SUBCRITICAL WATER MEDIATED HYDROLYSIS OF CIDER LEES AS A ROUTE FOR RECOVERY OF HIGH VALUE COMPOUNDS

by

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Abstract

Spent cider lees(yeast), a by-product of the cider industry, was subjected to subcritical water in a batch process over a temperature range of 100 °C to 300 °C. The efficacy of subcritical water to convert the waste biomass into a mixture of valuable compounds was investigated by studying formed products in the extracts. At milder temperatures (up to 150°C), yeast intracellular contents such as proteins were released. Also, phenolic compounds which were previously adsorbed on cell walls during fermentation start to release, which was shown for the first time, leading to an increase in the antioxidant activity of the extracts (maximum 80 g ascorbic acid equivalent in 100 ml). The release of phenolics was compared to organic solvent extraction and the same level of extraction was observed in subcritical water treatment. At higher temperatures, polymers of the yeast cell wall started to solubilise producing monosaccharides which later were converted to hydroxymethyl furfural (HMF). Kinetics of HMF synthesis were determined using first order kinetics suggesting that milder conditions are required for HMF production when compared to the same process using cellulose as substrate. Results support the potentials of the studied feedstock as a substrate for numerous bio-based chemicals.

To My Parents and
Maryam, My Adored Wife

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Conducting experiments in supercritical systems is a tricky business and it is thanks to the support of our group members. Thanks in particular to Dr. Teijun Lu for his technical knowledge on any equipment in the lab, especially the high pressure systems. I also want to thank the technicians Elaine and Hazel in Biochemical Engineering and Mr. Graham Burns and Dr. Peter Ashton in Chromatography and Mass spectroscopy labs in Chemistry for their assistance with analysing hundereds of samples. For having a nice time in Birmingham and in the supercritical fluids group in University of Birmingham, my thanks go to my friends Muhammad, Naeema, Mauricio, Pom, Raslan and Ricardo.

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Chapter 1

INTRODUCTION

1.1 Background and aims

Current trends on climate change and depletion of natural resources has accelerated the research on possible alternatives for energy and chemicals in the recent decades. While studies highlight the growing demand for renewable resources, the search for sustainable sources of energy and derivatives is still evolving. The quest for alternative sources arises mainly from the limited supplies of hydrocarbon sources, the negative impacts of their consumption and its detrimental effect on environment and also the political instability associated with energy supply. Even though, fossil fuels have been an efficient option for energy and material production, which is based on petroleum chemistry and has been developed and optimized since 19th century (Lyko et al. 2009).

In practice, our options are only restricted to resources which are cheap, abundant, easily renewable and have the potentials of deriving other materials and products from them. In addition, carbon-neutrality will be valued as it has been demonstrated in the praised energy supplies such as nuclear, wind, solar and hydroelectric. All of these options come

with some hurdles and disadvantages while they meet the required considerations for carbon-neutrality as the net production of carbon is zero after being consumed (S. Chen 2008).

The major feasible and sustainable resource for energy and chemical derivatives with zero or reduced carbon emission is biomass. It is an ideal renewable resource as it can be replenished over a relatively short timescale compared to other limited resources such as crude oil, coal, and natural gas (Clark & Deswarte 2008). Recent estimates show that nature produces more than 170 billion metric tons of biomass per year by photosynthesis while carbohydrates form 75% of it (Corma et al. 2007). Since biomass is also used as food, or constitutes part of the human and animal food chain, it is imperative to employ all possible techniques to use it in the most efficient manner possible or preferably use the non-food biomass resources (such as wood and agricultural wastes).

Current technologies to convert biomass to fuel are mainly based on three methods: First, fermentation wherein the sugars in biomass are converted to ethanol. Second, biodiesel process where oil fraction of biomass is converted to alkyl esters and finally gasification which results in the accumulation of fuel gases such as hydrogen. Most of the existing biomass conversion plants are based on one main product, e.g. biodiesel or bioethanol. Using the same approach which was undertaken during the development of oil refineries, more efficient processes will be obtained where biomass is converted to a variety of potential components other than fuels, such as chemicals or feedstocks for other syntheses steps (Lyko et al. 2009).

In this project, subcritical water at temperatures below 300 °C was investigated as a milder reaction medium for the conversion of cider lees waste to valuable products (monosaccharides, phenolic and platform chemicals). The hydrolytic condition leads to the disruption of the yeast cell walls and the release of intracellular nutrients or may be used for

the production of other intermediates (amino acids, nucleotides, lipids). The proposed process was further studied by characterisation and measurement of the generated compounds to measure the possibility of replacing traditional disposal techniques (i.e. incineration or drying), while providing extra ervenues by manufacturing new products.

The crude substrate in this project was mainly comprised from yeast cells which about 20% of its dry mass is formed of cell wall. The slightly different molecular structure in the yeast β-glucan, compared to the one in cellulose, was believed to demand milder conditions for its breakdown and conversion to other monomers. In addition, the application of crude biomass was used in order to enhance the current knowledge of hydroxymethyl furfural (HMF) synthesis from different and non-treated feedstocks. This was complimentary to the extensive researches available on applying similar processes to pure monomers for HMF production with limited application for large scale production (Bicker et al. 2003; Asghari & H. Yoshida 2006; Claude Moreau et al. 1996; Ishida & Seri 1996; C. V. McNeff et al. 2010; Claude Moreau et al. 1996).

The motivation for this research has also been to asses the hydrothermal process (referred in this work as Sub-critical water mediated hydrolysis) of cider lees, as a route to obtain higher value chemicals and extracts and also an alternative waste treatment to the land disposal and incineration. To investigate the possibility of reusing the extracts and feasibility of the process for the production of valuable chemicals, it was of special interest to study the properties of the extracts using different analytical techniques. The main analytical steps include carbohydrate measurement and identification of their derivatives and also other valuable compounds which have been released from biomass (such as proteins, polyphenolics and phenolic monomers) with possible application in food and cosmetic industries.

1.2 Structure of the thesis

Although each result chapter provides an introduction to the discussed topic, a comprehensive review of the current knowledge and recent progresses in hydrothermal conversion of biomass has been provided in the review of previous studies (chapter 2). This chapter provides a detailed overview of the current biomass conversion processes used with the focus on sub- and supercritical water.

The results of subcritical mediated hydrolysis of cider lees and the chemical composition and physical properties of the extracts were discussed in chapter 3. This chapter covers the results from total protein measurement, total organic carbon (TOC), total mass balance for the liquid and solid fractions of hydrolysates, total phenolic content (TPC) at different temperatures and times

The spent yeast cells were subjected to autolysis reaction and the results were discussed in chapter 4. Vitamin B3 was determined in autolysed samples which was in contrast to the subcritical water hydrolysis extracts. The main objective of this chapter was to find out if vitamin B3 can be derived from this feedstock using subcritical water mediated hydrolysis as yeasts are a known to be a good source of vitamin B3 and its derivatives

The prospects of using subcritical water to convert cider lees to chemicals was studied in more detail in chapter 5. It was shown that due to the presence of a carbohydrate backbone in the yeast cell wall and the hydrolytic nature of the reaction medium, degraded products (monosaccharides) and other dehydrated derivatives (e.g. HMF) could be obtained. The conversion and yield of solid solubilization were determined and used to study the kinetics of the hydroxymethylfurfural formation. The results from this chapter were compared to other studies where other biomass sources such as cellulose were used. It was proposed that the milder hydrolysis conditions obtained for yeast cell hydrolysis was due to the different types

of glycosidic bonds in glucan chain, and hence more flexible structure in the yeast cell walls when compared to cellulose.

In chapter 6 the phenolic compounds which were identified in chapter 3 were further studied and characterised. It was shown that the phenolic compounds which had been adsorbed by the yeast cell walls during the fermentation. These phenolic compounds can be released in subcritical water medium and the degree of desorption can be related to the reaction temperature. The release of these compounds were also compared to a typical organic solvent extraction and the results were discussed.

Chapter 7 is the overall conclusion and future work chapter which considers all the discussed chapters and propose additional or complimentary steps which can support this work by providing more data and study the alternative applications for this type of biomass

Chapter 2

LITERATURE REVIEW

2.1 Supercritical Fluids and Their Applications

Based on the Gibbs phase rule, a pure component can be observed in three different phases, which is determined by only two independent state variables. However, a pure component whose temperature and pressure are above its critical temperature (T_c) and critical pressure (P_c) is in supercritical state which is not a liquid nor a gas (Fig 2.1). The critical temperature is the maximum temperature at which a liquid can be formed by isothermal compression and the critical pressure is the highest vapour pressure that can be exerted by the liquid (T. Clifford 1998).

At supercritical state, fluids (liquids or liquefied gases) attain peculiar properties which can not be observed in other regions of their phase diagram. The physical properties of the fluids, such as heat capacities C_p and C_v, thermal conductivity, Prandtl number, thermal conductivity, change dramatically and become very large. However, viscosity and density of liquids become similar to gases which make them more applicable for extraction purposes. These properties are easily adjustable by change in the pressure or temperature of the system.

Supercritical fluid (SCF) extraction has been widely studied and applied for different purposes in the last three decades. The main industries which have observed the application

of these technologies use SCF in the extraction of hops, decaffeination of coffee, isolation of flavours and antioxidants from plants and natural resources (T. Clifford 1998). Carbon dioxide (CO_2) plays the main role in this technology due to its attractive properties as a non-toxic, cheap and abundant solvent which requires lower energy and operating costs due to its easily achievable critical point.

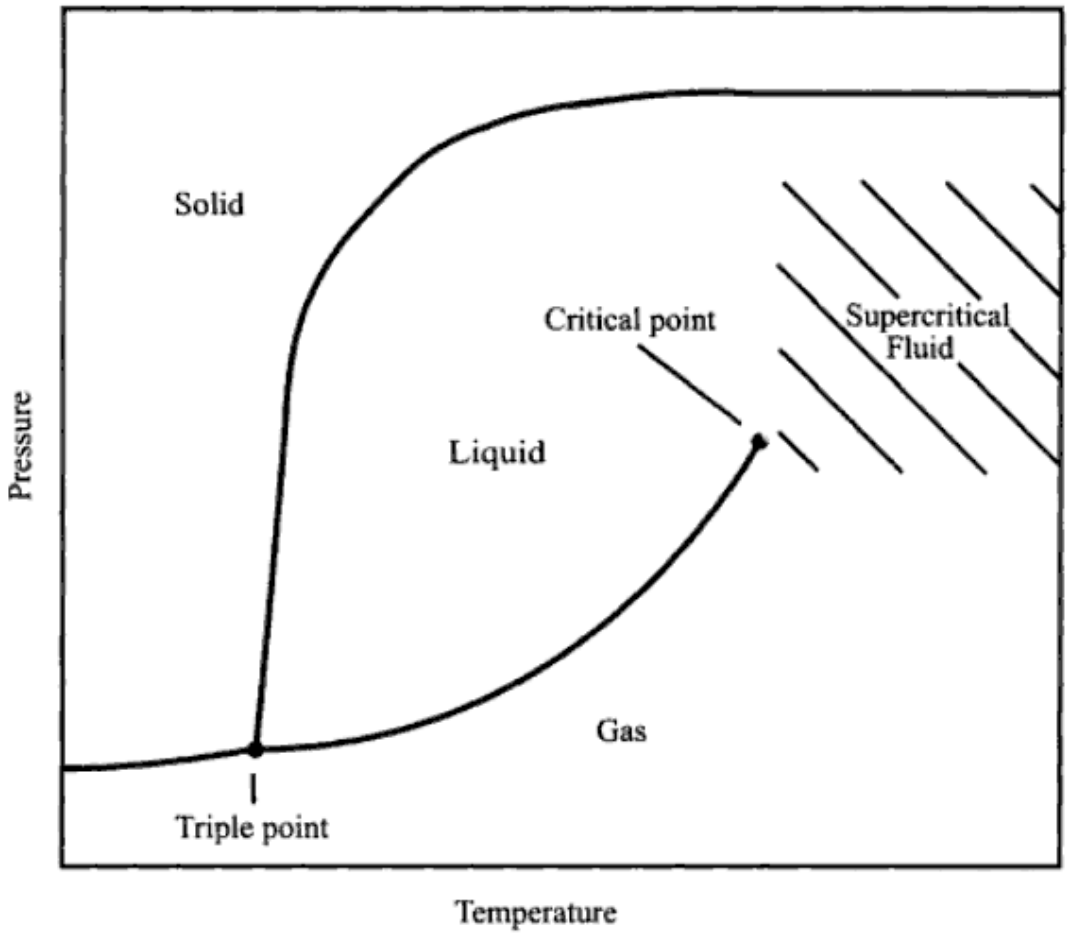


Figure 2.1 The phase diagram of a single substance (Clifford, T., 1998)

In addition to the extraction, other applications for SCF's which have been extensively investigated and developed are as below:

- Material and Polymer Production
- Food Processing
- Supercritical Fluid Chromatography
- Reactions
- Environmental Applications

Different chemicals have been investigated for their specific characteristics in their supercritical region. Besides CO_2, water is one of the most studied liquids and its application when is hot and compressed dates back to early 70 s in the last century. Due to its important role in this project, as the main reaction medium in the experiments, subcritical water properties and its applications are reviewed and compared to supercritical states.

