.683	0.004
4.33	0.469	0.011	9.50	0.686	0.003
4.50	0.480	0.011	9.67	0.690	0.004
4.67	0.490	0.01	9.83	0.693	0.003
4.83	0.501	0.011	10.00	0.697	0.004
5.00	0.510	0.009			

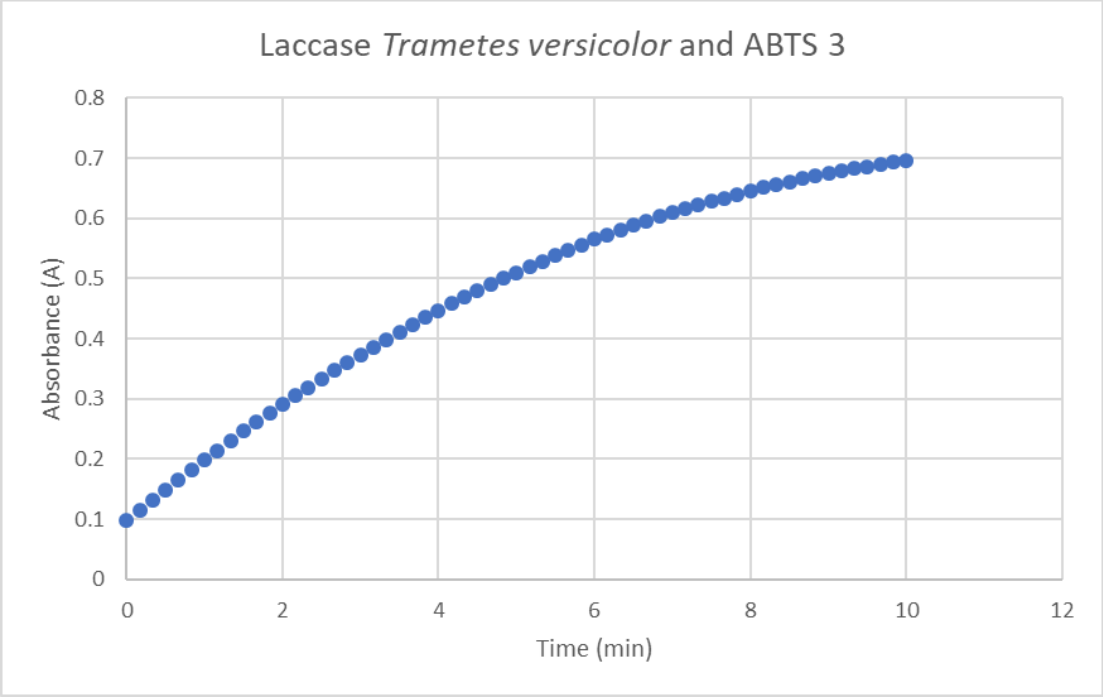

Figure 4.5: Absorbance vs time for ABTS 3.

The activity of laccase in each three cases was calculated using the highest ΔA at the start of the 10 minutes. The calculation of activity with ABTS 1 is shown below as an example:

$$\Delta A = 0.015$$

$$\frac{\Delta A}{min} = \frac{0.015}{0.17} = 0.088$$

The concentration per minute is calculated using the Lambert Beer Law:

$$A = \varepsilon \cdot l \cdot c$$

$$\frac{c}{min} = \frac{A}{\varepsilon \cdot l} = \frac{0.082}{36000 \cdot 1} = 2.451 \cdot 10^{-6} \frac{mol}{L \cdot \text{min}}$$

Activity is then calculated by multiplying the concentration per minute by the total volume in the cuvette and dividing by the mass of enzyme present in each cuvette (the concentration of enzyme in the cuvette).

$$Activity = \frac{2.451 \cdot 10^{-6} \frac{mol}{L \cdot \text{min}} \cdot 10^{6} \frac{\mu mol}{mol} \cdot 0.003L}{0.05\ mg\ enzyme} = 0.147 \frac{Units}{mg\ enzyme}$$

Results for all three runs can be found below in Table 4.6. An average was calculated using the three values, along with the standard deviation.

Table 4.6: Activity of laccase using ABTS as a substrate.

	ΔA (Au)	$\Delta C/t$ (mol/L·min)	**Activity (Units/mg)**
ABTS 1	0.015	$2.451 \cdot 10^{-6}$	0.147
ABTS 2	0.017	$2.778 \cdot 10^{-6}$	0.167
ABTS 3	0.017	$2.778 \cdot 10^{-6}$	0.157
Average	-	-	0.160
Standard deviation	-	-	0.011

The coefficient of variation was calculated by dividing the standard deviation by the average value.

Coefficient of variation	0.071

4.2.2 Average activity and standard deviation with syringaldazine as a substrate

Results were processed in the same way as for ABTS, and are shown in Table 4.10. Data collected is shown in Table 4.7, Table 4.8 and Table 4.9. Figures 4.6 to 4.8 show the corresponding representations.

Table 4.7: Absorption for syringaldazine 1.

Time (min)	**Abs (A)**	**Delta**	**Time (min)**	**Abs (A)**	**Delta**
0	0.014	-	5.17	0.704	0.011
0.17	0.038	0.024	5.33	0.715	0.011
0.33	0.064	0.026	5.50	0.725	0.010
0.50	0.093	0.029	5.67	0.734	0.009
0.67	0.123	0.030	5.83	0.743	0.009
0.83	0.153	0.030	6.00	0.752	0.009
1.00	0.182	0.029	6.17	0.759	0.007
1.17	0.211	0.029	6.33	0.765	0.006
1.33	0.239	0.028	6.50	0.771	0.006
1.50	0.267	0.028	6.67	0.776	0.005
1.67	0.295	0.028	6.83	0.782	0.006
1.83	0.321	0.026	7.00	0.789	0.007
2.00	0.348	0.027	7.17	0.795	0.006

2.17	0.373	0.025	7.33	0.799	0.004
2.33	0.397	0.024	7.50	0.804	0.005
2.50	0.421	0.024	7.67	0.808	0.004
2.67	0.445	0.024	7.83	0.811	0.003
2.83	0.468	0.023	8.00	0.814	0.003
3.00	0.491	0.023	8.17	0.817	0.003
3.17	0.512	0.021	8.33	0.818	0.001
3.33	0.533	0.021	8.50	0.820	0.002
3.50	0.552	0.019	8.67	0.821	0.001
3.67	0.571	0.019	8.83	0.822	0.001
3.83	0.589	0.018	9.00	0.823	0.001
4.00	0.606	0.017	9.17	0.823	0
4.17	0.623	0.017	9.33	0.823	0
4.33	0.638	0.015	9.50	0.823	0
4.50	0.653	0.015	9.67	0.824	0.001
4.67	0.667	0.014	9.83	0.824	0
4.83	0.680	0.013	10.00	0.823	-0.001
5.00	0.693	0.013			

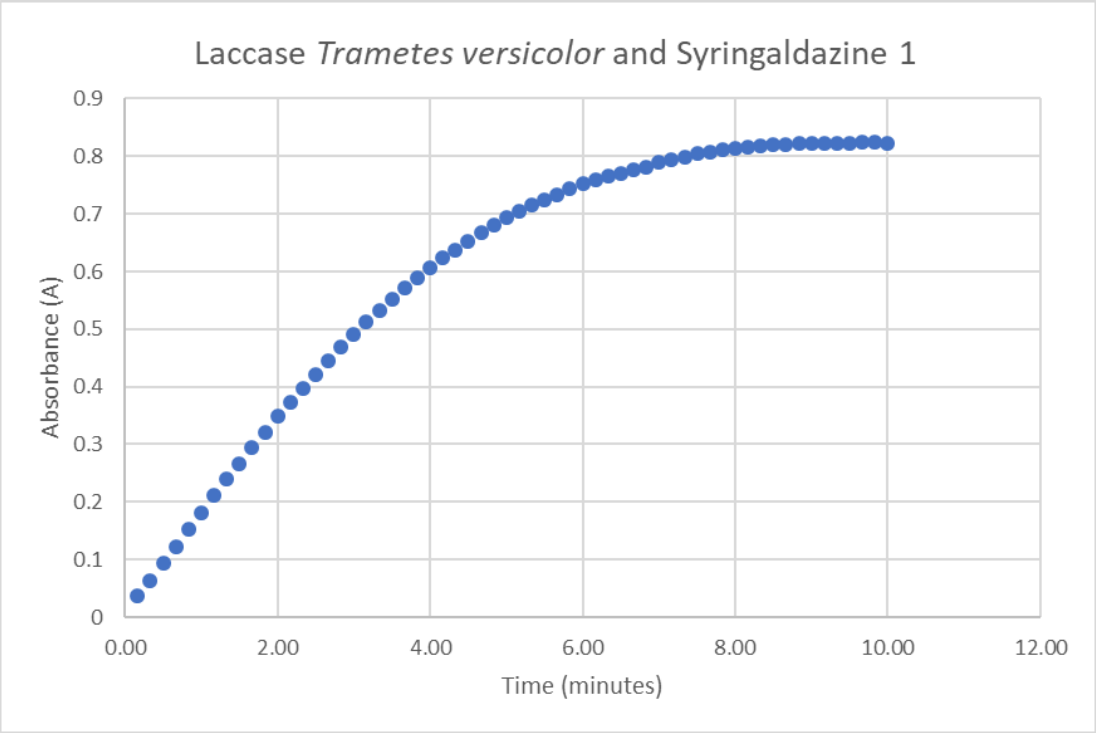

Figure 4.6: Absorbance vs time for syringaldazine 1.

Table 4.8: Absorption for syringaldazine 2.

Time (min)	Abs (A)	Delta	Time (min)	Abs (A)	Delta
0	0.005	-	5.17	0.832	0.016
0.17	0.027	0.022	5.33	0.848	0.016
0.33	0.056	0.029	5.50	0.864	0.016
0.50	0.088	0.032	5.67	0.879	0.015
0.67	0.121	0.033	5.83	0.889	0.010
0.83	0.155	0.034	6.00	0.904	0.015
1.00	0.189	0.034	6.17	0.917	0.013
1.17	0.222	0.033	6.33	0.931	0.014
1.33	0.256	0.034	6.50	0.941	0.010
1.50	0.289	0.033	6.67	0.950	0.009
1.67	0.321	0.032	6.83	0.959	0.009
1.83	0.352	0.031	7.00	0.966	0.007
2.00	0.383	0.031	7.17	0.975	0.009
2.17	0.413	0.03	7.33	0.983	0.008
2.33	0.443	0.03	7.50	0.989	0.006
2.50	0.473	0.03	7.67	0.996	0.007
2.67	0.502	0.029	7.83	1.002	0.006
2.83	0.530	0.028	8.00	1.008	0.006
3.00	0.557	0.027	8.17	1.014	0.006
3.17	0.583	0.026	8.33	1.019	0.005
3.33	0.607	0.024	8.50	1.021	0.002
3.50	0.631	0.024	8.67	1.025	0.004
3.67	0.654	0.023	8.83	1.028	0.003
3.83	0.676	0.022	9.00	1.028	0
4.00	0.698	0.022	9.17	1.029	0.001
4.17	0.720	0.022	9.33	1.031	0.002
4.33	0.740	0.020	9.50	1.032	0.001
4.50	0.759	0.019	9.67	1.032	0
4.67	0.778	0.019	9.83	1.033	0.001
4.83	0.797	0.019	10.00	1.033	0
5.00	0.816	0.019			

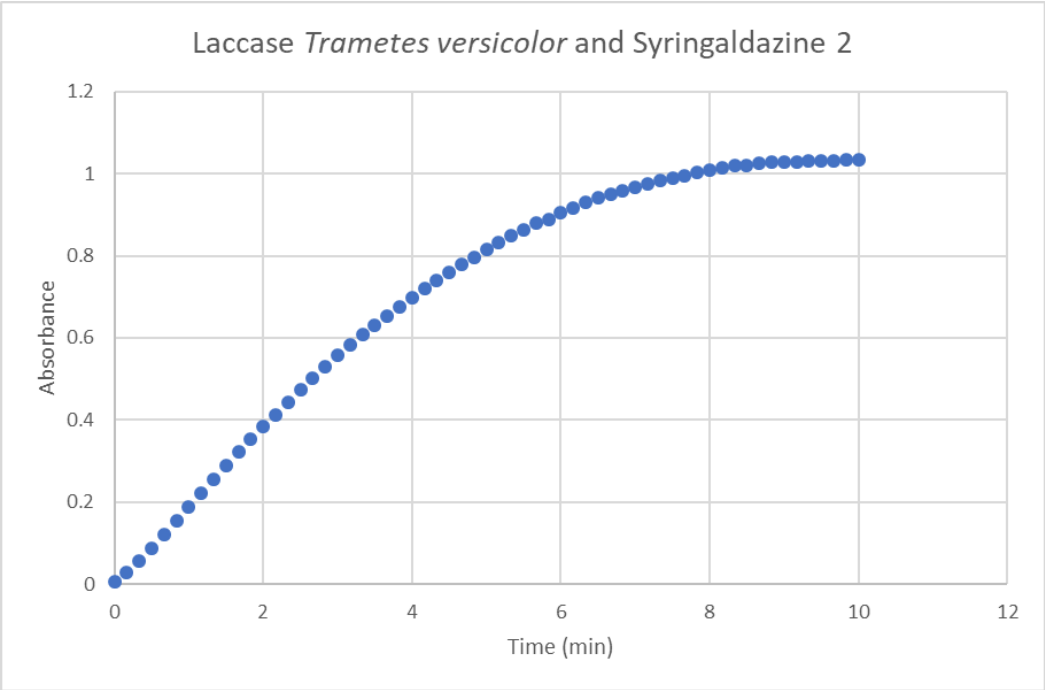

Figure 4.7: Absorbance vs time for syringaldazine 2.

Table 4.9: Absorption for syringaldazine 3.

Time (min)	Abs (A)	Delta	Time (min)	Abs (A)	Delta
0.00	0.231	-	5.17	1.086	0.021
0.17	0.247	0.016	5.33	1.109	0.023
0.33	0.270	0.023	5.50	1.135	0.026
0.50	0.296	0.026	5.67	1.156	0.021
0.67	0.324	0.028	5.83	1.176	0.020
0.83	0.354	0.030	6.00	1.197	0.021
1.00	0.385	0.031	6.17	1.219	0.022
1.17	0.417	0.032	6.33	1.239	0.020
1.33	0.448	0.031	6.50	1.260	0.021
1.50	0.479	0.031	6.67	1.280	0.020
1.67	0.511	0.032	6.83	1.297	0.017
1.83	0.540	0.029	7.00	1.314	0.017
2.00	0.570	0.030	7.17	1.328	0.014
2.17	0.601	0.031	7.33	1.345	0.017
2.33	0.631	0.030	7.50	1.361	0.016
2.50	0.660	0.029	7.67	1.373	0.012
2.67	0.687	0.027	7.83	1.385	0.012
2.83	0.716	0.029	8.00	1.396	0.011
3.00	0.745	0.029	8.17	1.410	0.014
3.17	0.775	0.030	8.33	1.424	0.014
3.33	0.804	0.029	8.50	1.437	0.013
3.50	0.832	0.028	8.67	1.443	0.006
3.67	0.861	0.029	8.83	1.453	0.010
3.83	0.890	0.029	9.00	1.464	0.011
4.00	0.919	0.029	9.17	1.476	0.012
4.17	0.946	0.027	9.33	1.488	0.012
4.33	0.972	0.026	9.50	1.502	0.014

4.50	0.998	0.026	9.67	1.513	0.011
4.67	1.022	0.024	9.83	1.520	0.007
4.83	1.043	0.021	10.00	1.527	0.007
5.00	1.065	0.022			

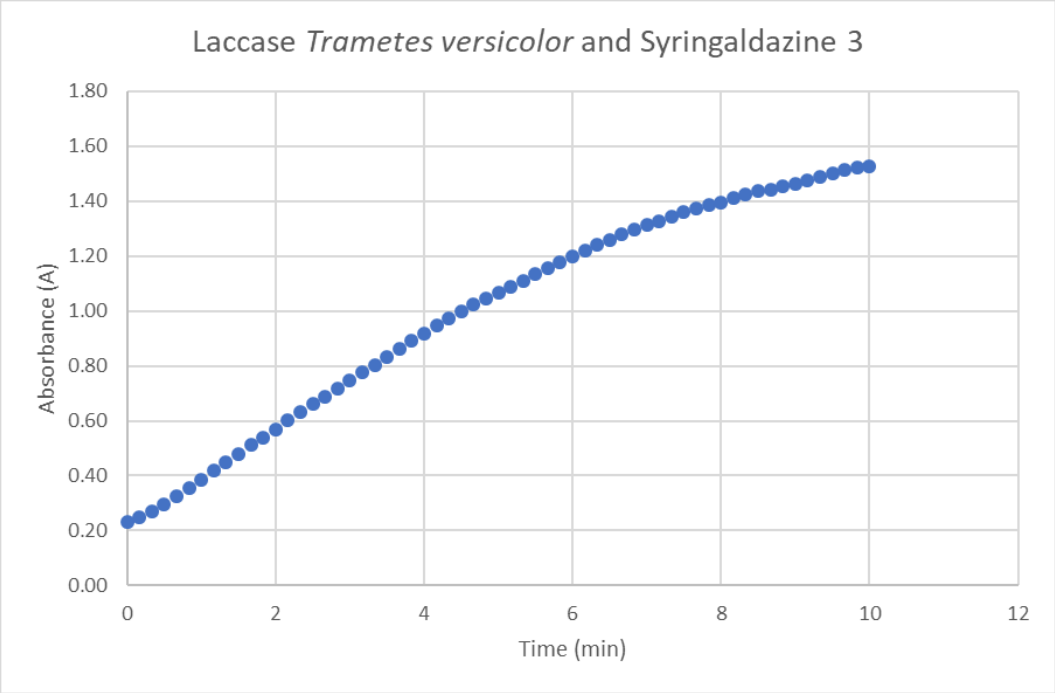

Figure 4.8: Absorbance vs time for syringaldazine 3.

Table 4.10: Activity of laccase using syringaldazine as a substrate.

	ΔA (Au)	$\Delta C/t$ (mol/L·min)	**Activity (Units/mg)**
syringaldazine 1	0.030	$3.529 \cdot 10^{-6}$	0.212
syringaldazine 2	0.034	$4 \cdot 10^{-6}$	0.240
syringaldazine 3	0.032	$3.765 \cdot 10^{-6}$	0.226
Average	-	-	0.226
Standard deviation	-	-	0.014

The coefficient of variation was calculated by dividing the standard deviation by the average value.

Coefficient of variation	0.063

4.3 Detoxification of the liquid fraction

Results for the concentration of the different compounds measured during experiment 1 are shown below. The individual concentrations in function of time are discussed in this section while the concentrations of each compound in all three experiments are compared in 'Chapter 5: Discussion'.

As explained above, each experimental set up was repeated three times, therefore the concentration values shown in the graphs below correspond to an average of those three values.

4.3.1 Sugars and organic acids concentration in function of time in experiment 1

Glucose, xylose, formic acid and acetic acid concentrations in function of time where all measured in the same way for all three experiments, as stated in section 3.6.

Firstly, as shown in Figure 4.9, glucose was only detected in Erlenmeyers containing cellulase. As expected, glucose concentration increases with an increase in time, as the cellulase has had more time to degrade the substrate. The day 1 sample was taken immediately after the addition of the cellulase, so oligosaccharides that have not yet been degraded into monosaccharides will be present, as they are released quickly after the addition of the enzyme.

On the other hand, the combination of cellulase and laccase enzymes resulted in a glucose concentration that was below the detection limit. This can be attributed to the inhibition effect laccase has on cellulase enzymes, as described in similar studies such as Oliva-Taravilla et al. [22]

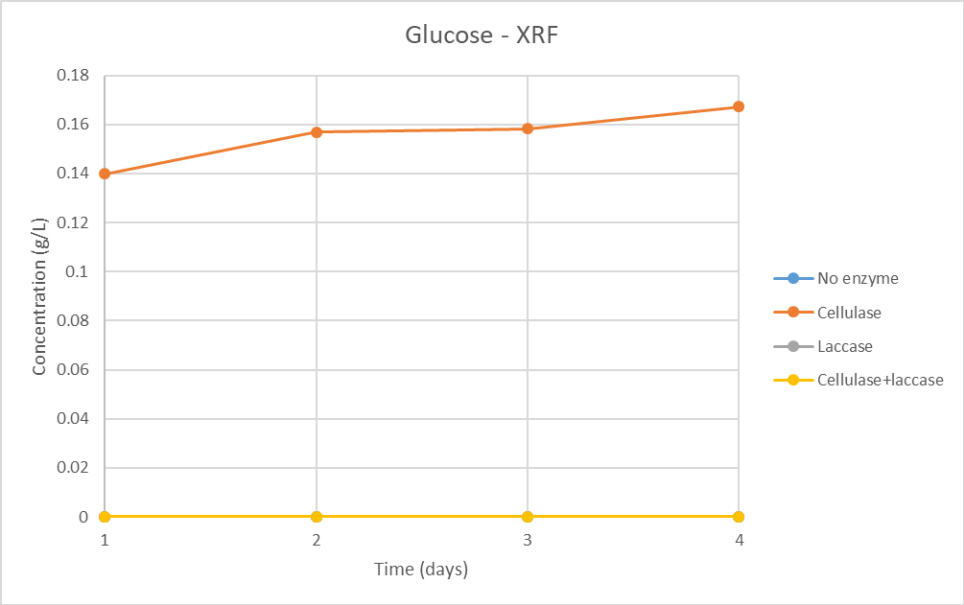

Figure 4.9: Glucose concentration in function of time in experiment 1.

Xylose concentration with respect to time increased much more significantly than glucose concentration in the Erlenmeyers where cellulase was present, as shown in Figure 4.10. This is due to the fact that, as mentioned in chapter 2, after the steam explosion takes place, the liquid fraction is mostly made up of degraded and liquefied hemicellulose. This liquid fraction is in fact known as the Xylose Rich Fraction (XRF). Just as with glucose, the steep increase in xylose concentration in the first two days, can be attributed to the fact that day one samples were collected immediately after the cellulase was added and therefore, oligosaccharides were present. By the time the day two sample was taken, a higher number of oligosaccharides had been broken down into monosaccharides.

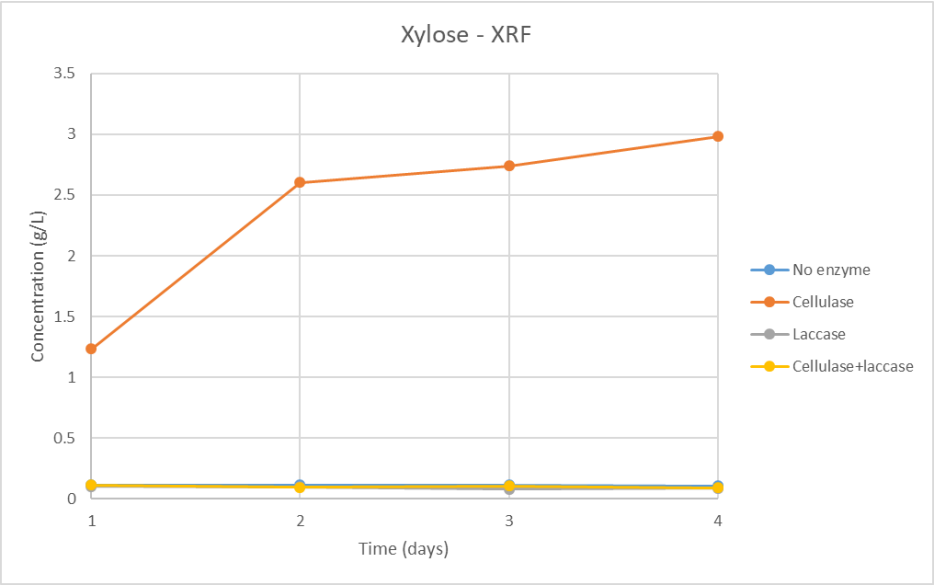

Figure 4.10: Xylose concentration in function of time in experiment 1.

All Erlenmeyers in experiment 1 contained formic acid. However, all Erlenmeyers containing enzymes saw a slight decrease in formic acid concentration, while the Erlenmeyer with no enzymes had an overall increase.

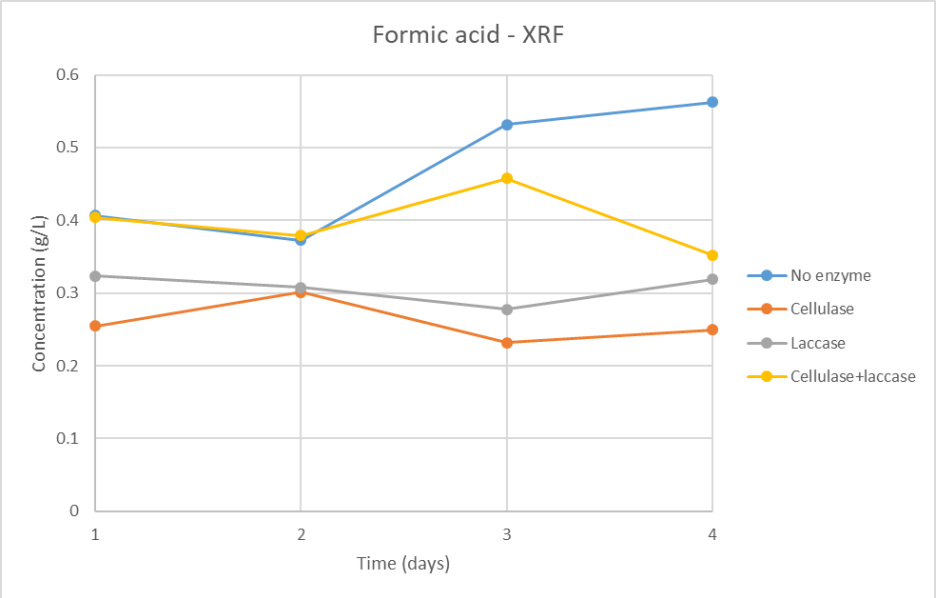

Figure 4.11: Formic acid concentration in function of time in experiment 1.

Finally, acetic acid was also present in all experiment 1 Erlenmeyers and at a higher concentration than formic acid in the presence of cellulase. Its concentration was also higher when only cellulase was present compared to the other cases. This can be attributed to the combined effect of the laccase inhibition on cellulase discussed above, and the fact that cellulase is a complex of different enzymes as mentioned in section 2.1.3.1. This complex of different enzymes can break the ether bond of the acetylgroup found in hemicellulose, increasing the concentration of acetic acid.

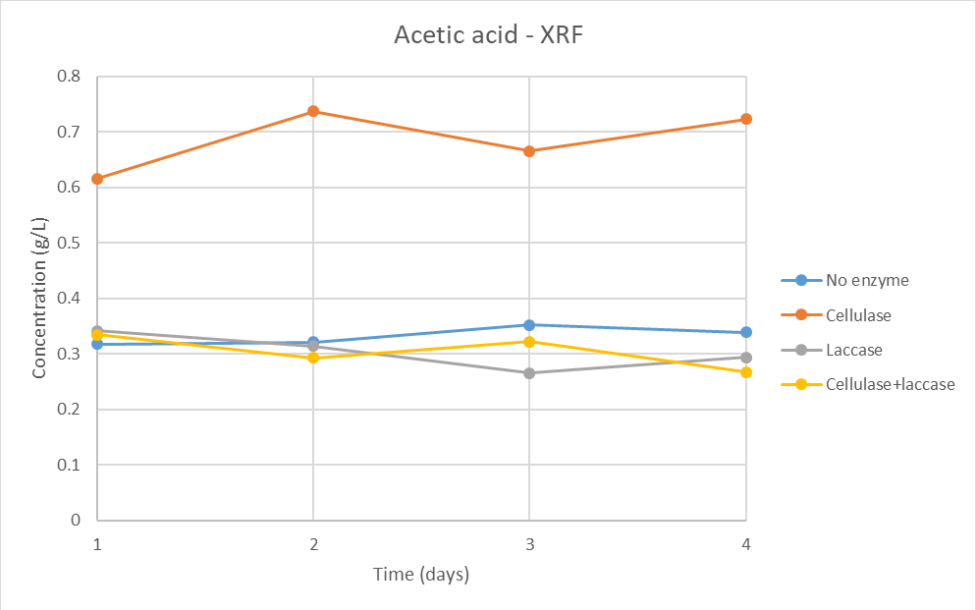

Figure 4.12: Acetic acid concentration in function of time in experiment 1.

4.3.2 Aromatic compounds concentration in function of time in experiment 1

The furanic and phenolic compounds concentration was measured as stated in section 3.6 and the results are displayed in Figure 4.13 to Figure 4.19.

The concentration of 5-hydroxymethylfurfural increases in the Erlenmeyers containing cellulase as shown in Figure 4.13, due to the hemicellulose degradation effect of the enzymes in the cellulase complex. However, the concentration in all other Erlenmeyers decreases, especially in those containing laccase. This is in line with what is expected, as laccase polymerises phenolic inhibitors into harmless oligomers/polymers.