2.2 Subcritical and Supercritical Water

2.2.1 Introduction

Comparing to other supercritical fluids, supercritical water is a recent addition to the list of intensively studied supercritical fluids (Afonso & Crespo 2005). Water has a high critical temperature (T=647.1 K =374 °C) and pressure (p=22 MPa) where in most of industrial applications may be regarded out of reach due to the cost of the high pressure apparatus and the required energy. Traditionally, the main area of application for high pressure steam has been the power plant cycles or hydrothermal reactions for water at superheated conditions. However, its unique properties at these conditions have been considered attractive for many applications such as alternative fuels, coal and biomass conversion and waste disposal. These latest applications were mainly started in the late 70s when the first oil crisis occurred and at the same time, there was increasing concerns about the environmental impacts of traditional chemical processes and application of supercritical fluids (G. Brunner 2009).

2.2.2 Properties of near critical water

Subcritical water (which also has been referred in literature as hot compressed water (HCW), superheated water, high temperature water (HTW), pressurised hot water) exhibits properties which are different from those of water at ambient temperature. Water changes from a solvent for ionic species to a solvent for non-ionic species when it pressurized and heated from ambient to near its critical point and beyond (G. Brunner 2009). These changes can be divided into two categories of microscopic and macroscopic properties. When compared this way, these changes may seem contradictory sometimes. For example, water becomes more nonpolar at these conditions while the individual molecules are still polar. This, however, has resulted in specific properties of water make it suitable for some reactions (Kruse & Dinjus 2007b). These properties are discussed further in following sections.

2.2.2.1 Microscopic properties

Hydrogen bonds

Increasing the temperature of compressed water as liquid, will result in lower hydrogen bonds between water molecules which has been demonstrated previously by Coitisno *et al.* (Coitino et al. 1994). The breakdown of hydrogen network, which is also in direct relation to density (Fig. 2.2), increases the dynamics of water molecules, cause the formation of "clusters" of water molecules and leads to an increased mobility of single water molecules (Akiya & Savage 2002).

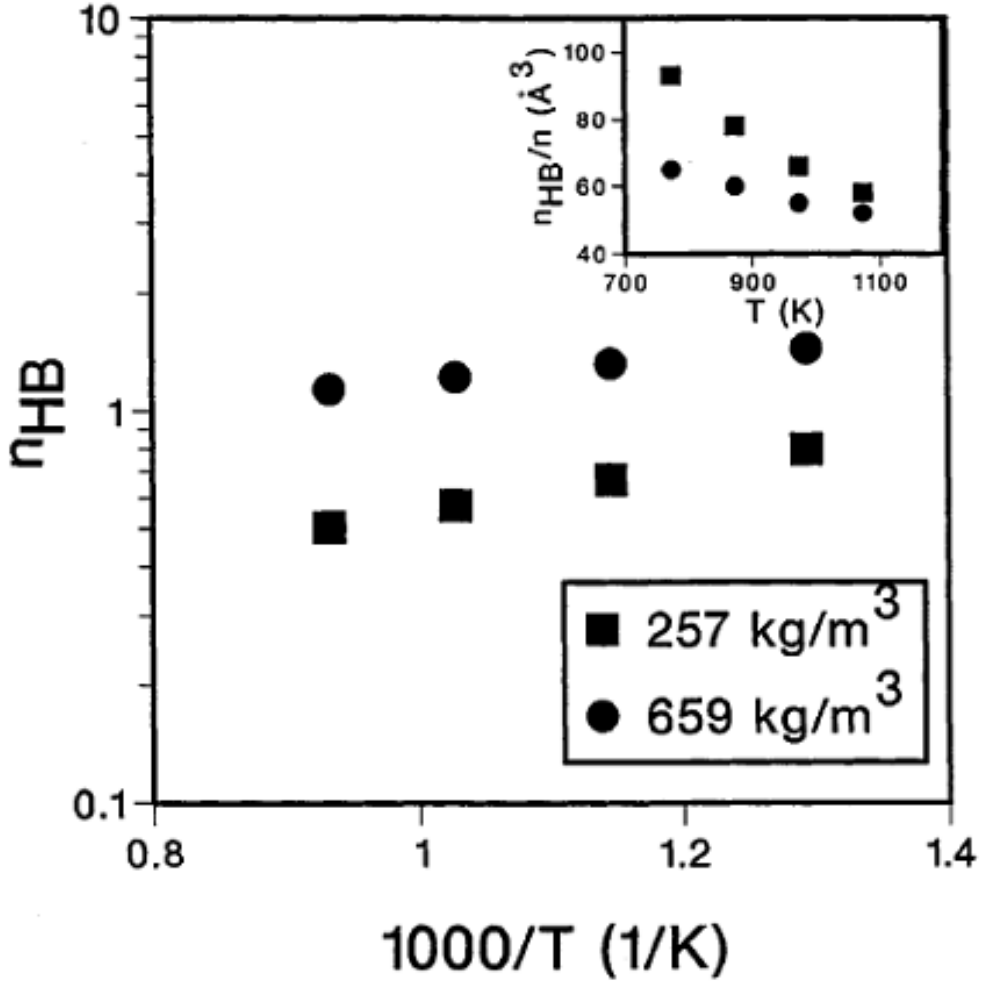


Figure 2.2. Number of hydrogen bonds per water molecule (adapted from (Akiya & Savage 2002)

Ultimately, this results in the higher self diffusivity of molecules, increased energy transfer between solutes and solvent and also higher reactivity of H^+ and OH^- ions (Kruse & Dinjus 2007b).

Collision frequencies

High pressure can change the reaction rates of different compounds in the water, though its effect can be different in various cases. For examples, free radicals may experience improved rates at higher pressures due to the superior energy equilibration rate which is caused by the higher collision frequency (Hippler 1997). As long as the pressure in the supercritical fluid system is maintained below 1000 atm, it can be assumed that the rate

of diffusion of free radicals dominates higher pressure effects and are higher than in liquids (Subramaniam & McHugh 1986). On the other hand, reactions which occur with higher-molecule mass compounds, e.g. reactions taking place during pyrolysis, are affected by the cage-effect which is a result of applying high pressure to water. Due to this effect, reaction rates are shown to be affected and decrease slightly (Ederer et al. 1999).

Dipole moment

The permanent dipole of the water molecule makes it very reactive in many reactions such as hydrolysis. It has been shown that it can take part during the activation steps of the reactions such as oxidation of the organic compounds (Kruse & Dinjus 2007b). After being heated and compressed, however, water structure can be influenced by the change in the function of pair correlation which can be due to the modification in the tetrahedral coordination and at the same time higher numbers of water molecule clusters. In practice, the structure of water at such conditions is an average of these two parameters.

2.2.2.2 Macroscopic properties

Similar to other fluids that approach their critical point, the properties of the vapour phase and liquid phase become very similar and finally identical. Supercritical water, however, shows various densities at different conditions which range from liquid-like to gas-

like values while there is no phase change throughout these conditions. Figure 2.3 indicates how these properties change while Table 2.1 listed some exact values for subcritical and supercritical water at selected conditions.

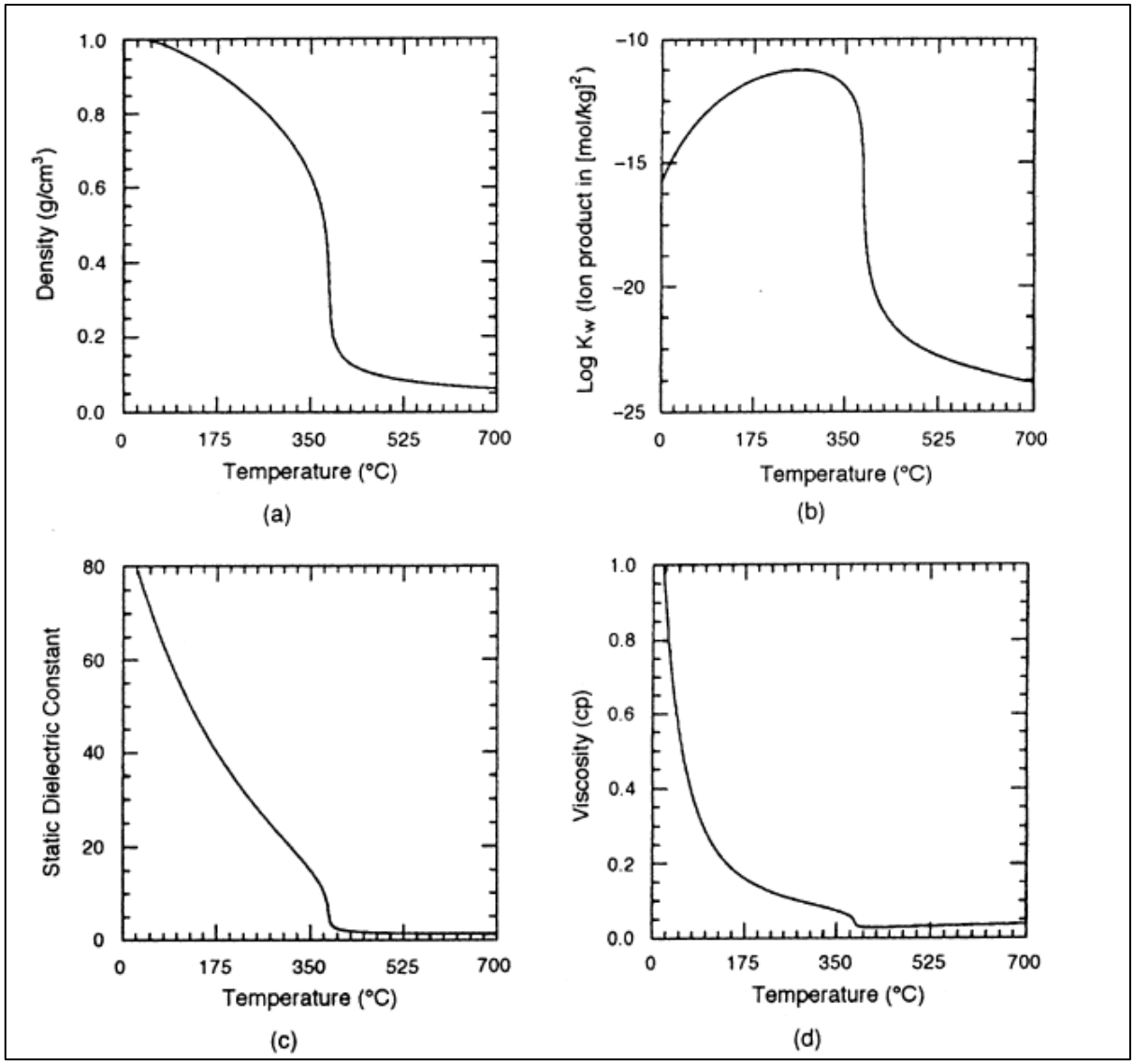


Figure 2.3. Properties of pure water at 250 bar as a function of temperature (Akiya & Savage 2002)

Table 2.1. Properties of water under different conditions (adapted from (Y. Yu et al. 2008))

Water Condition	ordinary(STP)	subcritical	supercritical	
temperature *T* (°C)	25	250	400	400
pressure *p* (MPa)	0.1	5	25	50
density ρ (g cm^{-3})	1	0.80	0.17	0.58
dielectric constant ε	78.5	27.1	5.9	10.5
pK_w^*	14.0	11.2	19.4	11.9

***** ionic product of water

Dielectric constant

Dielectric constant (or relative static permittivity) of a material is a measure of the extent to which it concentrates electrostatic lines of flux. During a reaction, a high relative dielectric constant reduces the activation energy of a reaction with transition state of higher polarity compared to the initial state (Kruse & Dinjus 2007b). The change in the extent of hydrogen bonding affects the dielectric constant of water which is demonstrated in Fig 2.3.c. For example, the dielectric constant of water at ambient temperature (25 °C) is 81 which drops to 21 at 300 °C and further decreased to only 4.1 at 500 °C which is very similar to non polar organic solvents such as pyridine and methylene chloride. This means that water at these conditions shows higher solvent strength towards organic and nonpolar compounds. The drop in dielectric constant will affect the miscibility of water as well, which is discussed below.

Miscibility

Water is known to be a poor miscible liquid with gases, organic solvents and hydrocarbons while it is a good solvent for ionic salts because of its significant dielectric constant (Kruse & Dinjus 2007a). However, this changes at near critical conditions as its density decreases and becomes a poor solvent for salts and becomes miscible with many organic compounds and gases. The relation between solvent properties and its temperature and pressure can be employed to precipitate particles or progressing homogenous reactions of organic compounds with gases such as oxidation of organic compounds with oxygen and air (Kruse & Dinjus 2007b).

Ionic product

The ionic product of water increases with temperature up to 10^{-11} near its critical temperature while it drops at temperatures more than critical temperature and increases with pressure. In other words, subcritical or supercritical water can act as a weak acid or alkali catalyst and promote the rates of various reactions which can be catalyzed by acid or alkali to some extent.

2.3 Sub- and Supercritical Water Applications

2.3.1 Extraction

In addition to synthetic reactions, subcritical water has been reported as an excellent solvent for extraction of various components from biological matrixes. This is mainly due to the enhanced solubility of nonpolar substances at elevated temperatures which improves its extraction power. Water at these conditions can also facilitate the extraction process by reducing and cleavage of bonds and binding forces in the sample matrix. Subcritical water extraction has been applied in cleaning of the soil and removing PAH (polycyclic aromatic

hydrocarbon) and metals (Hashimoto 2004; G. Brunner 2009). In biological samples, it has been extensively studied for the extraction of different nutrients and extracts such as lipophilic products from vegetable oils and seeds (J. W. King 2008), polyphenolics from fruits and rice bran (Rangsriwong et al. 2009; Pourali et al. 2010), antioxidants from the leaves of eucalyptus (Kulkarni et al. 2007) or rosemary plant (Ibañez et al. 2003), proteins and amino acids from rice bran (Sereewatthanawut et al. 2008) and flavour compounds from rosemary (Basile et al. 1998). In few studies, this process has been successfully coupled to other auxiliary steps such as ultrasonic (Roldán-Gutiérrez et al. 2008) or microwave systems (Raman & Gaikar 2002) to enhance the extraction from biological samples.

Subcritical water extraction has the main advantage as all other aqueous reactions where water replaces organic solvents and offers environmental and cost benefits to the process. In particular, subcritical water has unique characteristics such as high density, high reactivity, strong solubility and supplementary catalytic activity

2.3.2 Reaction Medium

The unique properties of water at high temperature provide an excellent solvent for many applications. It is a benign, cheap, abundant and environmental friendly solvent which can show tendency toward becoming nonpolar at high temperatures and pressures. Two major types of reactions which take place in super- and subcritical water are oxidisation and hydrolysis that have been widely used in waste and biomass conversions (Lamoolphak et al. 2007). Oxidation takes place in supercritical water and is employed in a process called supercritical water oxidation (SCWO) to destroy toxic organic compounds in industrial wastes. Hydrolysis, on the other hand, can occur in milder conditions (subcritical water) and is the cleavage of ether or ester bonds by addition of one water molecule which breaks down certain polymer molecules. Due to the existing ether bonds in carbohydrates, the hydrolysis

of carbohydrate can be easily carried out in hydrothermal conditions. As a reaction medium, subcritical water can provide an opportunity to conduct chemistry in a homogenous phase that would occur in heterogeneous mediums under conventional conditions (Y. Yu et al. 2008). This implies that there is the chance of changing reaction dominant entities from ionic compounds to free radicals by modifying water density.

The role of subcritical and supercritical water in organic reactions can be classified into two main subgroups:

- Water as a reactant/product, which includes the role of the individual molecules in the reaction as a reactant or product such as hydrolysis, hydration, hydrogen exchange and free-radical oxidation
- Water as catalyst, which includes its application in reactions that acts as a source of acid or alkali and modifies the transition states. Examples of this type of application can be various organic reactions such as alcohol dehydration process, Friedel-Crafts alkylation and aldol condensation.

In many applications, water can act as both a catalyst and reactant such as hydrothermal treatment, which was addressed in our project and will be discussed in detail in following sections.

2.4 Sub- and Supercritical Water and Biomass Transformation

2.4.1 Biomass as a source of energy and chemicals

While coal and crude oil resources come from carbon dioxide fixed by nature through photosynthesis, they are considered as limited and non-renewable. On the other hand, biomass and other resources like solar, wind and tides are renewable as they can be

replenished continuously and in a shorter time cycles while biomass provides a close-loop for CO_2 (Clark & Deswarte 2008).

The interest in alternative energy resources, especially in biomass increased during late 70's and early 80's due to the sudden increase in the fossil fuels price leading to more studies on alternative resources for fuels and chemicals. The United States Department of Energy (DOE) reported that the global bioenergy capacity is 293 x 10^{12} megajoules where only 2.86 x 10^{12} megajoules was available in the USA, most from the pulp and paper industry (Midgett 2008). Biomass conversion platforms are constantly increasing throughout the U.S. and world primarily by the production of bio-ethanol, biodiesel and bio-hydrogen.

An early example of these studies was carried out by the US Bureau of Mines in 1971 in order to investigate the utility of pressurized hot water to liquefy carbohydrates in the presence of Na_2CO_3 and CO (Appell et al. 1971). Applying catalysts to the reaction medium was carried out in earlier works to increase the yield of H_2 while later studies pointed out the negative effect of the addition of these compounds on the overall yield of the hydrogen (Molton et al. 1978). The next development was carried out in Pittsburgh Energy Research Centre (PERC) to study the oil-based and aqueous slurry liquefaction processes in early 1980's. Lawrence Berkeley Laboratory (LBR) process was a similar once-through process which its focus was to minimize the cost of recycling large quantities of oil, as it used water as the carrier in contrast to PERC which used oil. Another important development, using higher concentration of feeds, was Sludge to Oil Reactor System (STORS) where continuous processing of the biomass was developed in the Batelle Pacific Northwest laboratories. However, the low price of oil in the 80's curbed any significant technological and commercial advanced in this area (Midgett 2008).

Later during last years of 20th century, the research on biomass processing was emphasized again due to the further economical and environmental incentives. These studies were in parallel to the new developments in both homogeneous and heterogeneous catalytic processes (Baker et al. 1989; Douglas C. Elliott et al. 2006). The increasing number of works, which proceeds until now far, highlights two main perspectives:

- The potentials of biomass as a source of different chemicals which can be use either as the final product or intermediate molecules for other products.
- Improved reaction parameters and yield for the formation of biogas, bio-oil or liquid biofuels

Further development of using biomass for energy purposes and increasing interest in different derivable chemicals resulted in the formation of biorefinery concept. Applying biorefinery concept results in the integration of different material and energy streams that can be fractioned from the starting material, i.e. biomass feedstock. Different techniques have been proposed for the hydrolysis and conversion of wet and dry biomass and waste streams, mainly non-food plant materials such as straw and wood, in order to provide the necessary substrate for the secondary steps (such as bioethanol fermentation). Biomass hydrolysis can be carried out using chemicals such as acids or alkali, enzymes, hydrothermal treatment or the combination of these techniques like thermo-chemical routes. During hydrolysis in water, water can act as solvent, reactant and catalyst via self-dissociation though often is accompanied by other acid or alkali catalysts to inhibit undesired side reactions (Akiya & Savage 2002). In particular, the addition of CO_2 to the medium, which increases the hydronium ions, has been of interest in the application of several feedstocks (He et al. 2008).

The high temperature of water will promote the hydrolysis reaction into greater extents. At lower temperatures (subcritical region, 100-250 °C), the main focus is to fractionate the waste biopolymers to prepare a mixture of monomers or intermediates for further applications. Apart from the role of water which promotes hydrolysis, it has been reported that there are degrees of autocatalysis due to the generated acidic products (Knežević 2009).

At higher temperatures (250-400 °C), liquefaction is the main reaction which transforms wood or other cellulosic biomass sources to fuel gas and oil (G. Brunner 2009). When the medium is deprived from oxygen, the pyrolysis occurs resulting in bio-oil residues which can be used to derive valuable chemicals or alternative fuels (Yaman 2004; Demirbas 2000). Pyrolysis is favoured over ionic reactions at lower pressures, lower densities (gases) and higher temperatures (Knežević 2009). In the presence of nickel and alkali salts, methane is produced under such temperatures as well (eq. 1), which is for ideal conversion of glucose to methane:

$$C_6H_{12}O_6 \longrightarrow 3\,CH_4 + 3\,CO_2 \qquad (1)$$

Ultimately, the medium will be dominated by gasification reaction (conversion of organic compounds to hydrogen, carbon monoxide, carbon dioxide and methane) at higher temperatures (> 400°C) where hydrogen will be the main product in the supercritical water following equation (2) which is based on ideal conversion of glucose to hydrogen:

$$C_6H_{12}O_6 + 6H_2O \longrightarrow 12H_2 + 6CO_2 \qquad (2)$$

The presence of alkali salts often improves the hydrogen yield. Some efforts have also been made to carry out the hydrogen production at lower temperatures using different catalysts such as ZrO_2 (Watanabe et al. 2002) and reduced nickel (Ni) (Minowa & Ogi 1998).

Typically, one overall objective of the conversion of biomass into fuels is to remove oxygen from the feedstock (which is about 40-60 wt % in biomass where fuels and oils have typical values of 1% wt) (Peterson 2009). Oxygen removal under subcritical conditions can proceed via the following reactions: dehydration, decarboxylation and decarbonylation. The net effect of deoxygenation under these conditions is the CO_2 and H_2O formation (Knežević 2009). Oxygen removal occurs most readily via dehydration as oxygen can be eliminated as water or by decarboxylation which oxygen leaves the biomass as CO_2.

To provide a better understanding of the chemistry of subcritical water conversion of biomass and its potentials as a source of chemical derivatives, biomass and its chemistry are discussed below which concludes by an overview of the spent yeast, as the used biomass in this project.

2.4.2 Biomass: Chemistry and its Properties

Biomass can be described as any hydrocarbon compound which consists of carbon, hydrogen, oxygen and nitrogen and is available on a recurring basis (Fig 2.4) (Clark & Deswarte 2008).

While inorganic compounds are seldom identified in biomass, some types of biomass can carry significant proportions of these chemicals (up to 15% in herbaceous biomass) which accumulate as the ash fraction after burning. Due to the growing application of biomass in different industrial contexts, the term "industrial biomass" can be defined as "any organic matter that is available on a renewable or recurring basis (excluding old-growth timber), including dedicated energy crops and trees, agricultural food and feed crop residues, aquatic plants, wood and wood residues, animal wastes, wastes and co-wastes of food and feed processing and other waste materials usable for industrial purposes (energy fuels, chemicals, materials) (B. Kamm et al. 2006).

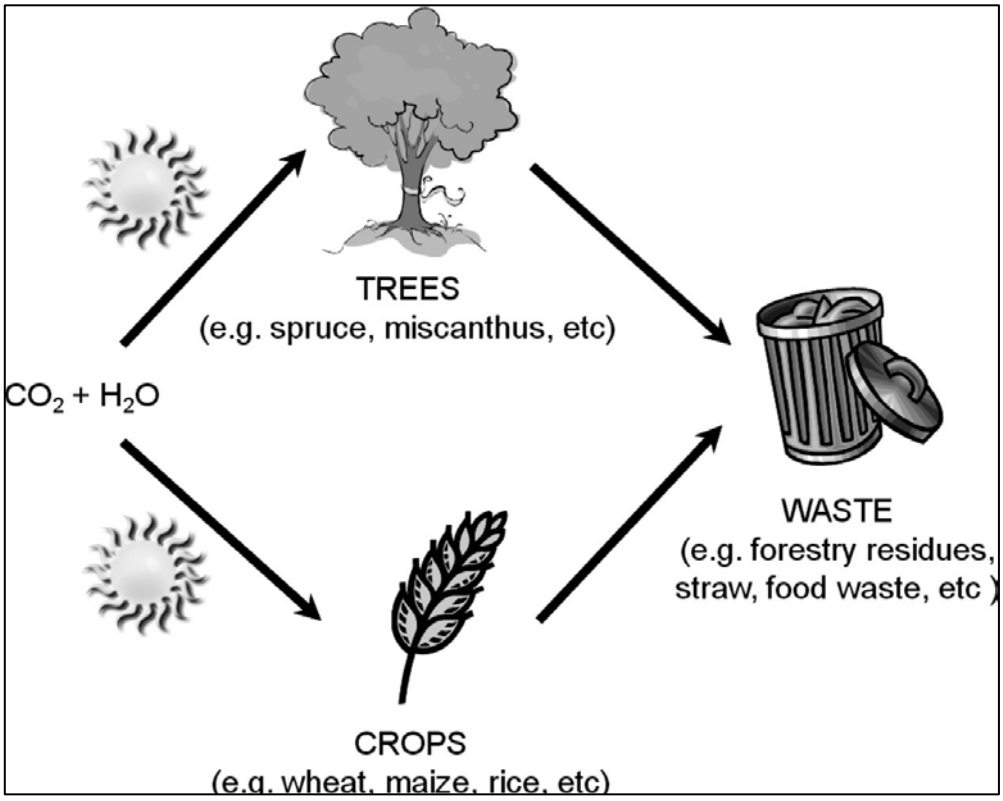


Figure 2.4. Different types of biomass (adapted from (Clark & Deswarte 2008)

A large proportion of the biomass is wet biomass which can contain up to 95% water and the direct use of this type of biomass is expected to be more feasible comparing to the technologies used for dry biomass conversion. Also, the conversion technologies usually benefits the wet wastes as they face a high cost of disposal via incineration, drying etc (Kruse & Dinjus 2007a).