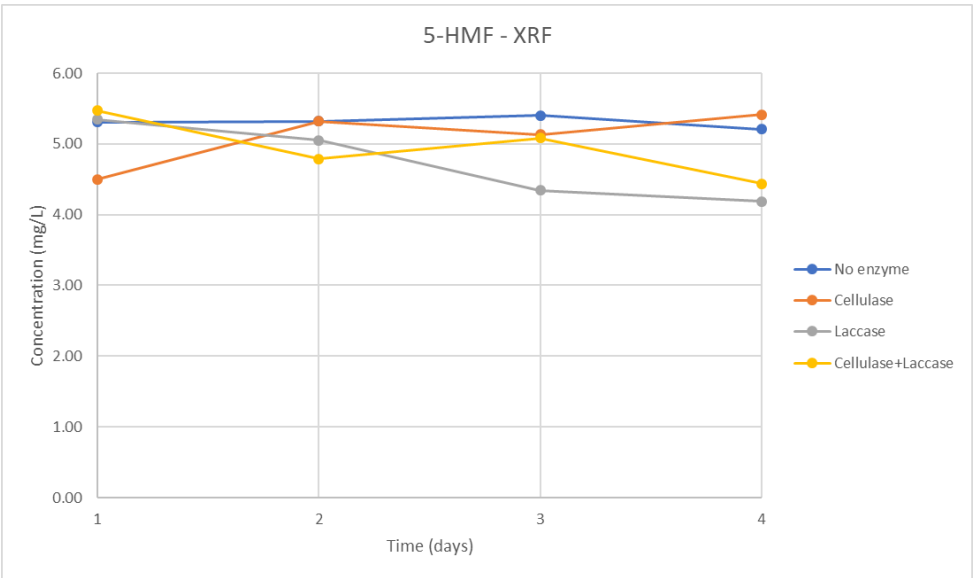

Figure 4.13: 5-hydroxymethylfurfural concentration in function of time in experiment 1.

The concentration of furfural and of 3,4-Dihydroxybenzaldehyde also decreases in experiment one, especially in the presence of laccase. In both cases, the Erlenmeyers with no enzymes show the highest values of concentration while the Erlenmeyers with laccase have the lowest inhibitor concentration.

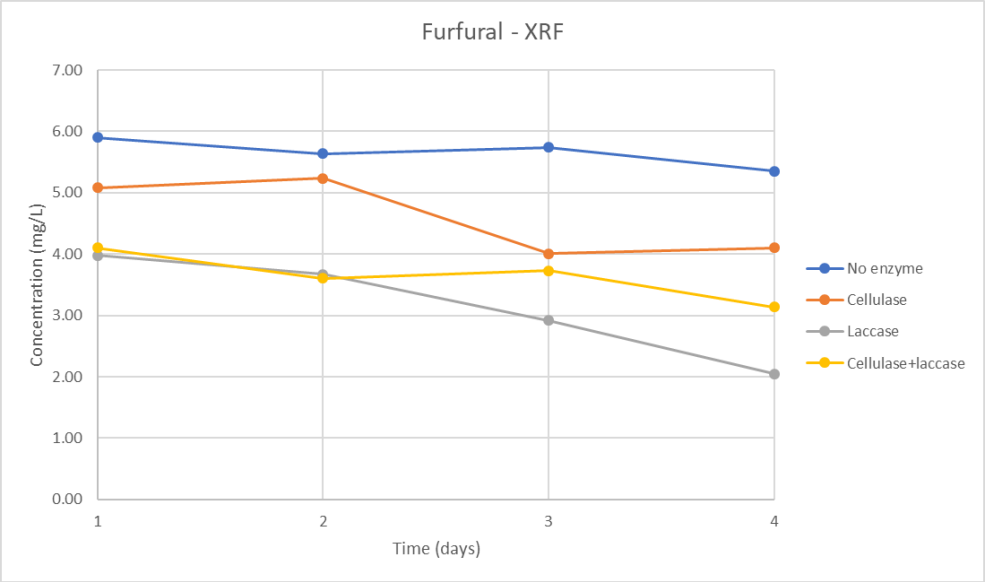

Figure 4.14: Furfural concentration in function of time in experiment 1.

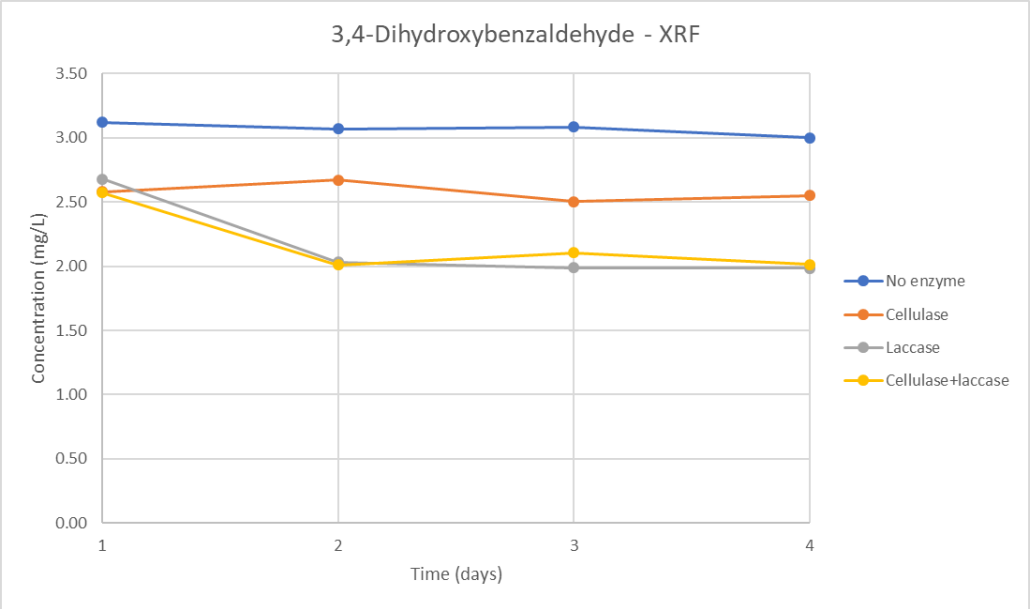

Figure 4.15: 3,4-Dihydroxybenzaldehyde concentration in function of time in experiment 1.

4-hydrobenzoic acid was only found to be present in the Erlenmeyer containing cellulase and laccase but only during the second day.

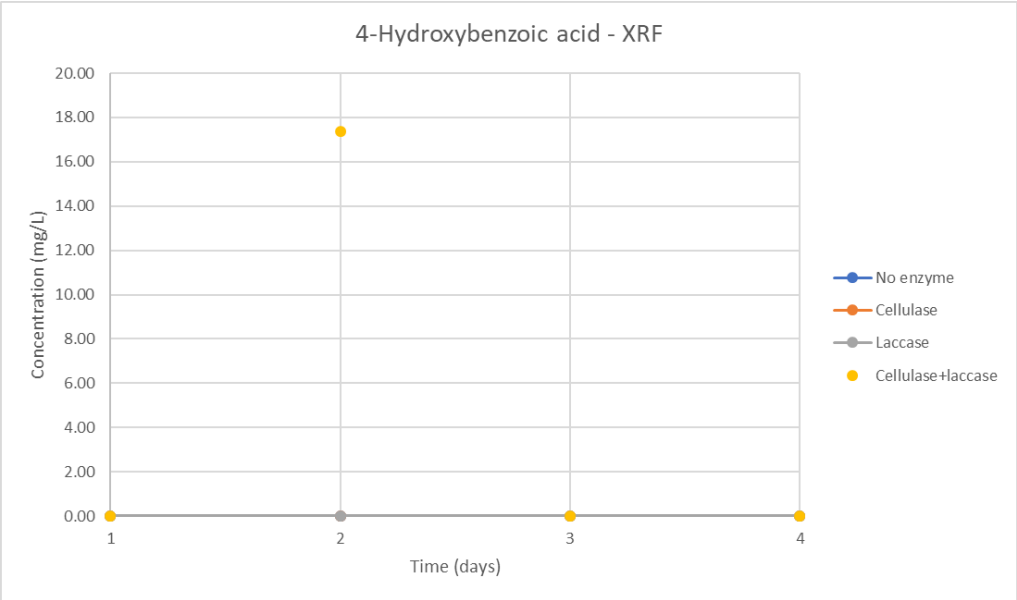

Figure 4.16: 4-Hydrobenzoic acid concentration in function of time in experiment 1.

Vanillic acid's concentration sees a decrease in time for all Erlenmeyers except for the ones with no enzyme. In this case, the lowest concentration is given by the addition of both cellulase and laccase.

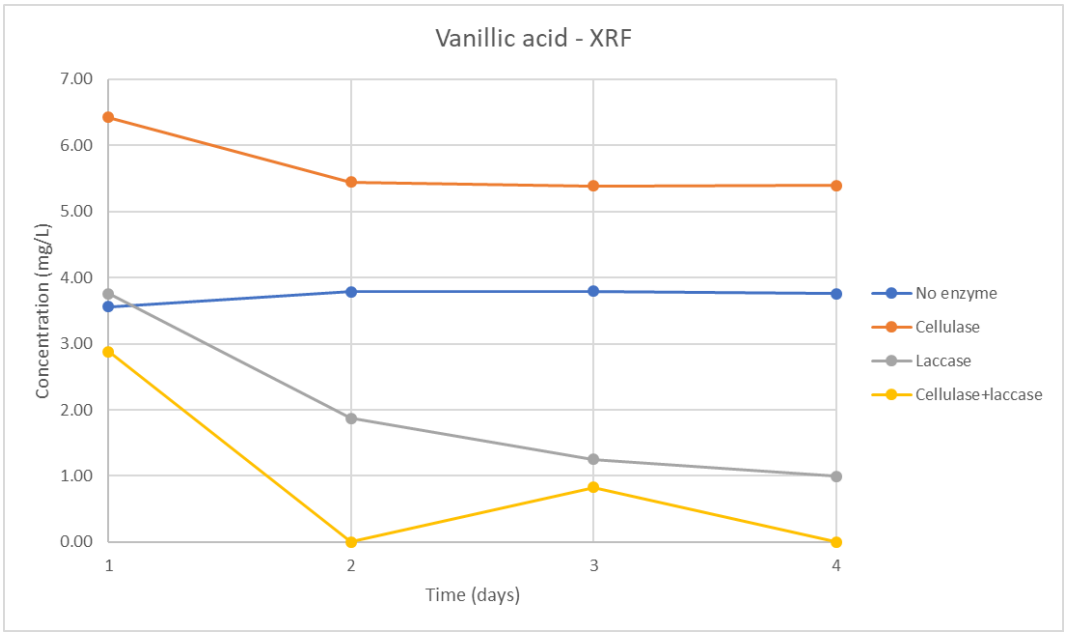

Figure 4.17: Vanillic acid concentration in function of time in experiment 1.

For syringic acid, concentration remains constant if no enzyme is added, increases in the presence of cellulase and decreases in a similar manner if laccase only or both laccase and cellulase are added.

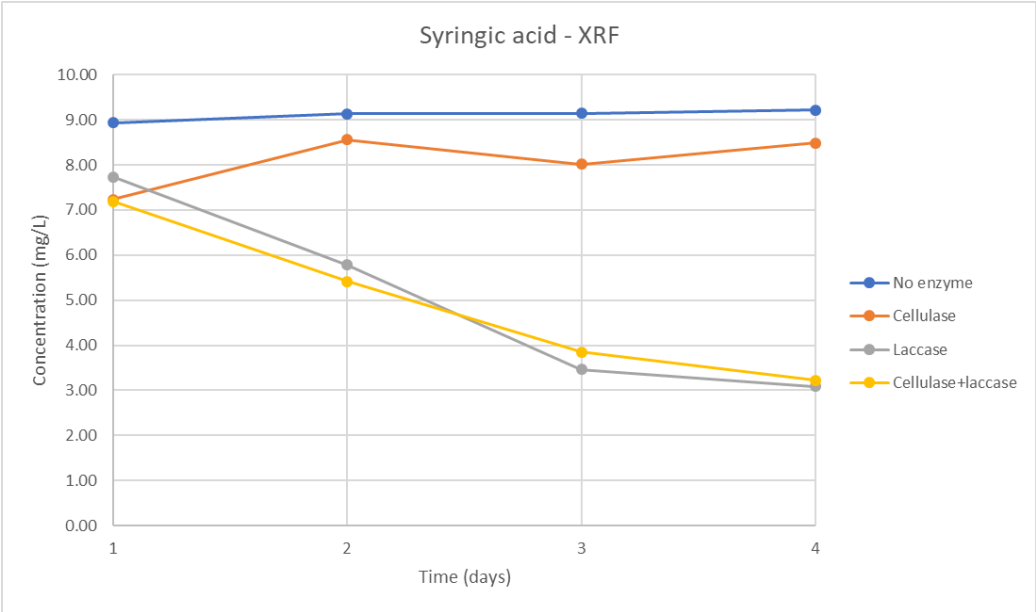

Figure 4.18: Syringic acid concentration in function of time in experiment 1.

No graph for Vanillin is shown, as the concentration was 0 g/L for all Erlenmeyers in experiment one.

The only Erlenmeyer with syringaldehyde present was the one with no enzymes. Its concentration rose slightly at first but then remained constant.

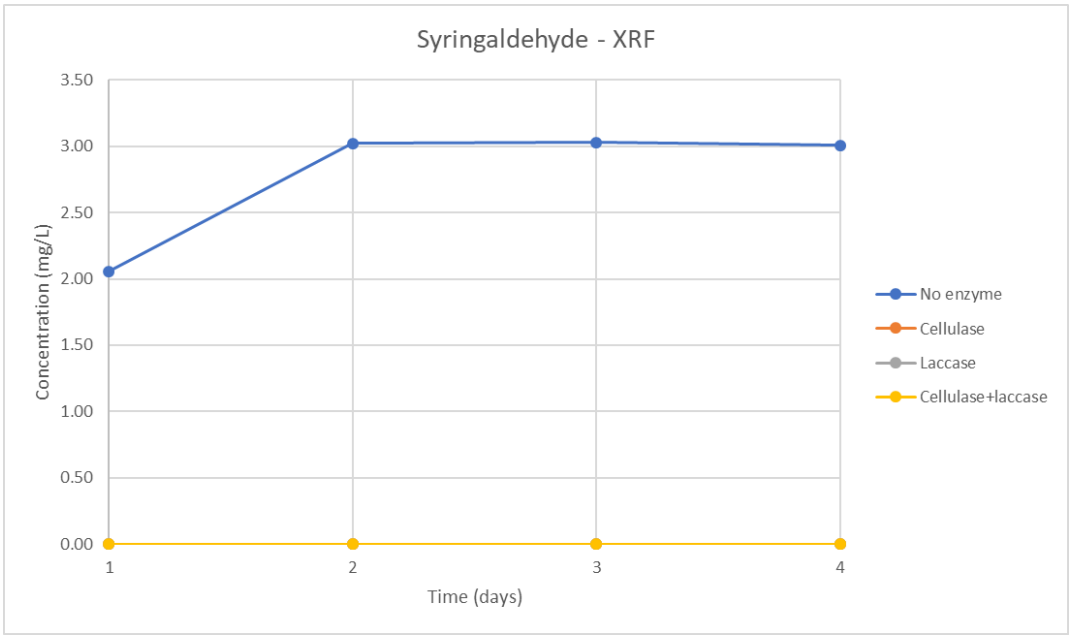

Figure 4.19: Syringaldehyde concentration in function of time in experiment 1.