The two most abundant types of biomass are wood (lignocellulosic biomass) and crops (starch based biomass such as maize, wheat and rice). The primary products which are accumulated in vegetable biomass are C_6- and C_5-sugars that form **cellulose** (by polymerization of glucose) and **hemicellulose** (a polymer of glucose and xylose). The other main component is **lignin** which is a highly cross-linked polymer built from phenolic groups and provides strength for the plant structure and decreases water permeation (Corma et al. 2007). The main components of biomass and their reaction intermediates in hydrothermal environment are provided in Table 2.2.

Cellulose is the most abundant chemical product in the world's continents with 50 billion tons of production/growth each year (Bobleter 1994). The repeating unit in cellulose chains are cellobiose (Figure 2.5).

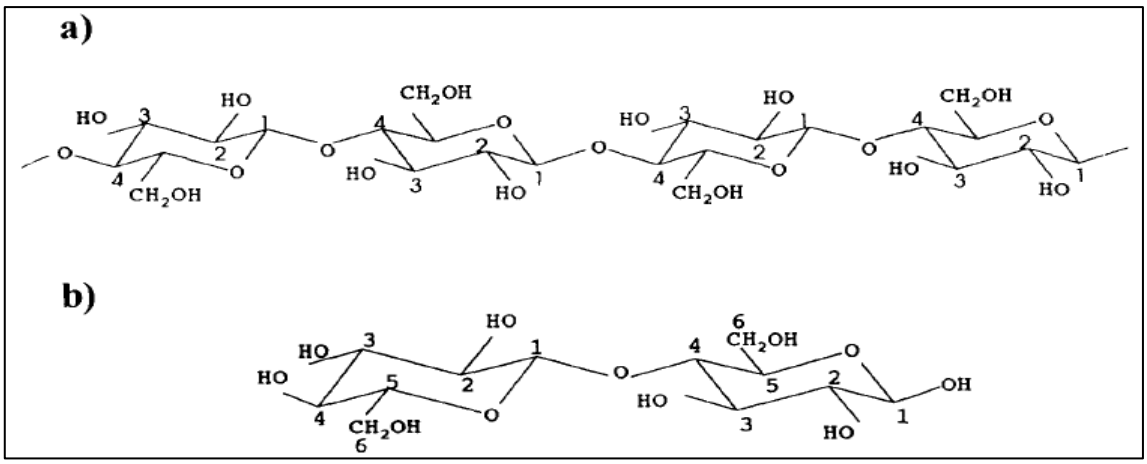


Figure 2.5. Stereo-chemical formulas of (a) cellulose and (b) cellobiose (Bobleter 1994)

In addition to these compounds, plants can make energy storage products such as lipids, sugars, starches or terpenes (which are rich in hydrogen and carbon). These resources are regarded as renewable due to their ability to regenerate by taking up CO_2 during their growth (photosynthesis) and then releasing it to the air after burning or decomposition (Clark & Deswarte 2008).

The worldwide production of biomass is around 3×10^{11} tons per annum where only 6 billion is currently used. From this small fraction, only 3 to 3.5% is employed in non-food applications like fuels and chemicals (B. Kamm et al. 2006). The biomass potential forecast in the EU countries is also demonstrated in Table 2.3 based on the type of biomass. Potentially, it is believed that biomass can provide up to 30% of energy generation of mankind if used in a sustainable way. Due to its short carbon cycle, biomass does not have a net contribution to the available carbon dioxide in the atmosphere (Knežević 2009).

Traditionally, food crops were being used to produce energy (e.g. biodiesel from vegetable oils) and chemicals (e.g. polylactic acid from corn). However, due to the increasing concerns on the competition of these routes with food supply chains, the economic and legislative drive has emphasized the importance of using alternative non-food resources such as wood, straw and energy crops (B. Kamm et al. 2006). The typical constitution of wood or grass biomass are approx. 50% cellulose, 25% hemicllulose, 20% lignin and some other materials such as minerals and organic acids. These ratios can vary significantly in different types of biomass resource which are summarized for some in Table 2.4.

Table 2.2. Chemical representatives of biomass feedstocks and reaction intermediates encountered in hydrothermal processing (adapted from (Peterson 2009))

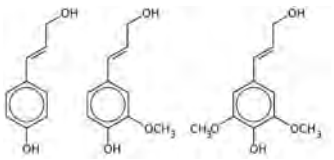
Substance	**Chemical formula**	**Structural information**
Feedstocks		
cellulose	$[C_6H_{10}O_5]_n$	n~500-10,000; β(1-4) linkages between glucose residues
hemicellulose	Typical monomers: $[C_6H_{10}O_5]$, $[C_6H8O_4]$	Branched with variable monosaccharide residues; dp ~ 500-3,000
lignin	Typical monomers 	Polymer of aromatic subunits in random structure; molecular weight:> 10,000
triacylglycerides	$R\text{-}CH_2CH\ (R^{'})CH_2\text{-}R^{''}$	R,R',R'' are fatty acids with ester linkages to the glycerol backbone
protein	$[NHCH\ (R)\ C(O)]_n$	Monomer is amino acid residues with various side groups; n~50-2000
Intermediates		
glucose	$C_6\ H_{12}\ O_6$	exists as 6-membered ring, 5-membered ring, and open chain
xylose	$C_5\ H_{10}\ O_5$	Exists as 6-membered ring, 5-membered ring , and open chain
amino acid	$H_2NCH(R)COOH$	R is the side group, varies from H to heterocyclic group
fatty acid	RCOO	R is any alkyl group, typically of 12-20 carbons with 0-4 double bonds
5-hydroxymethylfufural		

Table 2.3. Biomass potential in the EU (European Commission, 2006) (Clark & Deswarte 2008)

	Biomass Potentials (MTOE)*		
	2010	2020	2030
Organic Wastes	100	100	102
Energy Crops	43-46	76-94	102-142
Forest Products	43	39-45	39-72
Total	**186**	**215-239**	**243-316**

* Million tons of oil equivalent

Table 2.4. Chemical composition of selected plants in % of total dry matter (adapted from (Ortwin Bobleter 1994a))

Plant (scientific name)	Cellulose	Hemicellulose	Lignin	Ash
Hard woods:				
White poplar (*populus alba*)	49	25.6	23.1	0.2
Trembling aspen	49.4	30.9	18.1	0.4
Soft woods:				
White spruce (*p. glauca*)	44.8	30.9	27.1	0.3
Europ. spruce (*p. abies Karst*)	40.4	31.1	28.2	0.3
Ligno-cellulosic waste:				
Wheat straw	34.0	27.6	18.0	1.3
Maize stalk	38.0	26.0	11	3.0
Bagasse*	38.0	34.0	11	
Newspaper	50	20	30	

* Biomass remains of sugarcane stalks after their juices have been extracted

2.4.3 Biomass conversion in subcritical water

Biomass conversion using hydrothermal treatment is a thermo-chemical process during which water has the role of reaction medium and catalyst and can be used for different pure and mixed feedstocks. In general, this reaction can be carried out for different purposes to

obtain different products while both scenarios of catalytic and no catalytic conversion have been widely employed. The most commonly used process is the (acid) hydrolysis which have been largely used in wood and lignocellulosic hydrolysis plants worldwide. A comprehensive review by Bobleter demonstrates the hydrolysis of plant materials and the alterations that occur to polymers and intermediates in lignocellulosic biomass such as cellulose, hemicellulose and lignin (Bobleter 1994).

One benefit of sub-or supercritical water process for biomass conversion is that organic compounds, which are the precursors of tar, are solubilised in the medium. In other words, the collision frequency of the solutes with water molecules in an aqueous solution is much higher than that with a second organic molecule. As a result, they have a lower probability of "meeting" and forming polymerized structures like tar which leads to lower formation of coke and enhancement of the yield of desired products (Kruse & Dinjus 2007b).

A typical conversion process using subcritical water usually consists of initial treatment (pretreatment) of the feed before the main reaction step to convert the non-soluble condense polysaccharide chains to smaller monomers (i.e. fermentable sugars). In addition, the treated biomass would obtain the required properties such as flexible structure or absence of chemical barriers (e.g. lignin in lignocellulose), humidity, degree of fragmentation or rheological properties (Knežević 2009). The pretreatment step, however, is the biggest challenge in lignocellulosic biomass applications due to the presence of intermeshed cellulose and lignin polymers which are difficult to breakdown and demand high temperature water or higher concentration of catalysts.

2.4.4 Degradation of biomass components in subcritical water

An important aspect of hydrothermal conversion is that it can handle different mixed streams and a specific chemical feedstock is not mostly required. This is in contrast to most biofuels processes where glucose feedstocks are required for ethanol and triacylglycerides are required for biodiesel process (Peterson 2009). Based on the main composition of biomass, and also the shift of feedstocks from food crops to lignocellulosic wood and wastes, favoured the studies to model the hydrothermal conversion steps with natural biopolymers such as cellulose, starch, xylose etc. This is because biomass is a complex mixture of variable composition which makes the study of individual reaction path complicated. Hence, most of the studies are investigating the various reactions that these compounds undergo in such mediums.

Different fractions of lignocellulosic biomass (cellulose, hemicellulose and lignin) behave differently under subcritical water conditions. As an example, Mok and Antal (Mok & Antal 1992) showed that all the hemicellulose was extracted from woody and herbaceous biomass in noncatalysed water at 200 to 230 °C while just 4-22% of cellulose and 35-60% of lignin were extracted over the same time period (Peterson 2009). The conversion of lignocellulosic biomass in aqueous reactions has long been studied which have been perfectly reviewed in comprehensive papers by Bobleter (Bobleter 1994), Mosier (Mosier 2005) and more recently by Yu *et al*. (Y. Yu et al. 2008). The main objective of such technology is to depolymerise the glucan chains in cellulose or xylan in hemicellulose in order to produce monosaccharides for fermentative processes in cellulose-based ethanol or other biofuels (i.e. hydrogen) and chemicals. More review on the specific reactions of each component is provided below.

2.4.4.1 Cellulose

Cellulose is a polymer of glucoses which are linked with β-1-4 glycosidic bonds and form different intra- and inter-molecular hydrogen bonds which results in crystalline and water insoluble chain which is resistive to enzyme digestion. Both hydrogen and glycoside bonds can be hydrolysed under subcritical condition resulting in glucose monomers (Peterson 2009). Other common glycosidic bonds are β-1-3 and β-1-6 which are present in starch and yeast glucan and were addressed in this project as the main polysaccharide molecules (Glucan is an expression covering all glucose polymers).

2.4.4.1.1 Kinetics of cellulose hydrolysis under hydrothermal conditions

The first reaction step in cellulose conversion in sub- or supercritical water is the hydrolysis of cellulose to glucose. The resulting monosaccharides (glucose and fructose) are converted to different products such as furfural which goes via the formation of (5-hydroxymethylfurfural) HMF, methylfurfural and furfural (Kruse & Dinjus 2007b). The concentration of these compounds is significantly higher at subcritical region and in the presence of acids.

While the complete hydrolysis of cellulose or cellobiose under acidic conditions has been demonstrated previously (Torget et al. 2000), the question whether subcritical water differs from acidic or alkaline conditions has been frequently discussed (Sasaki et al. 1998). In an early work by Dunning and Lathrop in 1945, they showed that non-catalyzed hydrothermal conditions results in much lower hydrolysis rate comparing to acid catalyzed hydrolysis leading to lower glucose yields (<5%) (Dunning & Lathrop 1945). The decomposition of cellulose in supercritical water, however, has been shown to follow faster kinetics without the addition of any catalysts (Sasaki et al. 1998), but resulting in glucose derivatives such as formic acid and levulinic acid.

Experimental results suggest that the cellulose hydrolysis (i.e. hydrolysis of glycosidic bonds) is fastest in alkali hydrolysis while acidic and hydrothermal conditions have slower reaction rates respectively (Bobleter 1994). These three types of hydrolysis are shown and compared in Figure 2.6, based on most probable reaction pathways for cellobiose, which is the repeating unit of cellulose.

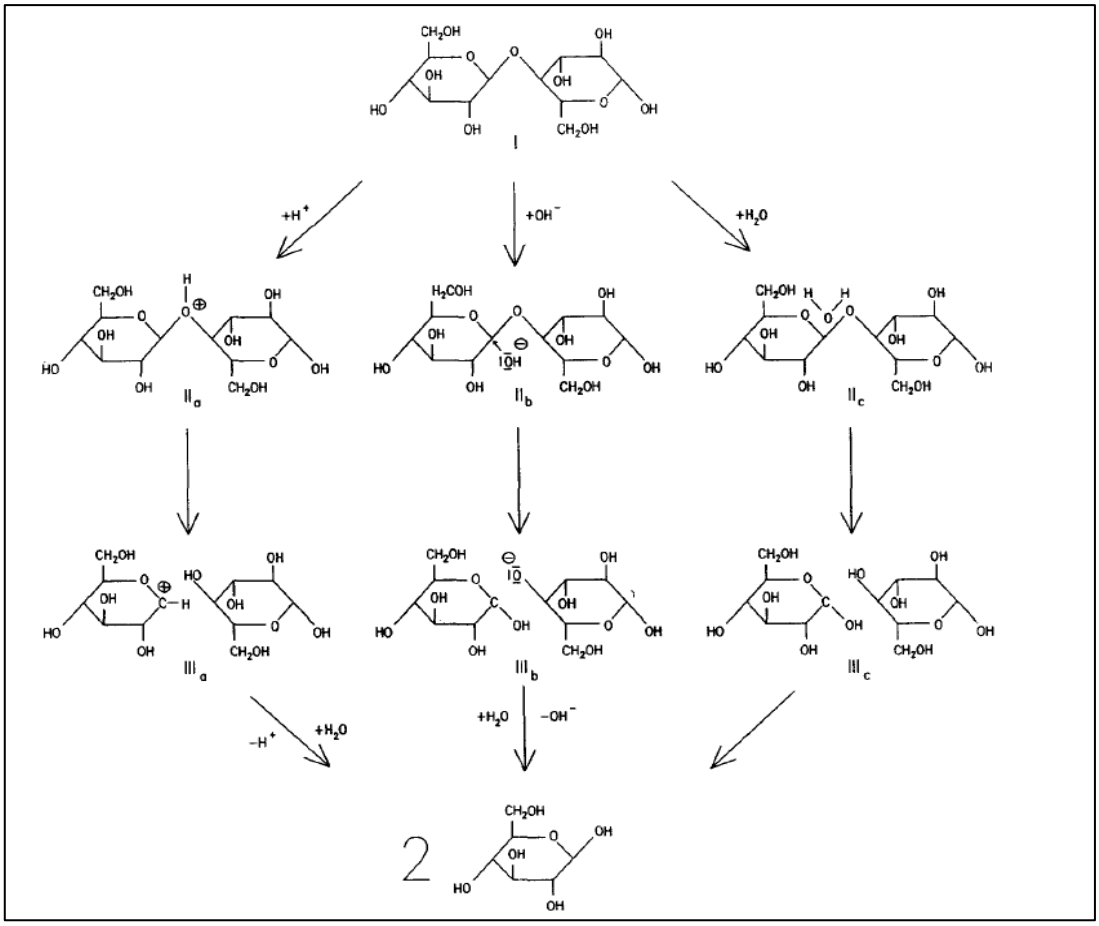


Figure 2.6. Mechanisms of cellubiose hydrolysis in acidic (H+), alkaline (OH-) and pure water and formation of glucose (Ortwin Bobleter 1994a)

A more detailed study on the hydrolysis of pure cellulose under subcritical and supercritical water was carried out by Sasaki *et al* (Sasaki et al. 1998). They used a flow type reactor to hydrolyze cellulose in very short times in water (temperature range from 290 to 400 °C at 25 MPa) and analysed the extracts for glucose, fructose and oligomers (cellobiose, cellotriose etc.). Using a maximum 10 wt% cellulose concentration in the reactor feed, they showed that at such conditions, around 60% of the raw material has been hydrolysed at 350 °C after less than only 2 seconds which is much faster than milder subcritical conditions. The kinetics of the cellulose hydrolysis was determined and showed to be of first order up to the critical point of water, where it increases by one order of magnitude (Fig 2.7).

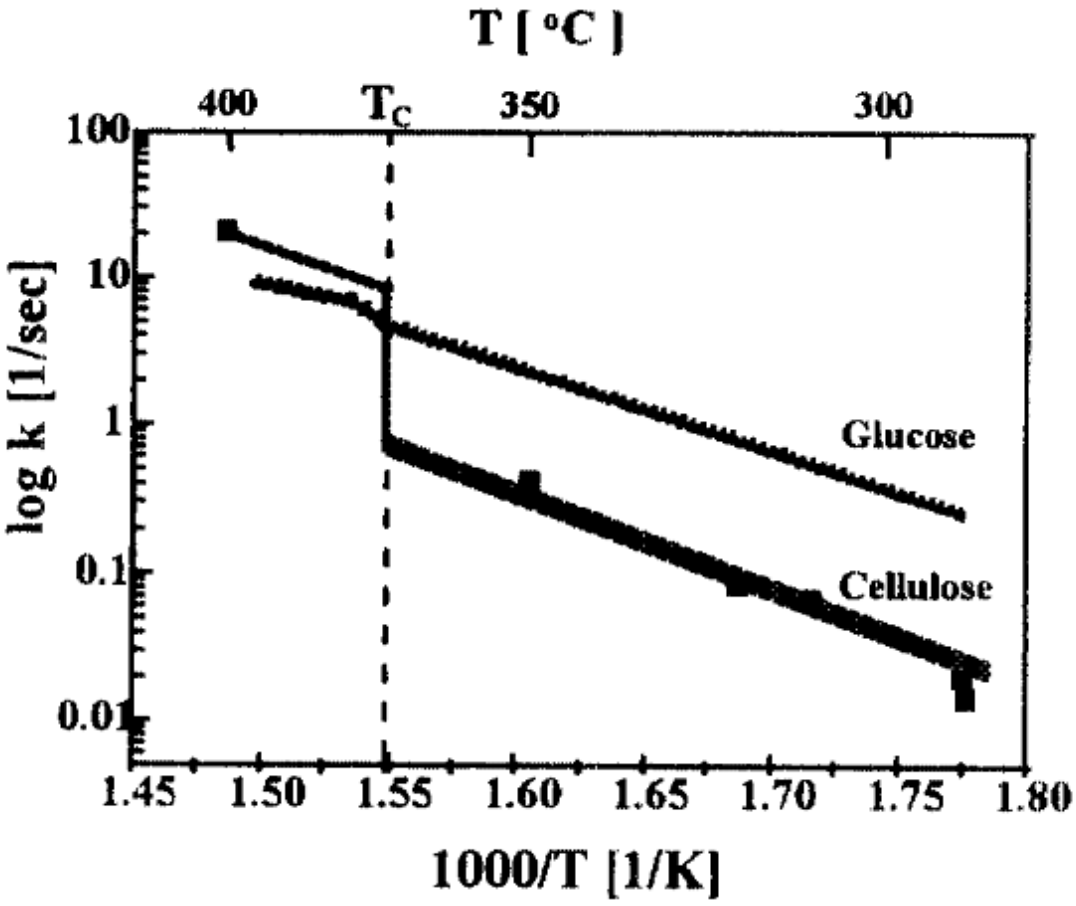


Figure 2.7. Arrhenius plot of rate constants for decomposition of cellulose and glucose in subcritical and supercritical water (Sasaki et al. 1998).

The reason for the jump at critical point has not been discussed in detail but only suggested that it could be due to the solubility of cellulose in supercritical water leading to its enhanced reactivity and conversion. In such conditions, the reaction changes from heterogeneous to homogenous due to the formation of oligomers, and leaded to higher reaction rates. For oligomer hydrolysis, the reaction rates have been reported as being in the same order over the range of temperatures from 290 to 400 °C (Sasaki et al. 1998). A detailed description of hydrolysis products was also carried out with the combination of carbon nuclear magnetic resonance (C-NMR), high performance liuiqd chromatography (HPLC) and fast atom bombardment mass spectrometry (FAB-MS) and demonstrated in Figure 2.8.

While most studies suggest that first order or pseudo-first order kinetics can describe the hydrolysis reaction of cellulose in subcritical conditions, there are some variations between the reported activation energy of the reaction (Fig. 2.7). The source of this variation, however, was mainly due to 1) different sources of cellulose that have been used in these studies and 2) different reaction configuration which have been employed. In the same study by Sasaki *et al*., authors estimated the activation energy in the flow system to be around 146 kJ/mol, though it increased to 548 kJ/mol at temperatures over critical point of water (Sasaki et al. 1998).

In a non-catalyzed medium and with cotton cellulose as the raw material, Schwald and Bobleter estimated the activation energy of 129.1 kJ/mol in the temperature range if 215 to 274 °C (Schwald & Bobleter 1989). In such conditions, authors could detect only a small amount of monomeric sugars as they had rapidly decomposed into furfural under static

conditions. Adschiri *et al*, however, obtained higher activation energy of 165 KJ/mol on powdered cellulose in a semi-batch process (T. Adschiri et al. 1993). This value was found to be 220 KJ/mol in an experiment carried out by Mochidzuki *et al*. in isothermal conditions (Mochidzuki et al. 2000). Table 2.5 lists and compares the reactor configurations, substrates and obtained values for the activation energy.

Table 2. 5. Activation energy for cellulose solubilisation from different studies

Research study	**Substrate**	**Reactor (process) configuration**	**Activation energy (kJ/mol)**
Sasaki *et al* (2000)	powdered microcrystalline cellulose	continuous	146
Adschiri *et al* (1993)	powdered plant cellulose	semi-batch	165
Schwald and Bobleter (1989)	cotton cellulose	batch	129.1
Mochidzuki *et al*(2000)	powdered cellulose	thermogravimetric batch reactor	220

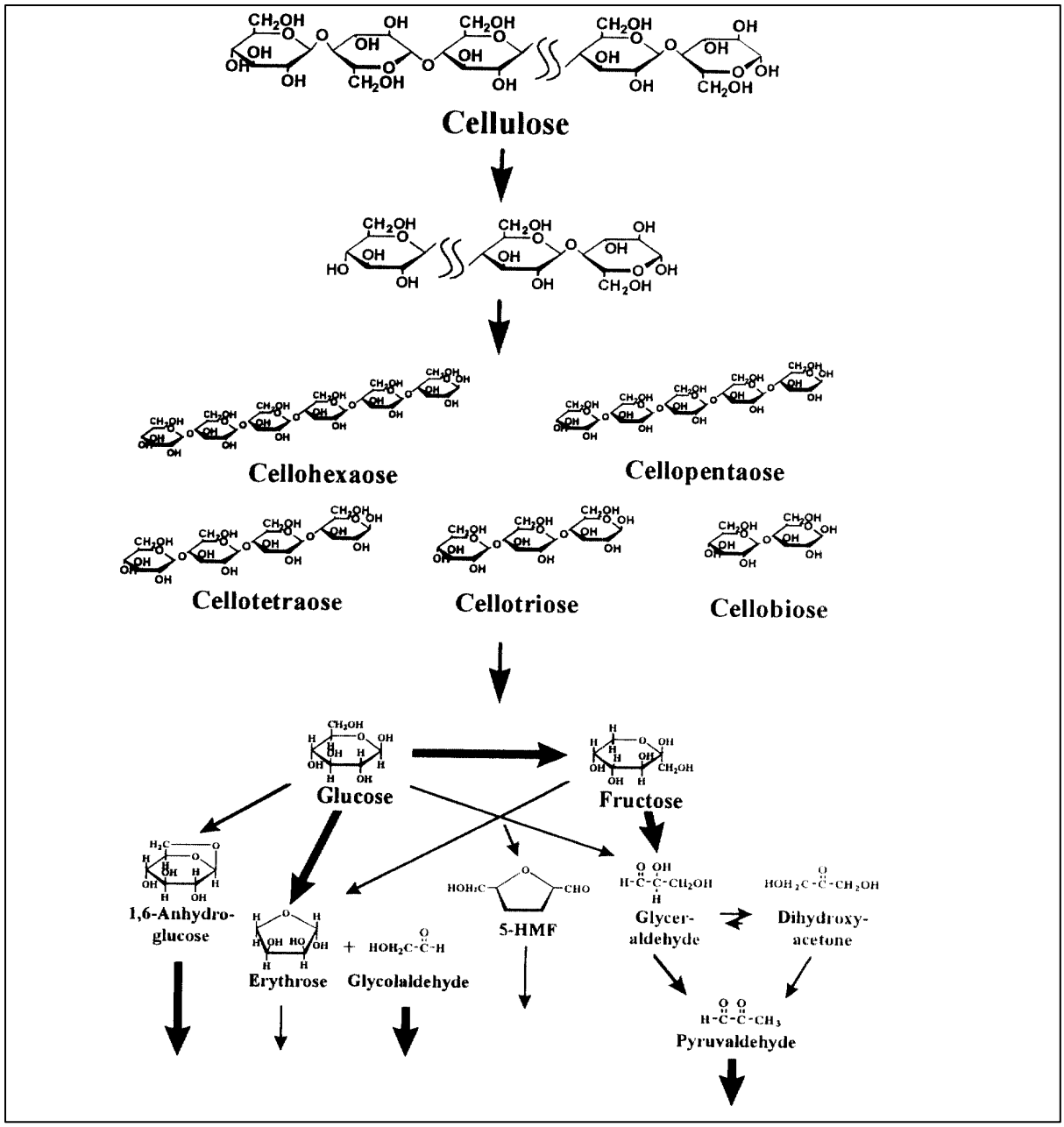


Figure 2.8. Different reaction pathways for the hydrolysis of cellulose in pure water (Sasaki et al. 1998)

The data of the reaction rates from different studies are overlaid and demonstrated in Figure 2.9 which suggests that the average activation energy is around 215 KJ/mol by fitting a straight line (Peterson 2009).