4.4 Detoxification of the solid fraction

4.4.1 Sugars and organic acids concentration in function of time in experiment 2

Glucose is only present in Erlenmeyers containing cellulose or cellulose and laccase. In both these cases, glucose concentration increases in a very similar way and reaches a similar final concentration, as reflected in Figure 4.20. However, while the Erlenmeyers with cellulase start with no glucose, the Erlenmeyers that also contain laccase in addition to glucose have a glucose starting concentration slightly above 0.

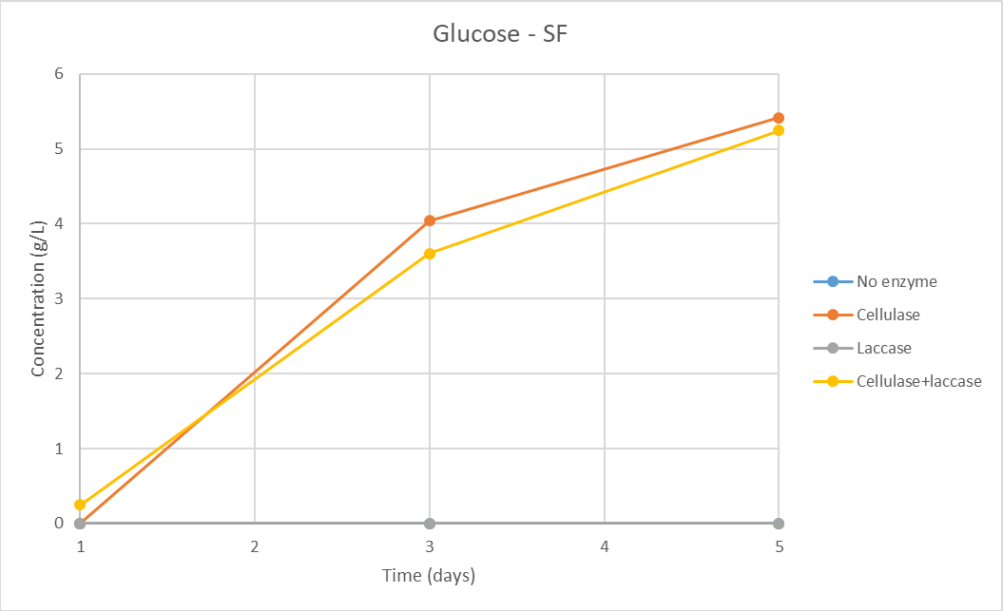

Figure 4.20: Glucose concentration in function of time in experiment 2.

Xylose concentration shows in Figure 4.21 a similar pattern to glucose concentration: xylose is only present in Erlenmeyers with cellulose or cellulose and laccase, in addition to increasing in concentration with an increase in time.

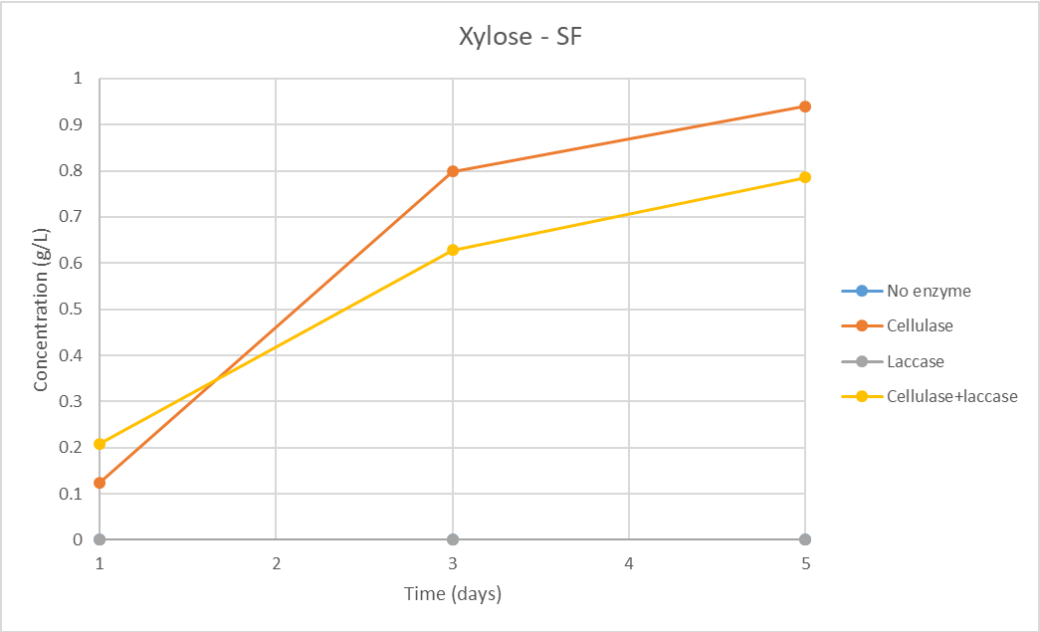

Figure 4.21: Xylose concentration in function of time in experiment 2.

Similarly to experiment 1, it can be observed in Figure 4.22 that the concentration of formic acid increases in the Erlenmeyer with no enzymes. In all other Erlenmeyers, the overall concentration after five days remains roughly unchanged.

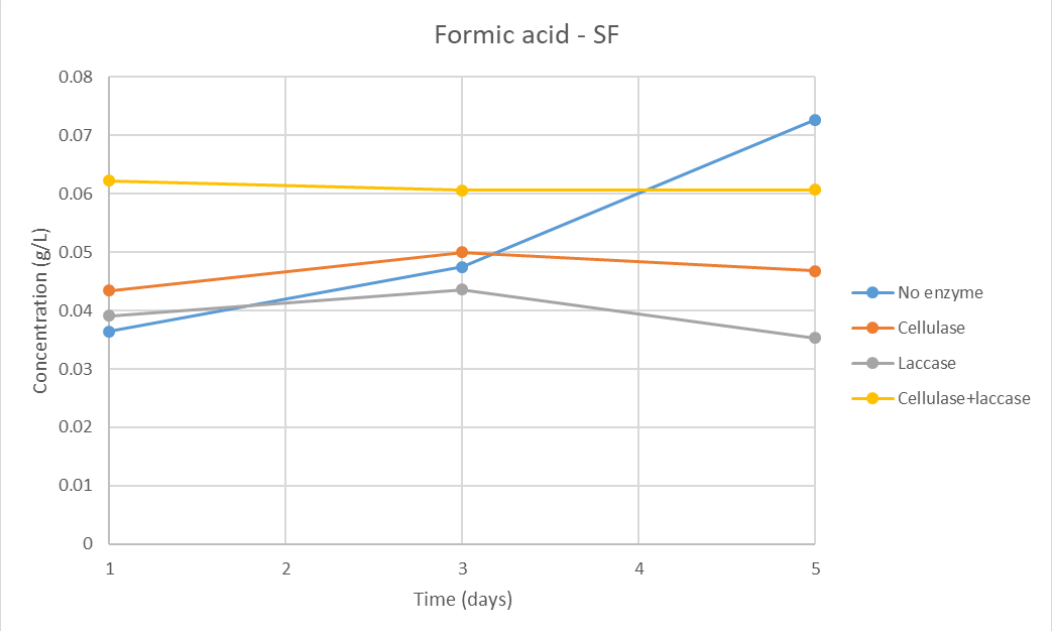

Figure 4.22: Formic acid concentration in function of time in experiment 2.

Acetic acid concentration shows a clear increase in the Erlenmeyers with cellulase and those with cellulase and laccase. On the other hand, there is an overall decrease in the concentration of acetic acid in the other two Erlenmeyers which contain either laccase or no enzymes.

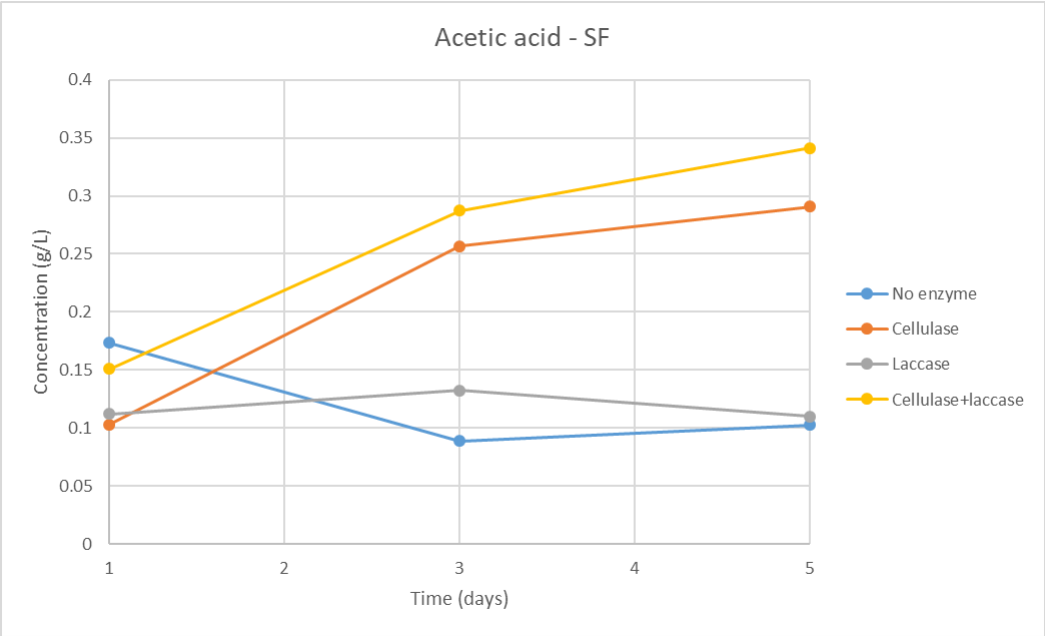

Figure 4.23: Acetic acid concentration in function of time in experiment 2.

4.4.2 Aromatic compounds concentration in function of time in experiment 2

In general, the concentration of most furanic and phenolic compounds in experiment 2 remained relatively constant overall, as can be seen in Figures 4.24 to 4.28.

For the Erlenmeyers with laccase, the concentration of 5-HMF, furfural and 3,4-dihydroxybenzaldehyde all decrease by the third day but then go back to day 1 values.

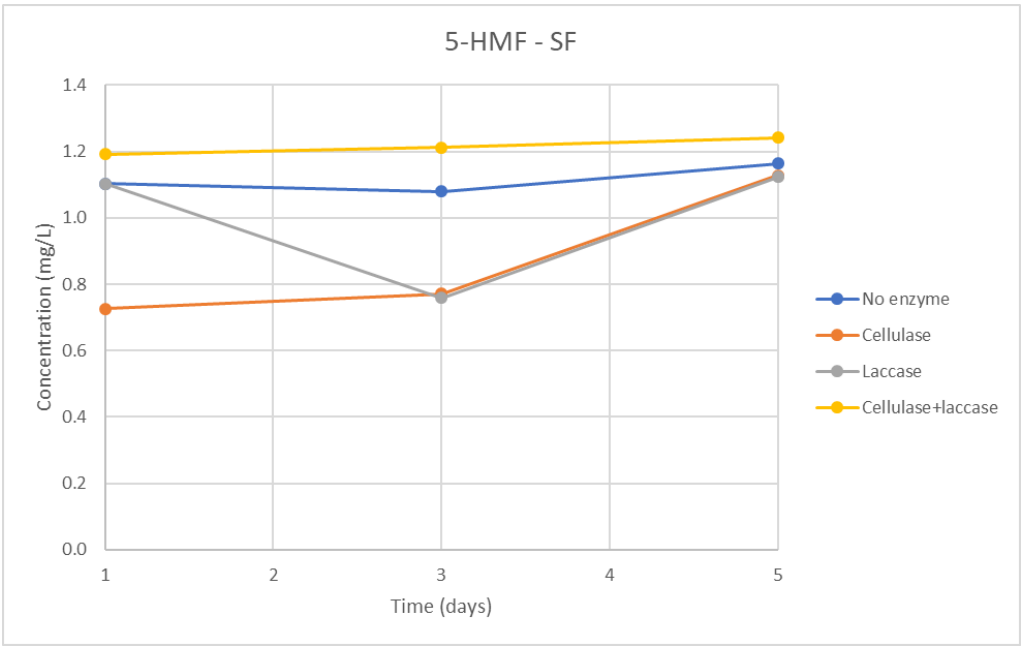

Figure 4.24: 5-hydroxymethylfurfural concentration in function of time in experiment 2.

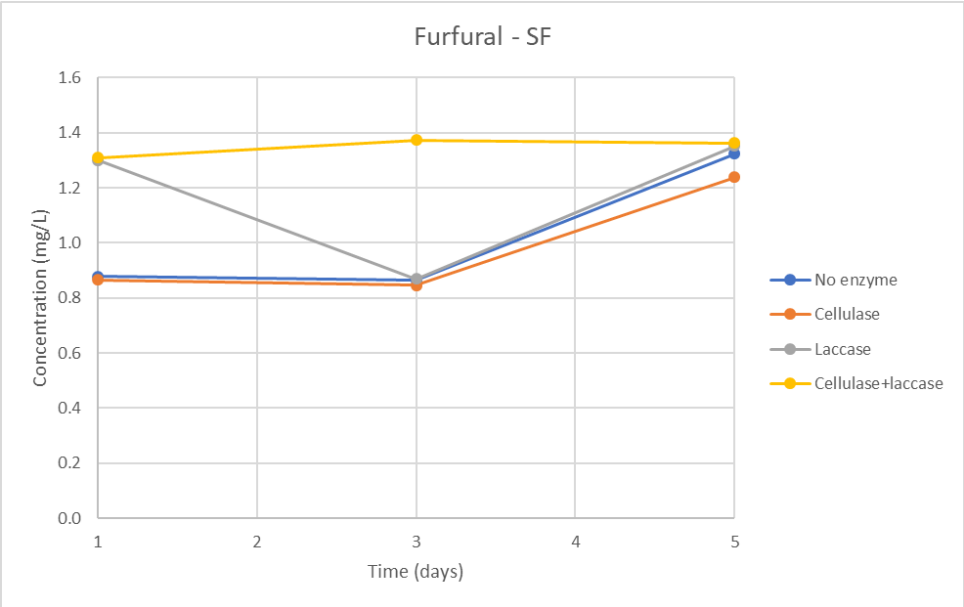

Figure 4.25: Furfural concentration in function of time in experiment 2.