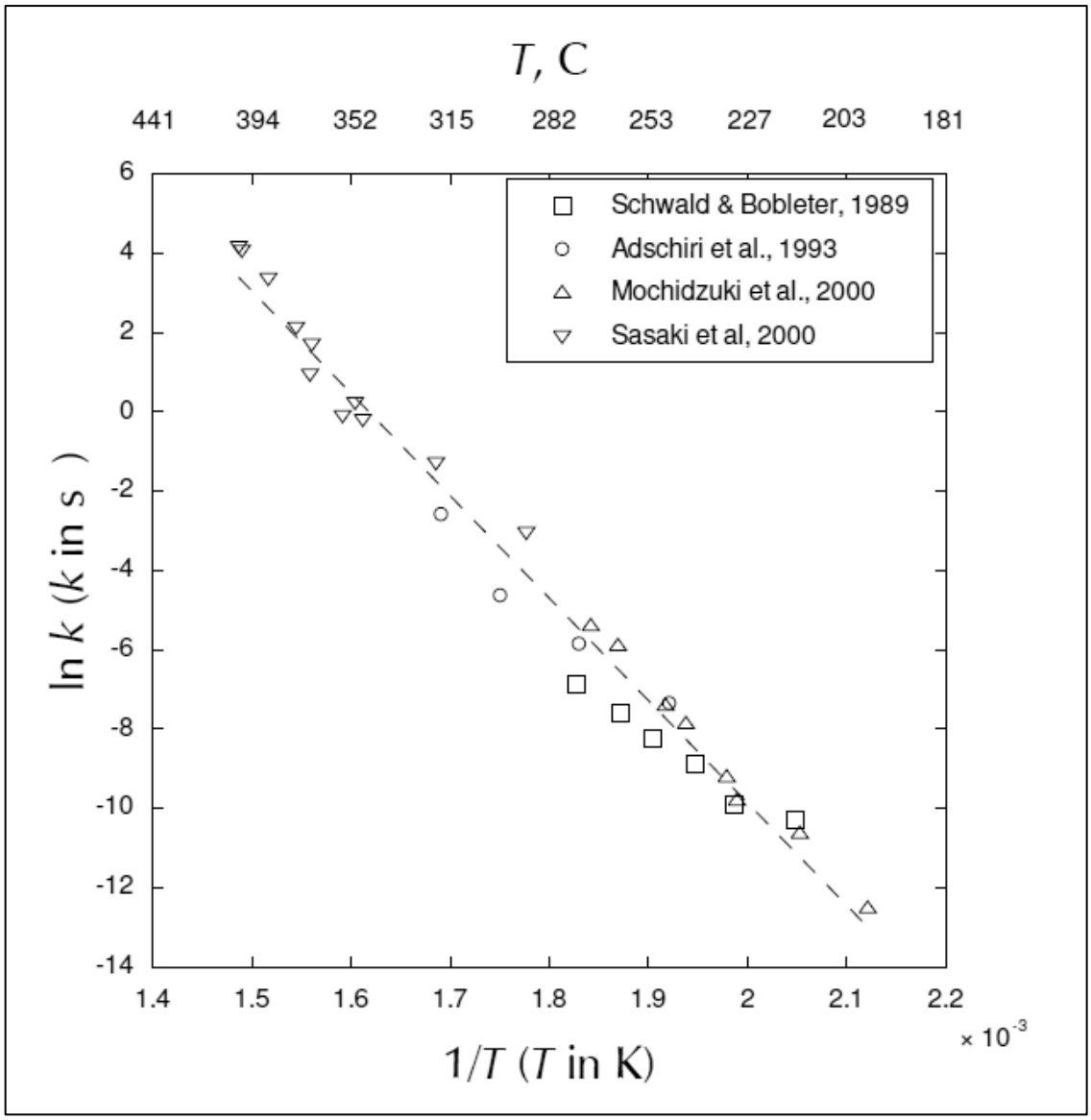


Figure 2.9. Arrhenius plot for cellulose decomposition in different studies (Peterson 2009)

In another study by Kabyemela *et al*., cellobiose decomposition was studied in sub- and supercritical water at temperatures between 300 to 400 °C and pressures from 25 to 45 MPa and short residence times (0.004 – 2s). Cellobiose was found to decompose via two pathways of hydrolysis of the glycosidic bonds and pyrolysis of the reducing end (Kabyemela et al. 1999). Comparing the reaction rates for these two pathways, authors suggested that the activation energy for cellobiose hydrolysis was 108.6 kJ/mol, and for the two pyrolysis were 110.5 kJ/mol and 106.1 kJ/mol.

Monosaccharide production is mainly the ultimate goal for hydrolysis of cellulosic biomass as the secondary processes, such as bioethanol fermentation, can use monosaccharides as the substrate for the ethanol production. The suggested reactions that take place at elevated temperatures (>250 °C) decompose glucose to other by-products very quickly which may not be of interest of the overall process (Sasaki et al. 2000). In many cases, these secondary products, such 5-HMF, can act as inhibitory factors or toxic chemicals for yeast cells which negatively affect the fermentation process and ethanol yield (Franden et al. 2009). Hence, the controlled hydrolysis of cellulose biomass should be considered with employing either additives or catalysts. Because low temperature aqueous medium (<250 °C) can only hydrolyze the cellulose in significantly low rates, it is not considered an optimum conversion process for bioethanol production from cellulose.

Due to the importance of the secondary reactions for glucose in hydrothermal conditions and the range of different products that can be derived from it, these reactions are also discussed in detail in following sections.

2.4.4.1.2 Glucose hydrolysis in subcritical water-Reactions pathways and kinetics

Glucose reactions in high temperature aqueous environment have been studied for well over a century. An important pathway is the isomerisation of the glucose to fructose which typically occurs under mild acidic conditions. Isomerization occurs mainly through the Lobry de Bruyn-Alberda van Ekenstein (LBAE) transformation which is a series if enediol transformations (Kabyemela et al. 1999). As a result, when glucose or fructose are solubilised in water, they can change to minimum six different forms (Figure 2.10) of monosaccharides and they follow the same general reaction pathway in hydrothermal systems (Peterson 2009).

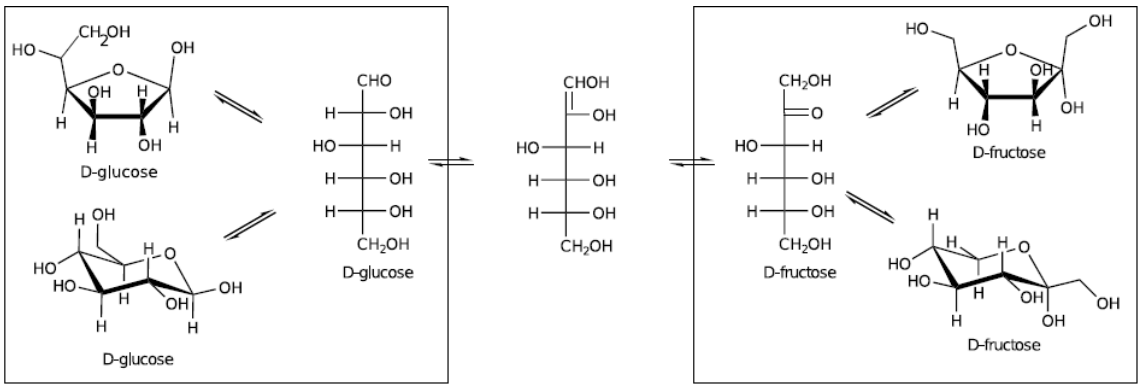


Figure 2.10. Isomerisation of glucose and fructose in water and their different forms (adapted from (Peterson 2009)

The isomerisation of glucose to fructose shows the importance of studying the reactions of fructose in similar conditions. In particular, it has been reported that fructose is more reactive compared to glucose and the rate of glucose isomerisation to fructose is higher compared to the reverse reaction in hydrothermal reactions (Kabyemela et al. 1999). A generalized reaction pathway for glucose has been shown in Fig 2.11 which includes the reactions for fructose as well. However, the rate of inter-isomerisation to fructose is not as

high as degradation of both glucose and fructose. This was demonstrated in a work by Antal *et al*. where only small fructose (or glucose) was left after starting a reaction with pure glucose (or fructose) (M J Antal et al. 1990).

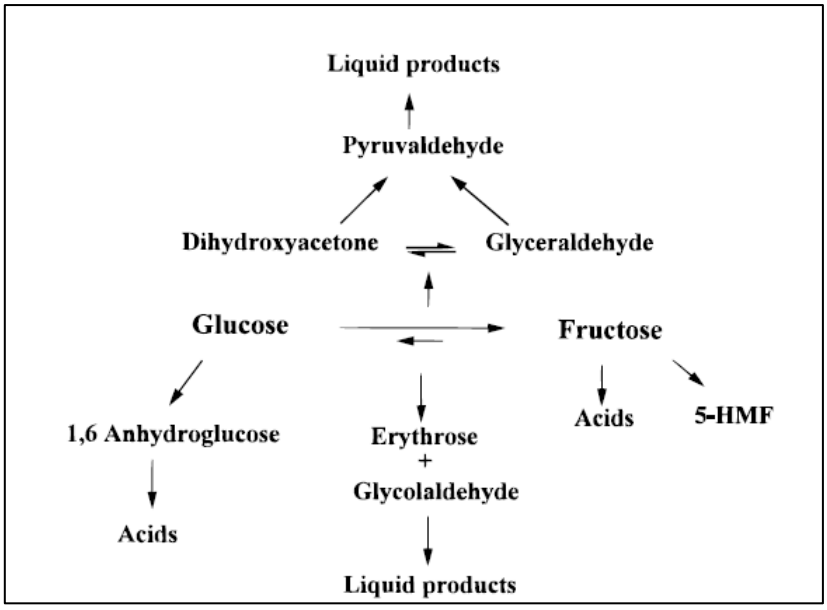


Figure 2.11. Generalized reaction pathway for glucose decomposition in subcritical and supercritical water conditions (B. Kabyemela et al. 1999)

The same results was also obtained in a research by Salak Asghari and Yoshida which accompanied by kinetics data suggesting that fructose reacts much faster than glucose in the presence of acid (at 340 °C, 52% of glucose was decomposed while the degradation was 98% for fructose) (Salak Asghari & H. Yoshida 2006).

The main products of glucose and fructose under such conditions are dehydration products such as HMF, 1.2.4-benzenetriol (BTO) and furfural. The dehydration of carbohydrates is promoted in subcritical water due to the high ionic product of the medium which enhances the reaction rate of this acid-catalyzed reaction (Kruse & Dinjus 2007a). Between these products, HMF production has been studied in more detail as it is one of the

major bio-based chemicals and can be employed as a precursor in the chemical and polymers industry (More detail in section 2.5.1). Dehydration reactions of glucose and fructose to 5-HMF and hydrolysis of 5-HMF show enhanced rated by the increase in water density at 400 °C (Aida et al. 2007).

A detailed reaction pathway of glucose and fructose in subcritical and supercritical water was elucidated by Kabyemela *et al*., in temperature range of 300-400 °C and temperature of 25-40 MPa for extremely short residence times between 0.02 and 2 s (Kabyemela et al. 1999). For glucose, the products were identified as fructose, a product of isomerisation, 1,6-anhydroglucose, a product of dehydration, and eruthrose and glyceraldehyde which are the products of a carbon-carbon bond cleavage. Detailed reaction pathways for the decomposition of glucose in sub- and supercritical water are shown in Figure 2.12 and the calculated reaction rates of each pathway are summarised in Table 2.7.

Despite different reaction conditions, most publications agree that glucose converts mostly to fragmentation products such as glycoladehyde, pyruvaldehyde and glyceraldehyde while fructose degrades to dehydrated products such as 5-hydroxymethlfufural (Peterson 2009). HMF formation from glucose, however, is mostly studied under acidic conditions as its yield significantly enhances in acidic pH's. HMF has been suggested as an important industrial building block and is in the list of top ten bio-based products which are suggested by Department of Energy, USA, in 2010 (Bozell & Petersen 2010). In addition, it has been reported that fructose can lead to 1,2,4-benzenitriol via HMF degradation which suggest that aromatic compounds can be produced from sugars as well as lignin (Luijkx et al. 1993). Another promising, green and biomass derived platform chemical from sugars is levulinic acid which is formed after degradation of HMF in acidic conditions. Direct conversion of glucose to levulinic acid has been reported by

Girisuta *et al*. in temperature range of 140-200 °C using 0.05-1 M sulphuric acid as catalyst (Claude Moreau et al. 1996). The highest yield was about 60% (mol/mol) with initial concentration of 0.1 M for glucose, 1 M sulphuric acid at 140 °C.

Temperature can have a profound impact on the reaction pathway as the main products of glucose hydrolysis are char (carbon) and liquid organics below the critical temperature while it is mostly converted to gases and partly liquid furans at temperatures above water's critical temperature (Michael Jerry Antal et al. 1991).