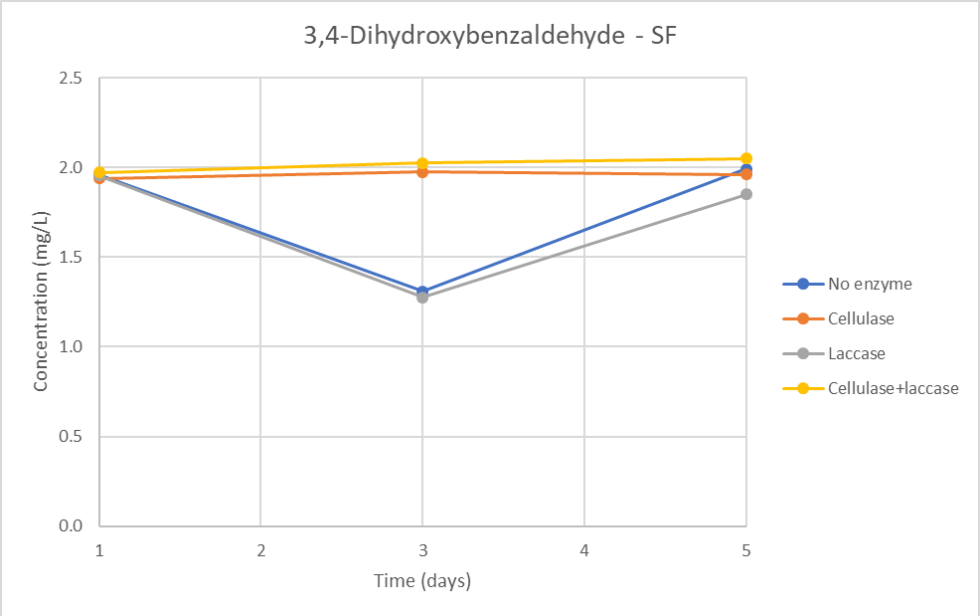

Figure 4.26: 3,4-Dihydroxybenzaldehyde concentration in function of time in experiment 2.

With reagards to vanillic acid, there is an increase in concentration in the Erlenmeyer containing cellulase and no enzyme while the Erlenmeyer with both cellulase and laccase suffers a slight decrease in concentration. No vanillic acid is present in the Erlenmeyer with laccase.

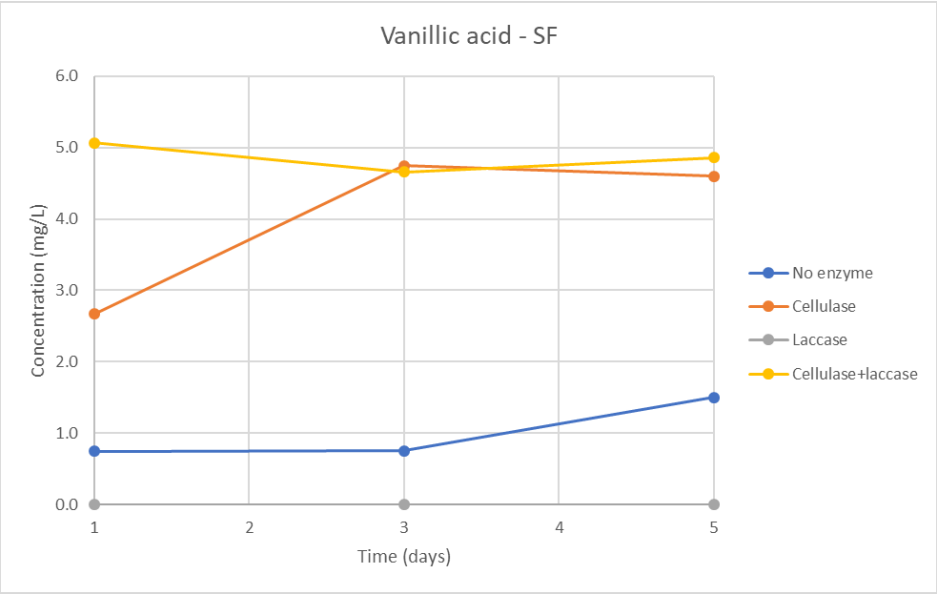

Figure 4.27: Vanillic acid concentration in function of time in experiment 2.

For syringic acid concentration, it is at its highest in experiment one in the Erlenmeyer with laccase, as the concentration in this Erlenmeyer first decreases but then increases by day 5.

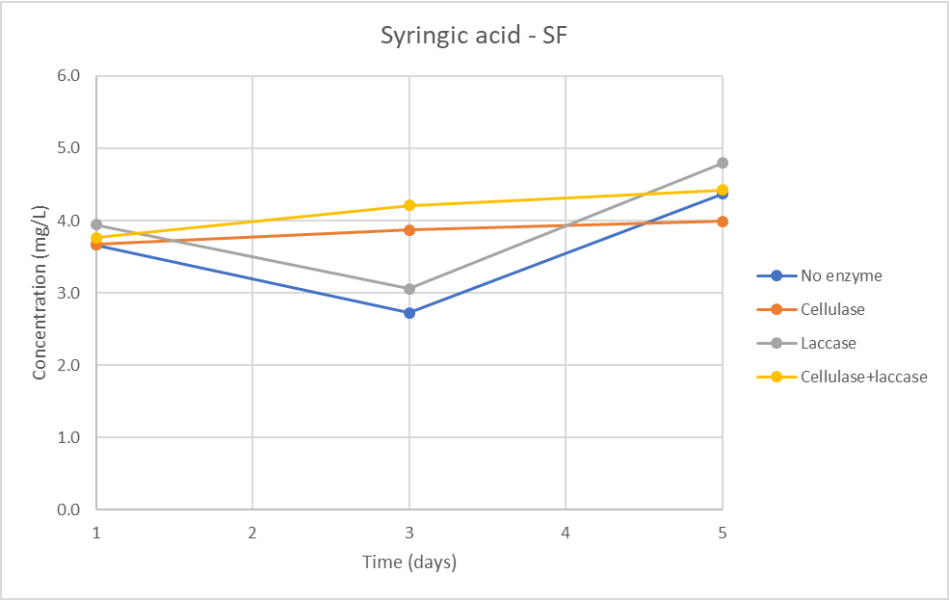

Figure 4.28: Syringic acid concentration in function of time in experiment 2.

No graphs are displayed for 4-hydroxybenzoic acid, vanillin or syringaldehyde as their concentrations were not detected by HPLC.

4.5 Detoxification of the liquid and solid fractions

4.5.1 Sugars and organic acids concentration in function of time in experiment 3

In the last experiment, glucose concentration shows a similarity with experiment 2. The Erlenmeyers containing cellulase had the most prominent increase in glucose concentration, whilst the ones with cellulase and laccase had a slightly smaller increase. This can be seen in Figure 4.29.

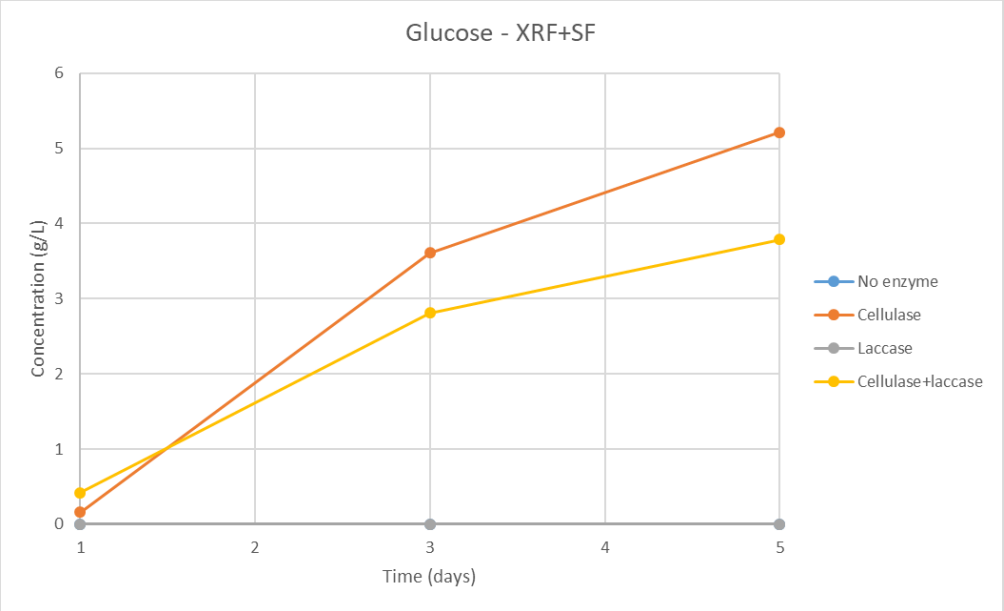

Figure 4.29: Glucose concentration in function of time in experiment 3.

Figure 4.30 shows the concentration of xylose in experiment 3 increases in a similar way to that of glucose for the Erlenmeyers with cellulase and cellulase and laccase. However, even if the starting concentration of xylose is higher than that of glucose, higher concentrations of glucose are reached by the end of the 5 days. The increase in concentration of glucose also shows a steeper trend than that of xylose from days three to five, which suggests that while cellulase has degraded most of the xylan from the hemicellulose in the liquid fraction by day 5, it still has glucan from the cellulose in the solid fraction to break down.

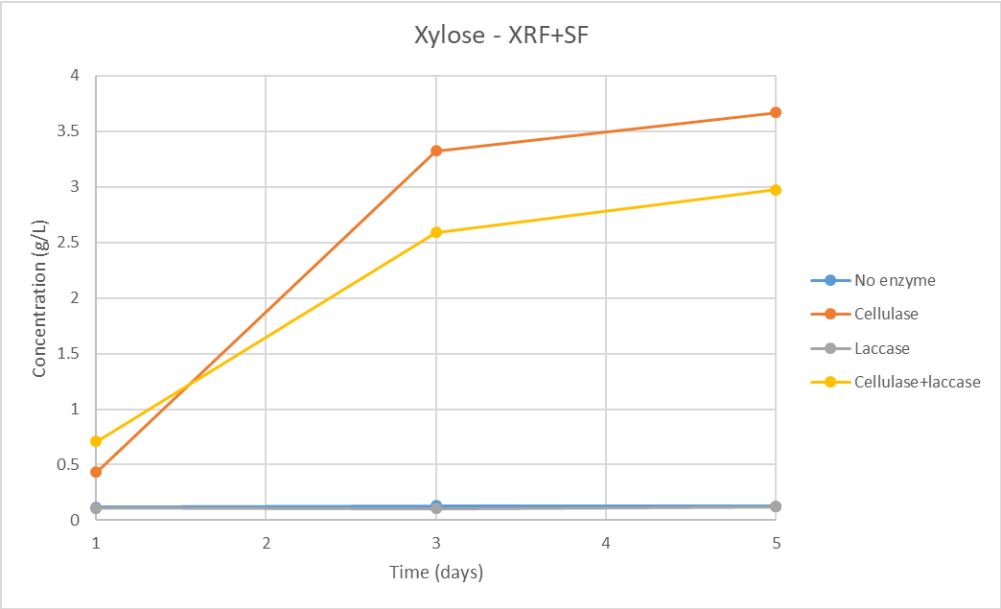

Figure 4.30: Xylose concentration in function of time in experiment 2.

Formic acid concentration can be seen in Figure 4.31 to remain relatively constant in the presence of cellulase and laccase and with no enzymes added. In the case of only cellulase present, there is a slight decrease in concentration while the presence of laccase has the opposite effect.

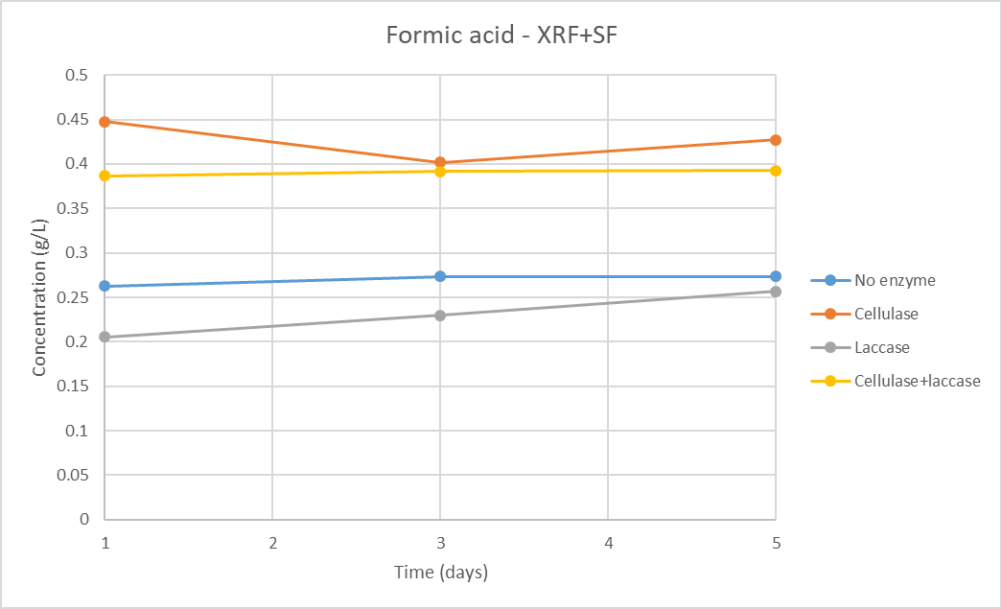

Figure 4.31: Formic acid concentration in function of time in experiment 3.

Finally, acetic acid concentration increases in the presence of cellulase and cellulase and laccase but remains constant with no enzymes or with only laccase, as shown in Figure 4.32.

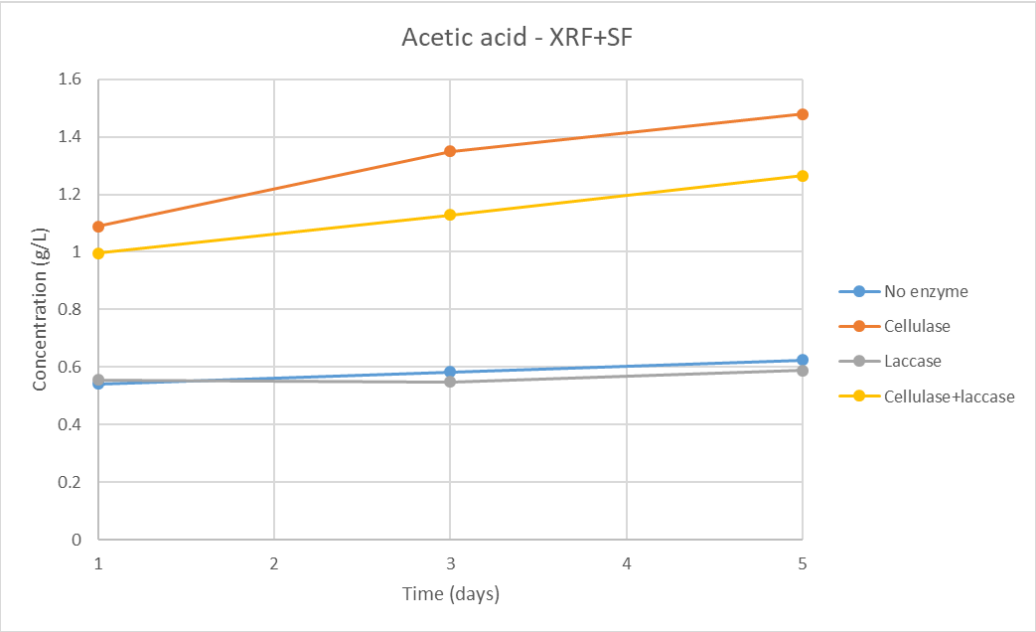

Figure 4.32: Acetic acid concentration in function of time in experiment 3.

4.5.2 Aromatic compounds concentration in function of time in experiment 3

The concentration of the phenolic and furanic compounds for experiment 3 are displayed on graphs in Figure 4.33 to 4.40.

The concentration of 5-HMF slightly increases overall, with the concentration found in the Erlenmeyers containing no enzyme, cellulase and laccase very similar in value and the concentration in the Erlenmeyer with both cellulase and laccase being lower.

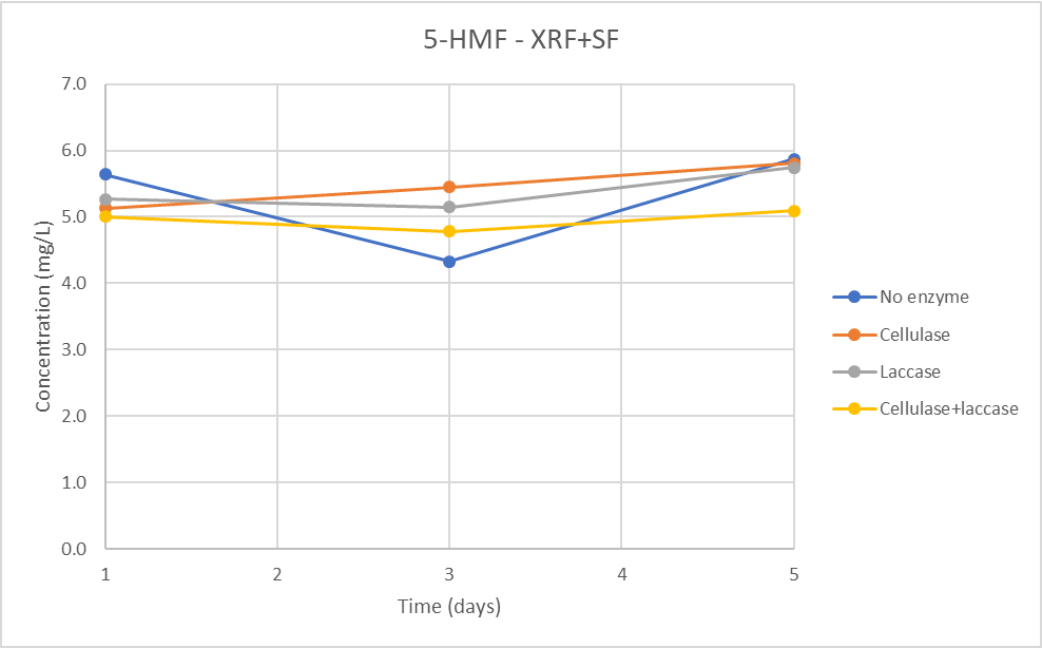

Figure 4.33: 5-hydroxymethylfurfural concentration in function of time in experiment 3.

Furfural concentration decreases in all Erlenmeyers, with the highest concentration being found in the Erlenmeyer with laccase added to it.

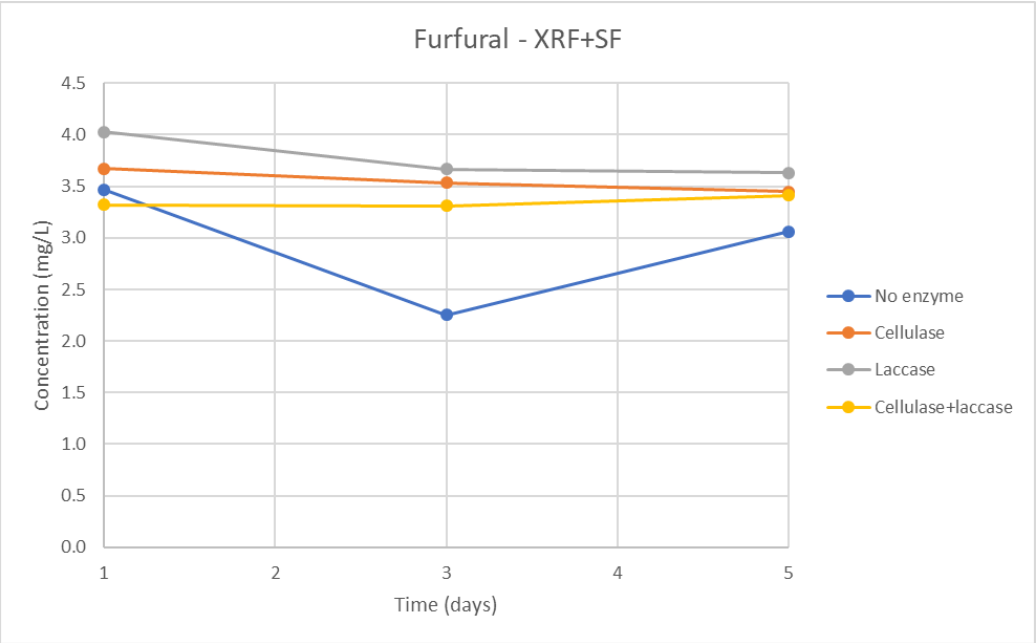

Figure 4.34: Furfural concentration in function of time in experiment 3.

For 3,4-dihydroxybenzaldehyde, no enzyme and the addition of cellulase both yield a constant concentration throughout the five days. On the other hand, Erlenmyers with both cellulase and laccase suffered a slight decrease in the concentration of 3,4-dihydroxybenzaldehyde and Erlenmyers with laccase had a more significant decrease.

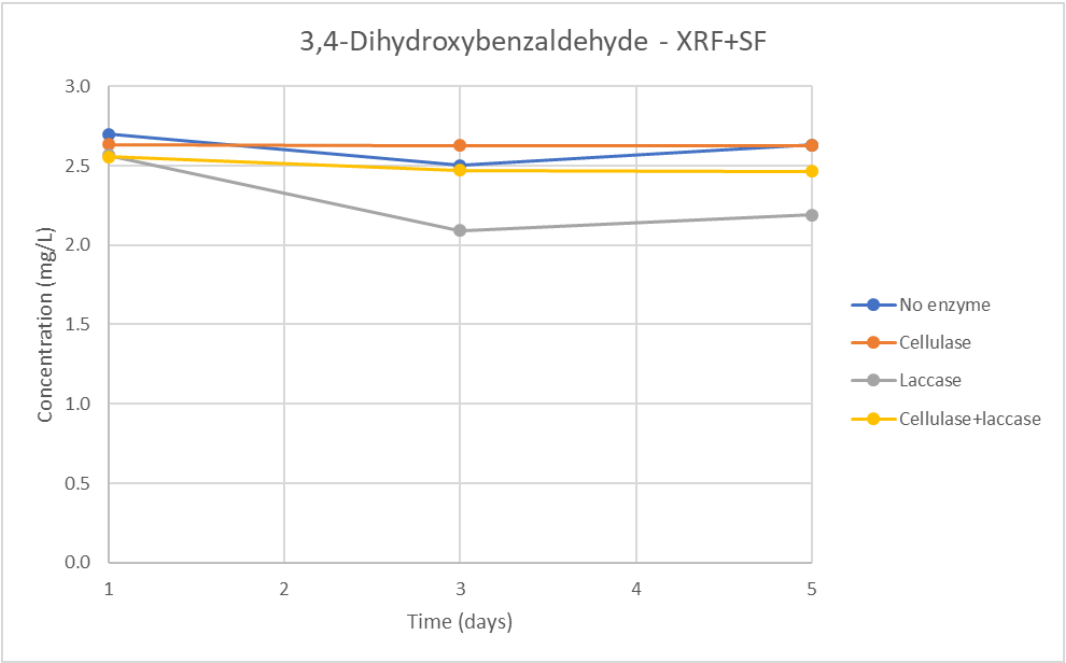

Figure 4.35: 3,4-Dihydroxybenzaldehyde concentration in function of time in experiment 3.

4-Hydrobenzoic acid was only present in Erlenmeyers with cellulase or with no enzyme. In the Erlenmeyers with cellulase, the concentration of 4-hydrobenzoic acid decreased but in the Erlenmeyers with no enzyme, 4-hydrobenzoic acid was eliminated by day 3.

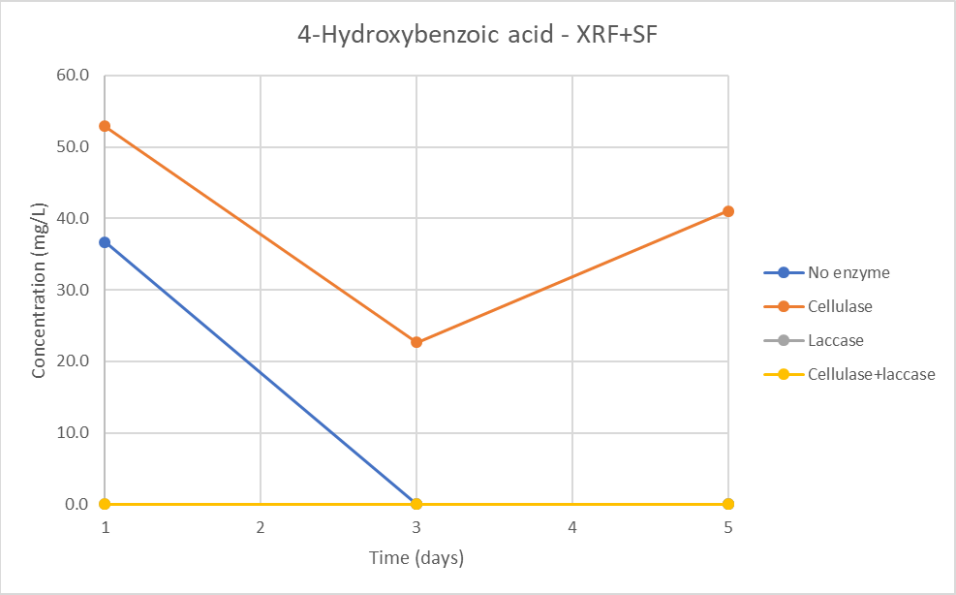

Figure 4.36: 4-Hydrobenzoic acid concentration in function of time in experiment 3.

With regards to vanillic acid in experiment 3, its concentration lowered overall, except for in the Erlenmeyer with no enzyme. The lowest concentration was found in the Erlenmeyer with only laccase and the highest in the one with both cellulase and laccase.

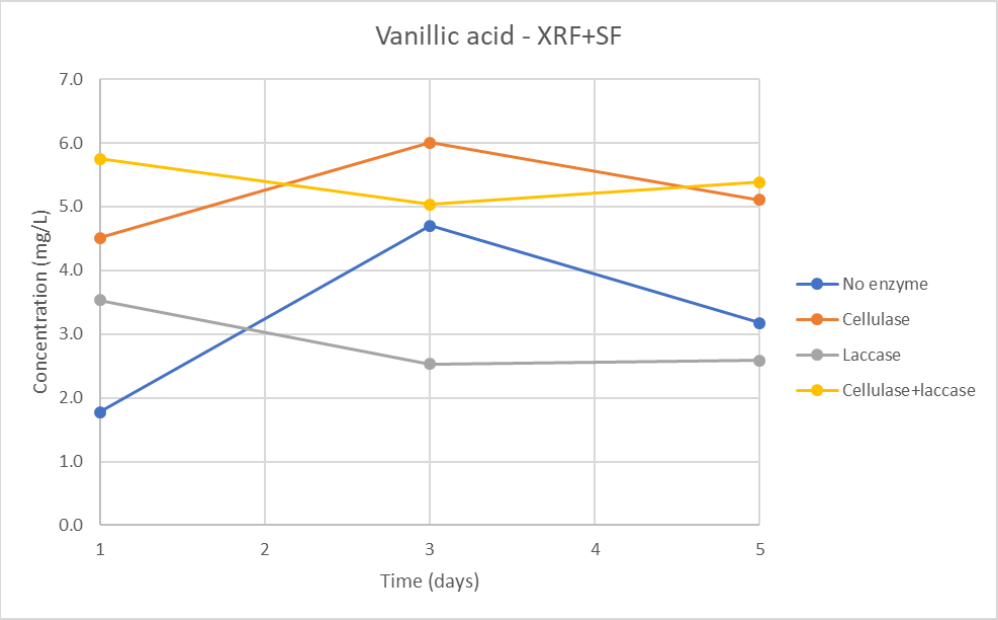

Figure 4.37: Vanillic acid concentration in function of time in experiment 3.

For syringic acid, while the concentration in the Erlenmeyers containing no enzyme, both cellulase and laccase and only cellulase increased overall by the fifth day, the concentration decreased slightly in the Erlenmeyers with only laccase.

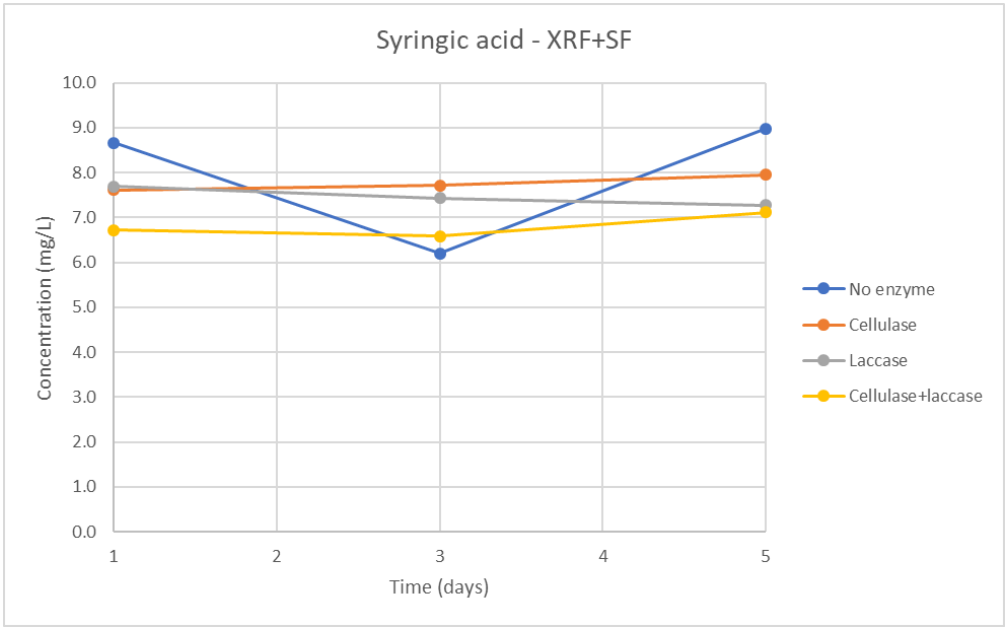

Figure 4.38: Syringic acid concentration in function of time in experiment 3.