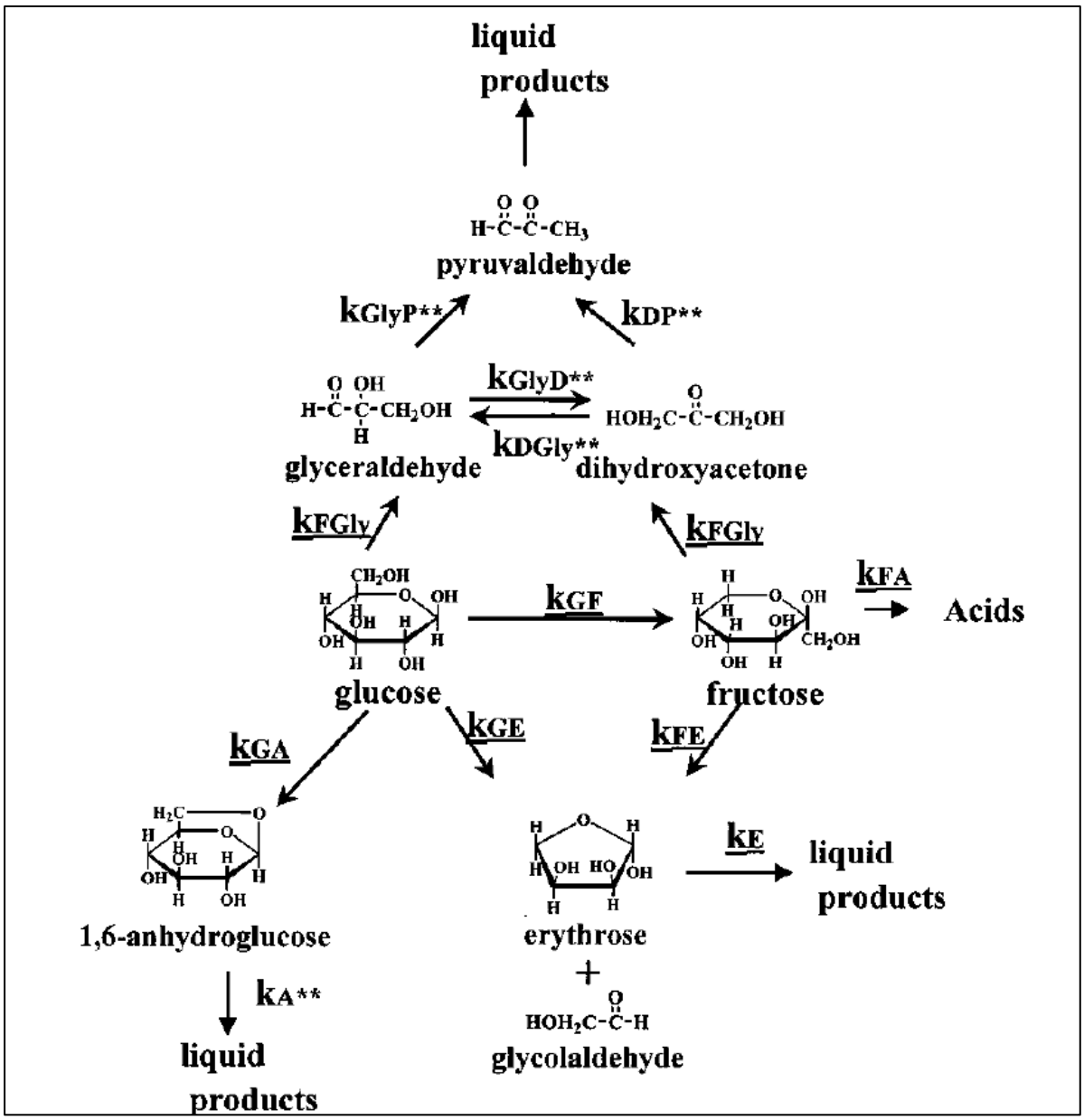


Figure 2.12. Kinetic reaction pathway of glucose decomposition in sub- and supercritical water (B. Kabyemela et al. 1999)

Table 2.6. Rate constants for the proposed glucose decomposition pathway (B. Kabyemela et al. 1999)

	Conditions	
rate constant (s^{-1})	300 °C, 25 MPa	350 °C, 25 MPa
k_{GA}	0.010 ± 0.001	0.020 ± 0.001
k_{E}	0.100 ± 0.004	0.550 ± 0.022
k_{A}	0.010 ± 0.001	0.040 ± 0.002
k_{FGLY}	0.100 ± 0.004	0.600 ± 0.024
k_{FACID}	0.180 ± 0.007	0.700 ± 0.028
k_{FE}	0.100 ± 0.004	0.800 ± 0.032
k_{GE}	0.210 ± 0.008	0.950 ± 0.038
k_{GGLY}	0.050 ± 0.002	0.200 ± 0.008
k_{GF}	0.200 ± 0.008	0.640 ± 0.026
$k_{GLYD}{}^{a}$	0.400 ± 0.016	1.380 ± 0.052
$k_{GLYP}{}^{a}$	0.190 ± 0.008	0.940 ± 0.038
$k_{DGLY}{}^{a}$	0.030 ± 0.001	0.200 ± 0.008
$k_{DP}{}^{a}$	0.170 ± 0.007	0.560 ± 0.022

	Conditions	
rate constant (s^{-1})	400 °C, 30 MPa	400 °C, 40 MPa
k_{GA}	0.080 ± 0.007	0.500 ± 0.045
k_{E}	5.000 ± 0.450	4.000 ± 0.360
k_{A}	0.310 ± 0.028	1.440 ± 0.130
k_{FGLY}	6.500 ± 0.585	5.000 ± 0.45
k_{FACID}	10.400 ± 0.936	4.800 ± 0.432
k_{FE}	8.000 ± 0.720	6.000 ± 0.540
k_{GE}	18.100 ± 1.629	12.000 ± 1.080
k_{GGLY}	1.000 ± 0.090	2.500 ± 0.225
k_{GF}	7.000 ± 0.630	6.000 ± 0.540
$k_{GLYD}{}^{a}$	7.150 ± 0.644	15.700 ± 1.413
$k_{GLYP}{}^{a}$	4.600 ± 0.414	7.420 ± 0.668
$k_{DGLY}{}^{a}$	1.040 ± 0.094	2.450 ± 0.794
$k_{DP}{}^{a}$	1.200 ± 0.108	2.550 ± 0.230

In most studies, first order reaction model has been successfully used to describe the kinetics of glucose decomposition in subcritical water with good fit to experimental results. An exception is a reaction order of 0.8 which was suggested in a continuous reactor at high temperatures (>250 °C) (Matsumura et al. 2006). Matsumura *et al*. also suggested that the reaction order would change at higher temperatures as the reaction field changes from ionic to radicalic conditions. Similar to cellulose, the activation energy has been widely measured and has been between was 114 kJ/mol (Knežević et al. 2009).

2.4.4.2 Hemicellulose

Hemicellulose are also high polymers built from sugar units but the backbone is mainly comprise of pentoses (e.g. xylans), or alternating units of mannose, glucose and galactose and significant side chains such as acetic acid, pentoses, hexoses (Bobleter 1994). The ratio of these monomers can vary in different feedstock sources. Due to the lack of an ordered glycosidic bonds, hemicellulose does not have a crystalline structure and thus, is more susceptible to hydrothermal extraction and hydrolysis (Peterson 2009). Hemicellulose is water soluble when it is separated from other plant fractions and this solubility is due to the presence of the side chains. To separate the hemicellulose, the lignin bonds should be cleaved which can be done with alkali solutions at lower temperatures (<140 °C) or only water at higher temperature (200 °C<) (Pedersen et al. 2010).

2.4.4.3 Lignin

Lignin, a second to cellulose as the most plentiful renewable carbon source, is a complex high molecular-weight polymer which in contrast to cellulose and hemicellulose has an aromatic structure. The main monomers in lignins are *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol which are all phenyl-propane derivatives (Table 2.2). Currently,

a limited supply of lignin is available as a by-product of the pulp and paper industry but this is subject to change due to increasing numbers of biomass-to ethanol plants which produce lignin as their by-product (B. Zhang et al. 2007). Although it commonly have been used for combustion to generate heat and power, its complex phenolic structure implies that it can be used as a source of various value-added products (B. Zhang et al. 2007).

2.5 Biorefineries and Bio-based Chemicals

A biorefinery is a facility that integrates biomass conversion processes and equipment to co-produce fuels, power, and chemicals from diverse biomass source. This definition is very close the developed oil refineries where various products (such as fuel and petrochemical intermediates and final products) are generated. In a bio-based economy, industrial biorefineries are regarded as the most promising route for the production of fuels and chemicals (B. Kamm et al. 2006). Biorefinery technology follows two main strategic goals: moving away from imported petroleum for western countries (an energy goal) and the establishment of robust bio-based industry (an economic goal) (Bozell & Petersen 2010). While the energy goal has already been widely investigated with the extensive research on bioethanol, biodiesel and hydrogen from biomass, fuels are considered as high volume-low cost commodities and they have low return on investments as a sole process. This problem is more pronounced in newer biofuel options such as algal oil that has not been able to present an economically feasible route for its production unless other streams from this biomass can be identified for additional revenues.

Bio-base chemicals, which are derived from biomass, are an attractive solution to alleviate our dependence on petroleum-based chemicals and improve the financial attributes of biorefinery plants by manufacturing more valuable products. In contrast to traditional organic synthesis chemistry, which one of its main aims is to construct complex structures

from simpler molecules, biomass conversion requires efficient, inexpensive and feasible routes to derive useful chemicals from complex structures which are present in the starting feedstocks. In addition, the research on bio-based chemicals can be considered as divergent as it is desirable to produce multiple outputs using numerous chemical transformations (i.e. bond making, hydrogenation, reduction and oxidation, dehydration etc.).

Recent researches have reported the potential chemical products or intermediates which can be derived from plant biomass (Marshall & Alaimo 2010). This has been accompanied by the growing interest of synthetic chemists to design reactions which deliver more complex structure from simple intermediate products. In terms of synthetic chemistry, however, the industrial use of carbohydrates are hindered based on the over-functionality of carbohydrates which is due to the large number of OH-groups that obtain almost the same chemical reactivity and makes selective chemistry very difficult to perform (Bicker et al. 2003). Yet, an important step to resolve this problem is to focus on the formation of carbon-carbon double bonds or carbonyl groups instead of hydroxyl groups in the carbon framework of carbohydrates. This approach and such a structure are believed to play a crucial role joining the carbohydrate resources to petrochemical industry (Bicker et al. 2003).

In a recent review by Bozell and Peterson from the US Department of Energy, an updated evaluation of potential target structures which can be derived from biorefinery was carried out (Bozell & Petersen 2010). A list of top 11 compounds, which follows their previous list of top ten compounds published in 2004, were selected based on meeting 9 different criteria (Table 2.7). In summary, these criteria were based on the level of attention of research community towards a compound, technology applicability and the possibility of deriving different derivatives, feasibility of high volume production, potential as being a platform and other technological chemical measures.

Table 2.7 New top chemical opportunities from biorefinery carbohydrates, criteria for their inclusion and resulting technology (adapted from (Bozell & Petersen 2010)

Compound	Illustrative general biorefinery technology needs
Ethanol	Selective alcohol dehydrations; improved biochemical production of alcohols from biomass (rate, yield, titer, product, pH, inhibitor tolerance); engineering of optimal fermentation organisms
Furans (HMF, FDCA)*	Selective dehydrations of carbohydrates; new catalysts and reaction media for dehydration; reactive separations; selective oxidations of alcohols; improved oxidation and dehydration catalysts; catalytic systems for reactions in aqueous solution
Glycerol and derivatives	Reactions in aqueous solution; selective reductions and oxidations of polyols improved biological conversions of polyols
Biohydrocarbons (Isoprene)	Improved biohydrocarbon production; engineering of organisms to convert sugars to hydrocarbons; optimizing rate, yield, titer, product tolerance
Lactic acid	Optimization of bioconversion of carbohydrates; bioprocesses with high rate, yield, titer, product, pH and inhibitor tolerance; engineering of organisms to produce single materials
Succinic acid	Bioconversion of carbohydrates; optimization of yield, rate, titer, separation; engineering of organisms for optimal production of target
Hydroxypropionic acid	Optimization of bioconversion of carbohydrates; bioprocesses with high rate, yield, titer, product and inhibitor tolerance; engineering of organisms to produce single materials; selective dehydrations of alcohols; selective reductions of carbonyl groups, new selective hydrogenation catalysts; chemical processes in aqueous solutions
Levulinic acid	Selective dehydrations of carbohydrates; improved separations of products; utility of co-product schemes by biorefinery; improved catalysts for selective carbohydrate conversion processes
Sorbitol	Selective hydrogenolysis of polyols; new catalysts for reduction of carbohydrate derivatives; selective dehydrations of polyols; comparative assessment of chemical and biochemical conversion technology; selective bond breaking/bond making technology for polyols
Xylitol	Selective hydrogenolysis of polyols; new catalysts for reduction of carbohydrate derivatives; selective dehydrations of polyols; comparative assessment of chemical and biochemical conversion technology; selective bond breaking/bond making technology for polyols

* *HMF (Hydroxymethylfurfural), FDCA(furandicarboxylic acid)*

While some of the introduced chemicals in Table 2.7 are based on reaction of sugar monomers in a chemical platform (e.g. HMF, levulinic acid and Xylitol), the suggested production route for others as ethanol, lactic acid, succinic acid and hydroxypropionic acid is fermentation as the most efficient option. Typically, the fermentation process is always coupled with making extensive amount of spent microorganisms (mostly yeast except in lactic acid process) which is the major waste from fermentation step. This comes on top of the large volumes of yeast waste which is currently produced in breweries and ethanol fermentation plants, and all are mostly incinerated without any further exploitation. This

highlights the potentials of a conversion process to transform this microbial biomass to other useful products, which can introduce supplementary product streams to the main process in the fermentation.