Vanillin was only detected by the HPLC in the Erlenmeyers containing no enzyme and only on the third day.

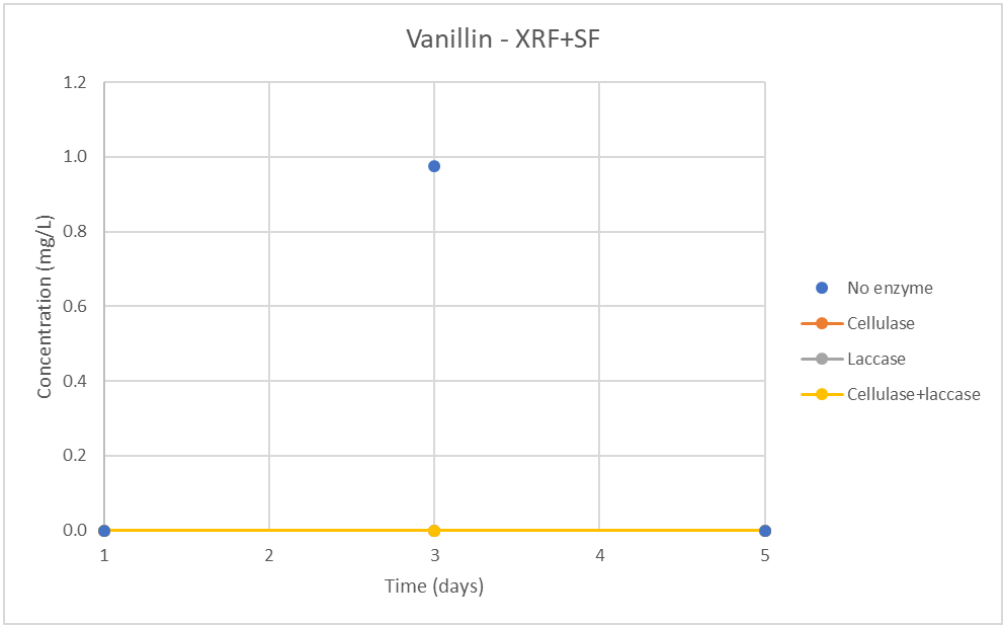

Figure 4.39: Vanillin concentration in function of time in experiment 3.

Syringaldehyde was only detected in the Erlenmeyers containing cellulase. Its concentration dropped to 0 g/L in the third day but by the fifth day it rose to higher levels than at the beginning.

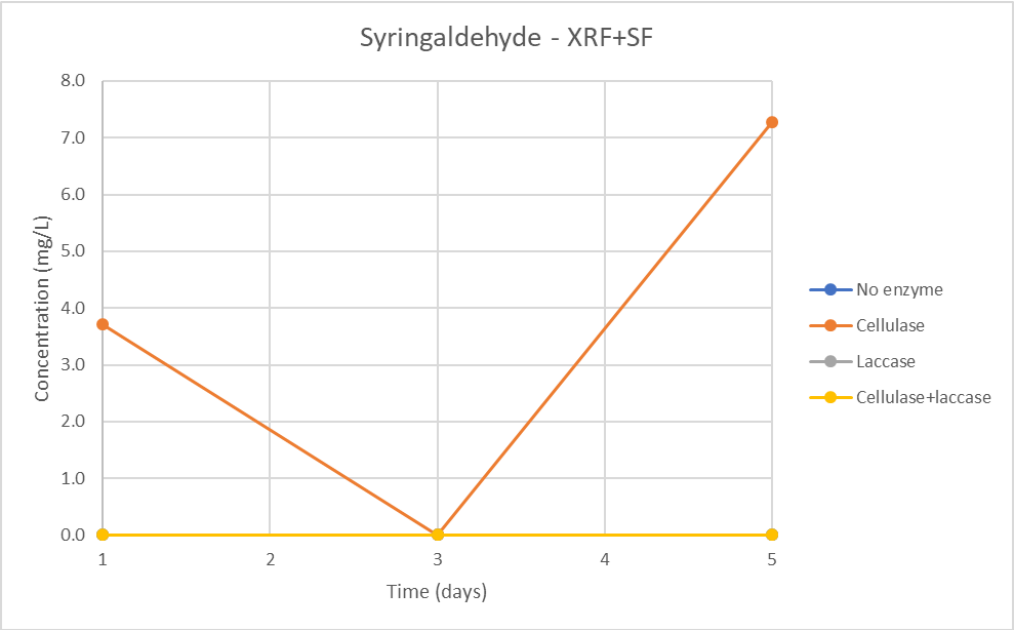

Figure 4.40: Syringaldehyde concentration in function of time in experiment 3.

Chapter 5: Discussion

5.1 Glucose concentration in function of time

In all three experiments, glucose was only detected by the HPLC in Erlenmeyers which contained cellulase or cellulase and laccase. This is as expected, as it is cellulase which is responsible for the production of glucose.

The lowest concentration of glucose was reached during experiment one, (0.167 g/L compared to 5.417 g/L in experiment two and 5.213 g/L in experiment three), where only the liquid fraction was present. This was expected, because during the steam explosion, it is mainly hemicellulose that is degraded and liquefied. On the other hand, the amount of cellulose degraded will depend on the conditions of temperature, corresponding pressure and incubation time of the steam explosion pretreatment. Therefore, the majority of the cellulose remains in the solid fraction after the steam explosion. The low concentration of glucose in experiment one is explained by the fact that the steam explosion corresponding to the liquid fraction was carried out at a low temperature as to leave the cellulose, for the most part, unaffected. In consequence, the amount of cellulose available for cellulase to degrade into oligomers was very limited.

Experiments 2 and 3 had a higher concentration of glucose due to the presence of the solid fraction. The solid fraction has a higher content of cellulose than the XRF, therefore when the SF is present, the cellulase enzyme has more substrate to produce glucose from.

With respect to the influence of laccase, no glucose was obtained when laccase was the only enzyme added. However, with both laccase and cellulase catalysing the breakdown reaction, glucose concentrations similar to those achieved only by cellulase were reached, especially in experiment 2 with only SF present and no XRF.

5.2 Xylose concentration in function of time

All Erlenmeyers showed detectable xylose concentrations except for the Erlenmeyers with no enzymes and with only laccase in experiment two (only SF, no XRF).

Xylose concentration followed practically the same pattern for the Erlenmeyers with laccase and for those with no enzyme. In the experiments with XRF, xylose concentration under these conditions remained constant and as mentioned above, xylose was not detected for the experiment with only SF. These is due to the fact that xylose comes from the degradation of hemicellulose by the enzyme cellulase. Therefore, if no cellulase is present, no xylose is produced. The reason why a small constant concentration is present in experiments with XRF and no cellulase, is that this liquid fraction contains the degraded hemicellulose from the steam explosion. Some of the hemicellulose was degraded into xylose during the steam explosion.

For the Erlenmeyers containing cellulase, xylose concentration increased in all three experiments. However, the experiments with XRF showed a much higher maximum concentration than the one with no XRF. Between experiment one and three, experiment three showed the highest xylose concentration in day 5 with 3.67 g/L. This is due to the fact that, as both the liquid and solid fractions were present in this experiment, the cellulase enzyme has more substrate to convert to xylose. Additionally, the starting concentrations of xylose was higher in the experiment with no SF. In this

experiment, more xylose than glucose was present by the end of the 5 days. This is again explained by the fact that the cellulose is mainly found in the SF while the liquid fraction is largely made up of monomeric sugars derived from hemicellulose degradation.

With regards to the Erlenmeyers containing cellulase as well as laccase, the concentration of xylose in the only SF and the XRF+SF experiments was similar to the concentration achieved by cellulase alone, although lower maximum concentrations were reached. In the experiment only containing XRF, the concentration of xylose remained constant.

5.3 Formic acid concentration in function of time

All Erlenmeyers in all three experiments contained formic acid. For the Erlenmeyers containing cellulase, formic acid concentration decreases slightly in both experiments containing the XRF, as it is a volatile organic compound so some of it is evaporating. On the other hand, formic acid concentration slightly increases in the one with no XRF present. This could be due to the fact that some of the formic acid produced during the steam explosion is captured in the cellulose matrix which makes up part of the solid fraction. This is then released into the liquid during hydrolysis.

The Erlenmeyers with no enzyme present show an increase in the concentration of formic acid, although the increase is much more noticeable in experiments one and two. The Erlenmeyers with only laccase all show different patterns. While concentration of formic acid remains more or less constant in experiment one, it decreases in time in experiment two and increases in experiment three. Finally, the Erlenmeyers with both cellulase and laccase have a decrease of the concentration in formic acid in function of time when only XRF is present and show a constant concentration when SF if present with or without XRF.

Results also show that the overall concentrations of formic acid reached are higher when XRF is present, and lower if there is only the solid fraction. This is due to the fact that, as mentioned in chapter 2, formic acid is an inhibitor derived from the degradation of furfural and 5-HMF, which in turn come from the degradation of hexose and pentose sugars found in hemicellulose and will be solubilised in the XRF.

5.4 Acetic acid concentration in function of time

Similarly to formic acid, acetic acid was also detected in all Erlenmeyers from all three experiments. In Erlenmeyers containing no enzyme, all experiments show different outcomes. For experiment one, there is a slight increase in concentration, while the concentration of acetic acid drops in experiment two and remains constant in experiment three. The addition of cellulase enzyme shows to increase the concentration of acetic acid in all three experiments. This same effect is seen for Erlenmeyers with both cellulase and laccase, except for experiment one where the concentration of acetic acid slightly decreases. This decrease could be explained by the fact that acetic acid is a volatile organic compound so some of it could have evaporated. With regards to the consequences of adding laccase, there is a small decrease in the concentration of acetic acid in experiment one, while the concentration remains constant in experiments two and three.

In a similar way to formic acid, the concentration of acetic acid is higher in the experiments containing XRF but, it is particularly higher in the third experiment, the one with both solid and liquid fractions. This is due to the fact that acetic acid is an inhibitor which comes from the degradation of hemicellulose and lignin. While hemicellulose is found in higher concentrations in the liquid fraction, lignin is part of the SF, this justifies acetic acid having significantly higher concentrations than formic acid in all three experiments but particularly in the experiments with SF present.

5.5 Aromatic compounds concentration in function of time

The concentration of phenolic compounds in the Erlenmeyers in experiments one, two and three, is very low (0-0.0064 g/L) compared with the concentration of sugars and organic acids (0-5.41g/L). As phenolic compounds are inhibitors for the enzymatic hydrolysis and the fermentation steps, ideally, their concentration should be as low as possible.

5.5.1 Erlenmeyers containing no enzymes

In experiment one, where only the XRF is present, the content of 5-HMF and syringic acid is practically constant throughout the five days. Vanillic acid and syringaldehyde concentrations increase at first but then also stabilise and remain constant. Constant concentrations are in line with what is expected, as no enzymes are present. Only the concentrations of furfural and 3,4-dihydroxybenzaldehyde decrease over the five-day period. 4-Hydrobenzoic acid and vanillin were not detected.

In experiment two, where only the solid fraction is present, 5-HMF slightly increases in concentration while syringic acid has a more noticeable overall increase. Furfural and vanillic acid concentrations behave in the same way, they remain constant until day three and increase from day three to five. On the other hand, the concentration of 3,4-dihydroxybenzaldehyde decreases rapidly from day one to three but then increases by day five to day one levels. 4-Hydrobenzoic acid, vanillin and syringaldehyde were not detected. Compared to experiment one, the concentration of all aromatic compounds but especially of 5-HMF and furfural was lower. This may be due to the different origin of phenolics and furans. As 5-HMF and furfural originate from xylose and glucose respectively and experiment two has no XRF, these compounds are more difficultly synthesised. Generally low concentrations in this experiment indicate that XRF is responsible for a large part of the aromatic compound concentration.

In experiment three, the Erlenmeyers containing XRF+SF, both 5-HMF and syringic acid show an overall increase in concentration after a drop from day one to three. Vanillic acid also increases in concentration by the end of the five days. On the other hand, furfural concentration decreases and 3,4-dihydroxybenzaldehyde remains constant. 4-hydrobenzoic acid is present at the start but by day three the concentration has dropped below detection levels for the HPLC.

5.5.2 Erlenmeyers containing cellulase

In the first experiment, the concentration of 5-HMF and of syringic acid increases and 3,4-dihydroxybenzaldehyde levels remain constant. In contrast, furfural concentration decreases, just as in the experiment containing no enzymes, but this time the decrease is at a faster rate. Vanillic acid

concentration drops from day one to day three and then remains constant at a higher value than in experiment one. 4-Hydrobenzoic acid, vanillin and syringaldehyde are not present.

In the second experiment, 5-HMF concentration increases to approximately the same levels as in the same experiment but with no enzyme. Syringic acid and vanillic acid both also increase but in the case of the former the resulting concentration is lower than that in achieved with no enzyme and in the case of the latter the final concentration is higher. Furfural concentration remains constant until day three when if increases and 3,4-dihydroxybenzaldehyde concentration remains constant. Just as mentioned above, generally concentrations of all inhibitors are lower in this second experiment that contains no XRF compared to experiment one that does.

In experiment three, the concentration of 5-HMF increases although not as much as in experiment two. In contrast, the concentration of furfural decreases slightly. It can be observed that while furfural concentration in the presence of cellulase decreases over time when XRF is also present, it increases when there is only SF. Like in experiment one and two, the concentration of 3,4-dihydroxybenzaldehyde also remains constant throughout experiment three when only cellulase is present. The concentration of vanillic acid with cellulase follows a similar patter as the one when no enzyme is present, the difference being that the final concentration reached is higher in the presence of cellulase. In contrast to experiments one and two, there is 4-hydrobenzoic acid present in experiment three and its concentration drops but then increases to an overall lower concentration. Finally, syringaldehyde has the biggest increase in concentration and vanillin is not present.

5.5.3 Erlenmeyers containing laccase

In the first experiment, 5-HMF, furfural, syringic acid, vanillic acid and 3,4-dihydroxybenzaldehyde all decrease in concentration to lower levels than those reached for the same experiment but with no enzyme or with only cellulase present. This is in accordance to what is expected, as laccase polymerises phenolic inhibitors, decreasing their concentration. No other compounds had a concentration high enough to be detected by HPLC.

In the second experiment, both 5-HMF and furfural follow the same pattern. Both have a starting concentration higher than the one in the same experiment but in the absence of laccase and both concentrations drop and then end up rising to virtually the same value as the starting concentration. 3,4-Dihydroxybenzaldehyde follows this pattern too but its final concentration on day five is lower than the starting concentration. Syringic acid concentration follows the same pattern as the one described for the same experiment but in the absence of enzymes although concentration values in the presence of laccase are shifted up slightly. All remaining compounds were not detected.

In the third experiment, 5-HMF slightly increases in concentration to approximately the same value as shown by the results for the same experiment but with no enzyme and with only cellulase. Furfural and 3,4-dihydroxybenzaldehyde decrease in concentration but while furfural has a final concentration higher than that for the same experiment but in the presence of no enzyme or of only cellulase, 3,4-dihydroxybenzaldehyde's final concentration is lower. Syringic acid and vanillic acid also have concentrations which decrease during the five-day period but vanillic acid's concentration reaches a plateau at day three. 4-Hydrobenzoic acid, vanillin and syringaldehyde are not present.

5.5.4 Erlenmeyers containing cellulase and laccase

In experiment one, similarly what happened in the same experiment but with the addition of only laccase as described in title 5.5.3, the concentrations of 5-HMF, furfural, syringic acid, vanillic acid and 3,4-dihydroxybenzaldehyde all decrease. However, they all decrease to levels lower than those achieved by the addition of only laccase except for vanillic acid, whose final concentration is higher with the addition of both cellulase and laccase. 4-Hydrobenzoic acid shows a concentration peak in day 3, while its concentration is not detectable any other day. This suggests a possible error in the sample. Vanillin and syringaldehyde are not present.

In experiment two, the concentration of 5-HMF increases slightly. Furfural has the same initial and final concentrations as the one shown by the presence of only laccase while syringic acid's initial and final concentrations are very similar to those obtained by the absence of enzymes. 3,4-Dihydroxybenzaldehyde concentration increases very slightly and ends up as the highest concentration for this inhibitor and opposite to that, the concentration of vanillic acid decreases but the final concentration is also the highest for the compound and in this experiment.

In experiment three, the concentration of 5-HMF remains constant and is the lowest concentration compared to the same experiment but with no enzyme, only cellulase or only laccase. Furfural and syringic acid both have a very slight increase in concentration. On the other hand, 3,4-dihydroxybenzaldehyde and vanillic acid both decrease in concentration. Vanillic acid concentration decreases in the same way as it does when only laccase is added, only that the concentrations are higher under the presence of both laccase and cellulase.

Chapter 6: Conclusion

The ultimate goal of the bachelor thesis was to investigate the best conditions for the detoxification of lignocellilosic hydrolysate. Ideally, the hydrolysate would contain the highest concentration possible of fermentable sugars and the concentration of inhibitors produced would be kept at a minimum in order to achieve the maximum yield of bioethanol possible. An increase in the efficiency of the production of second generation bioethanol, would increase its economic profitability, making it a viable alternative to other fuel sources which are more damaging to the environment.

The Erlenmeyers which had no enzymes added to them, behaved as expected. No glucose or xylose was produced due to the absence of cellulase and inhibitor concentration generally remained constant or saw a slight increase, with the exception of the volatile furans whose concentration decreased slightly when there was XRF present due to evaporation. The lowest concentration of both organic acids and phenolic compounds was found out to be produced when only the SF of the pretreated lignocellulosic biomass was present.

Upon adding the cellulase enzyme, the highest yield of glucose and xylose was achieved. Sugar concentrations were especially high when the enzyme was added to both XRF and the SF. However, this set up is also the one that yielded, in general, the highest concentrations of organic acid and phenolic inhibitors. It is also necessary to point out that the concentration of glucose reached when only the SF and cellulase were present, was almost as high as with the XRF+SF, and even though xylose concentration was much lower, the concentration of all inhibitors also decreased very significantly.

When only the laccase was added to the Erlenmeyers containing the pretreated lignocellulosic biomass, no xylose or glucose was produced, as no cellulase is present to degrade the cellulose and the hemicellulose. On the other hand, there was a clear detoxification effect by the laccase, as the concentration of phenolic compounds decreased. This effect was more clearly reflected in the results yielded by the concentration of phenolic compounds where only XRF was present. However, it has to be noted that the decrease in concentration of 5-HMF and furfural may be due, partly, to evaporation, as they are both volatile compounds. With respect to the effect of laccase on organic acid's concentrations, the effects were varied. In the experiment with only XRF an in the one with only SF, there was a very slight decrease in concentration which could be attributed to evaporation, as both acetic and formic acid are volatile organic compounds. However, when both XRF and SF were present, acetic and formic acid concentrations increased slightly. This could be due to the fact that the effect of evaporation was not big enough to compensate for the acetic and formic acid captured in the cellulose matrix during the steam explosion and released during hydrolysis.

Finally, the combination of both cellulase and laccase produced no glucose or xylose in the absence of the SF. In those Erlenmeyers with either the SF or XRF+SF, the increase in concentration of sugars over the five days was lower than that produced by only cellulase. Although, the final concentration of glucose reached when only SF was present, came very close in value to the concentration given by only cellulase. This decrease in glucose and xylose yield when laccase is combined with cellulase and there are phenolic inhibitors present, is in accordance to what has been observed in similar studies such as Oliva-Taravilla et al.[22]. The reason for this decrease in sugar yield is the inhibitory effect added by

the oxidation of lignin which is catalysed by laccase. There is an increase in the number of lignin-cellulose interactions and therefore, the cellulose is less available for the cellulase to catalyse its breakdown. In addition, laccase also enhances the synergetic effect of phenolic oligomers interacting with hydrolytic enzymes and specially endoglucanases, cellobiohydrolases and xylanases.

Regarding the effect of combining both enzymes on the inhibitor concentration, although the organic acids had a higher concentration than with cellulase with only the SF present and a lower concentration in the presence of both XRF and SF, the overall concentrations were still lower in the Erlenmeyers with SF only. Cellulase and laccase together, had the biggest detoxifying effect of phenolic inhibitors in the Erlenmeyers containing only the liquid fraction. However, despite this detoxifying effect in the liquid fraction, phenolic compound concentration was still lower when only SF was present, even if in this case, phenolic concentrations either stayed the same or increased slightly.

Chapter 7: References

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Chapter 8: Attachments

8.1 Calibration curves for sugars and organic acids

The calibration curves used to convert the area value given by the HPLC analysis of sugars and organic acid samples are shown below in Figures y to y.

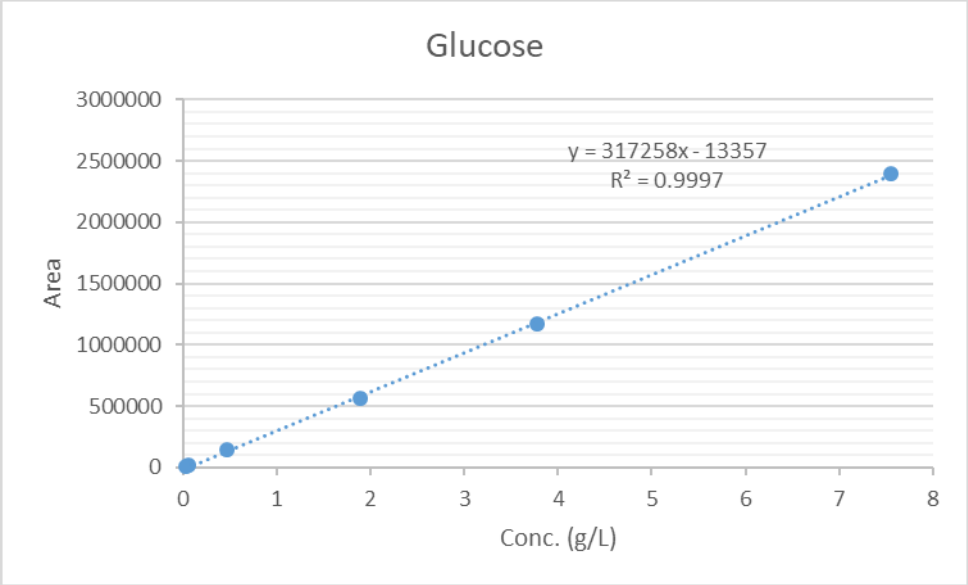

Figure 8.1: Calibration curve for glucose.

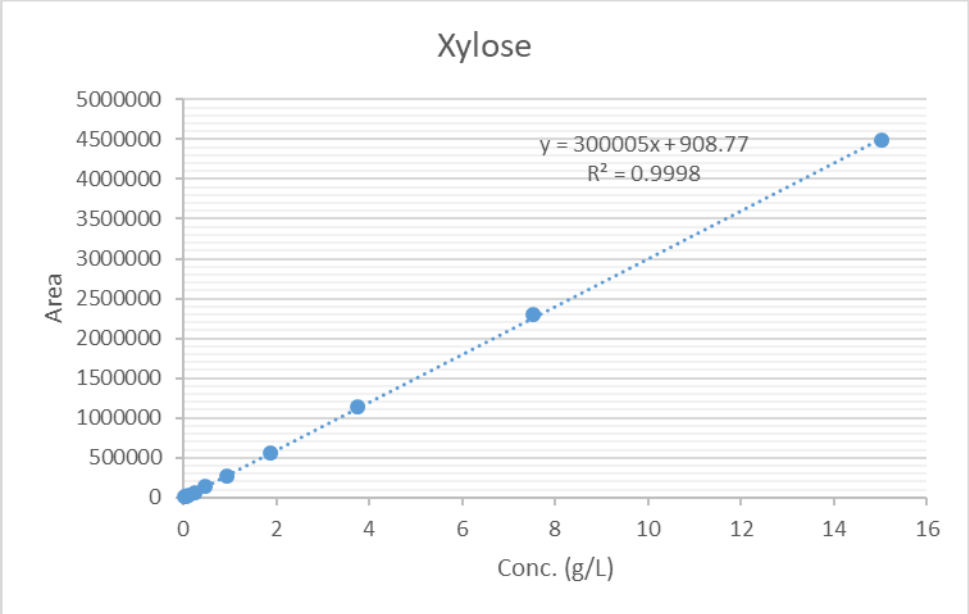

Figure 8.2: Calibration curve for xylose.

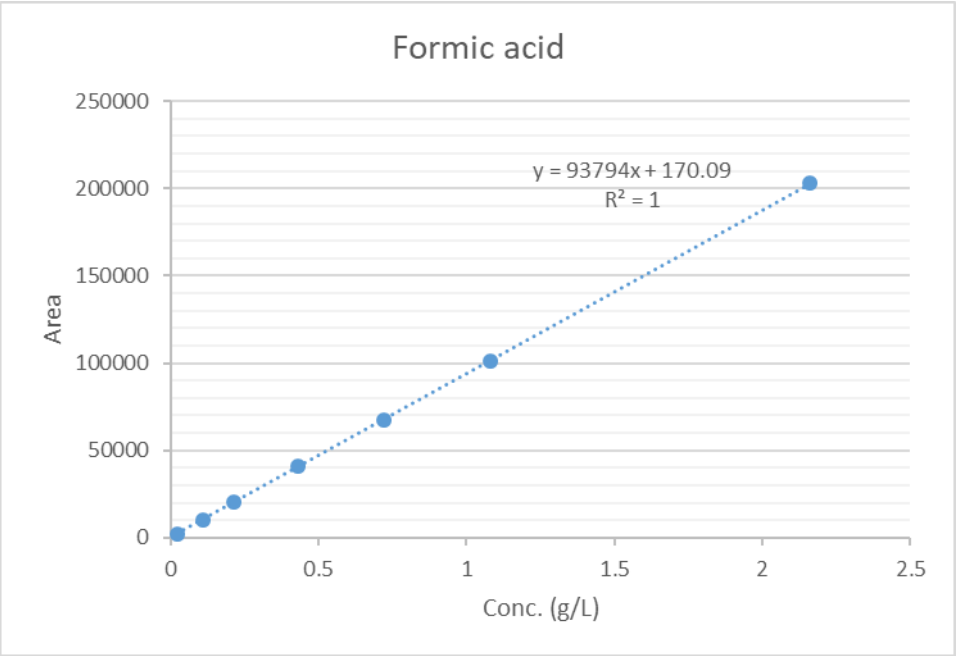

Figure 8.3: Calibration curve for formic acid.

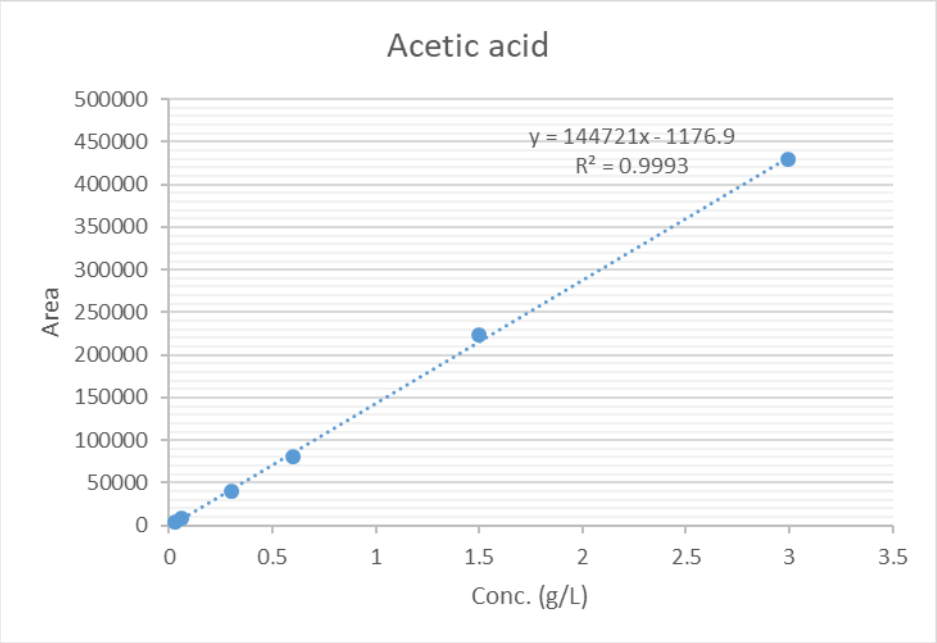

Figure 8.4: Calibration curve for acetic acid