2.5.1 Furan-related Derivatives

Sugar-derived furans have been widely studied in the research on dehydration of 5- and 6-carbon monosaccharides. This reaction has been of special interest as these sugar molecules can be derived from cellulosic biomass which introduces a potential green and renewable source for these compounds. The main products of these reactions are furfural and hydroxymethylfurfural which have been labelled as platform chemicals based on their potential to be converted to numerous chemical compounds. Traditionally, this reaction used to suffer from lack of yield and selectivity of the final products which these have been addressed in recent studies mainly by applying novel catalysts or solvents. Dimethyl furan (DMF) has been suggested as an attractive biobased fuel with better qualities than ethanol, as it have 40% higher energy density compared to ethanol, a higher boiling point (20 K) and also is not soluble in water (Roman-Leshkov et al. 2007). DMF can be produced from hydroxymethylfurfural (HMF) with CU-Ru/C catalyst in the presence of hydrogen (Kazi et al. 2011). In case of HMF, numerous systems have been used to improve the yield and feasibility of the process. The dehydration of hexoses to form HMF has been conducted in water (Roman-Leshkov et al. 2007), aprotic organic solvents (dimethylsulfoxide (DMSO)) (Claude Moreau et al. 1996) and biphasic systems (water/methylisobutylketone (MIBK)) (Seri et al. 2000) using catalysts such as organic acids (oxalic, maleic) (Szmant & Chundury 2007), inorganic acids (sulphuric, hydrochloric) (Ishida & Seri 1996) salts ($MgCl_2$) (Armaroli et al. 2000), organocatalysts (such as $LaCl_3$) (Carlini et al. 2004), solid acids (ion-exchange resin, VPO_4) (Carlini et al. 2004) and zeolites (Claude Moreau et al. 1994).

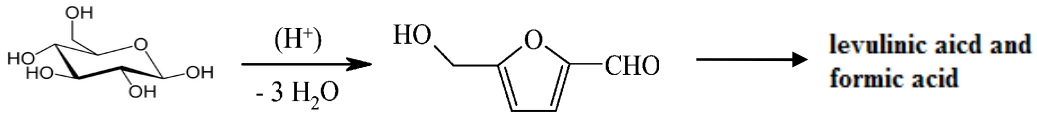
Preparation of HMF from fructose and glucose in ionic liquids have also been studied resulting in high yields (70%) using $CrCl_2$ catalyst in 1-ethyl-3-methylimadazolium chloride (C Moreau et al. 2006). The separation of product from the ionic liquids, however, has remained a challenge and may require several hours of extraction. The yield of HMF is higher when the used substrate is fructose, but glucose is more attractive as it is cheaper and more abundant comparing to fructose. The potentials of HMF production from biomass-derived glucose are further discussed in chapter 5 where the results of our study on production of HMF from spent yeast biomass have also been provided.

2.5.1.1 HMF: Chemistry and production routes

There is a growing research activity in the chemistry of furfural derived compounds such as 5-hydroxymethylfufural (HMF), 2.5-fruandicabaldehyde and 2.5-furan-dicarboxylix acid due to the numerous applications they can offer (Lewkowski 2003). HMF is a furan derivative and has a high reactivity and multi-functionality. It is an important intermediate chemical that can be employed to derive a range of chemicals which currently originate from petroleum and its derivatives. It is a complex primary aromatic alcohol, an aldehyde, and a furan ring system which has already been used in the production of resins (Zakrzewska et al. 0). But its greater potential is being a precursor of other 2,5-distributed furan derivatives such as 2,5-frurandicarboxylic acid (FDCA) via selective oxidation which can be used as a replacement for terephthalic acid in the production of polyesters (e.g. PET and PBT) (Chheda et al. 2007). Other important applications can be for the production of fine chemicals, pharmaceuticals, solvents or biofuels such as 2,5-dimethylfuran (DMF) (Bicker et al. 2003). But as this chemical has not been yet a high-volume chemical (in view of the current difficulties regarding its cost-effective production), the potential uses of HMF-

derived polymers and other low cost chemicals has not been fully addressed (Chheda et al. 2007).

The synthesis of HMF is based on the triple dehydration of hexoses using different substrates such as Hexose themselves, oligo- and polysaccharides. (Lewkowski 2003). Scheme 2.1 shows a general reaction pathway for the synthesis of HMF from hexose. Despite the simple pathway, HMF formation is complex and includes a series of side-reactions that can negatively the yield of the reaction. The decomposition to levulinic acid and formic acid and the polymerisation to humic acid are the most important side reactions (Lewkowski 2003).



Scheme 2.1 Synthesis of HMF from glucose and its derivative products

The HMF synthesis is more efficient and more selective when started from ketohexoses (i.e. fructose) than from aldohexoses (i.e. glucose). The dehydration of hexoses is commonly known as being catalyzed by protonic acids as well as by Lewis acids (Bicker et al. 2005).

The production of HMF via dehydration of hexoses has been studied in numerous solvents such as water (pure water or acidified water and additives) (Asghari & H. Yoshida 2006), biphasic systems (Chheda et al. 2007; Bicker et al. 2003), aprotic organic solvents such as dimethylsulfoxide (DMSO) (Musau & Munavu 1987), common organic solvents such as acetone (Bicker et al. 2005) and ionic liquids (Zakrzewska et al. 0) Different catalytic systems have also been employed for this reaction including inorganic and organic

acids (oxalic and maleic acid) (Salak Asghari & H. Yoshida 2006), aluminium salts (Tyrlik et al. 1999) and organocatalysts (Lanthanide chloride (Seri et al. 2000)).

Each of these systems, however, may be limited in terms of the yield, selectivity and formation of by-products or the cost of the process in larger scale. In aqueous systems, selectivity of the reaction is limited which leads to other by-products such as humic, especially at higher concentrations of fructose or glucose where the formation of polymerized humin is more facile (Bicker et al. 2005). Although using heterogeneous catalysts have shown to improve the selectivity for the HMF formation to more than (>80%), the conversion remained in the range of 25- 50% (Carlini et al. 2004). The HMF selectivity in different medium are compared in Figure 2.13.

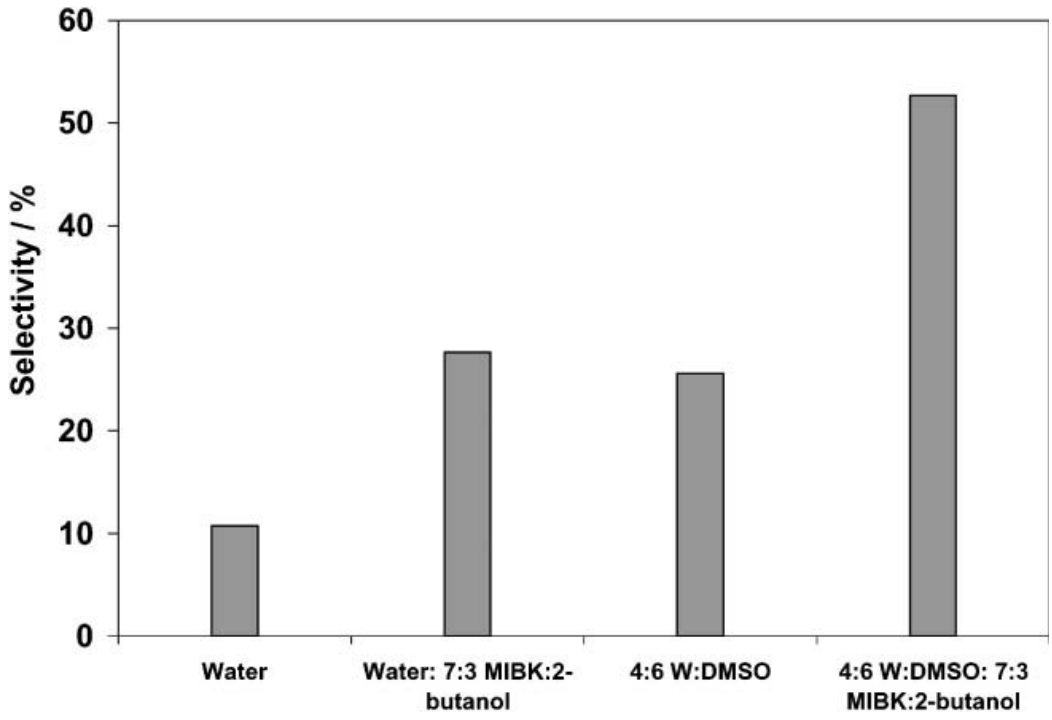


Figure 2.13 Effect of HMF selectivity of adding an extracting organic solvent to the aqueous phase for 10 wt% glucose dehydration 443 K using HCl as catalyst (Chheda et al. 2007)

Biphasic systems also suffer from using a large quantity of organic solvents and also the need to remove organic residues from the phase which is water in most cases. The high boiling point of the HMF and organic solvents such as dimethylsulfoxide (DMSO) also means that large mounts of energy is required to separate HMF from the organic phase after the reaction.

Although the fructose offers higher yields in most systems, glucose is the preferred feed as it is more abundant and readily available (Chheda et al. 2007), and hence has been considered in most studies for HMF production. Table 2.8 compares the yield of HMF production from glucose, cellulose and cellobiose in different solvent systems and catalysts. In aqueous system, the maximum yield was not more than 16% in the presence of TiO_2, while in water:DMSO:DCM system, HMF can be produced from glucose with a yield of up to 30%. An intrinsic drawback of direct conversion of glucose is the low selectivity of HMF production which is mostly lower than 50%.

In more novel systems, higher yields of HMF can be achieved. As an example, Li *et al.* obtained the yields of 60% and 90% from cellulose and glucose respectively in a microwave heated system and in the presence of $CrCl_3$ (C. Li et al. 2009).

Table 2. 8 Yield of HMF production from glucose in different systems

Raw material	Solvent	Catalyst	Yield (%)	Selectivity (%)	Temp (^{o}C)	time (min)	Ref
Glucose	Water	H_2SO_4	5	n.a	200	5	Watanabe *et al*., 2005
Glucose	Water	TiO_2	16	n.a	200	5	Watanabe *et al*., 2005
Glucose	Water	HCl	2	11	175	45	Chheda *et al*., 2006
Glucose	Water:DMSO	HCl	11	26	200	10	Chheda *et al*., 2006
Glucose	Water:DMSO:MIBK: 2-butanol	HCl	23	47	175	17	Chheda *et al*., 2006
Glucose	Water:DMSO:DCM	n.a.	30	48	175	270	Chheda *et al*., 2006
Cellobiose	Water:DMSO:DCM	n.a.	38	45	175	570	Chheda *et al*., 2006
Cellulose	EMIM	$CuCl_2/CrCl_2$	57	n.a	120	480	Su *et al*., 2009

Several studies have reported different building blocks and useful intermediates that can be derived from biomass. It is due to the presence of carbohydrates backbone which are made of hexoses (i.e. glucose or fructose) which are shown to have different functionalities. According to the report by the US Department of Energy, HMF is one of the top biomass derived chemicals which can be coupled to the existing biorefinery technology (Bozell & Petersen 2010). Due to its potential industrial application and possibility of being made from biomass, HMF has been referred as"sleeping giant" and a "key substance between carbohydrate chemistry and mineral oil-based industrial organic chemistry" (Zakrzewska et al. 0). Most of the studies on carbohydrate-based HMF synthesis are performed with modelling the real biomass with pure monomers (such as glucose and fructose) or polymers such as cellulose.

2.5.2 Brewer Spent Yeast and Its Applications

The sample which was used in this study was a yeast containing waste slurry from cider manufacturing process. The other waste in cider process is apple pomace, apple

residues after juice extraction, which was not addressed in this project.. While main constituent of the substrate was yeast cells, other constituents were apple juice, apple debris and related plant debris, culture medium additives and fermentation aids such as B vitamins (thiamine), sufur dioxide and sugar. Apart from the chemical composition or the strain of the yeast, the cider spent yeast is structurally similar to the one which is produced from the beer fermentation as the main components in both wastes are yeast. Cider spent yeast (lees) was used to be sold as animal feed (£2-£4 per kg for dried yeast) previously but current market is declining which resulted in the additional costs for drying and disposal of this waste.

The yeasts used in the brewery industry are from two main groups of bottom-fermenting and top-fermenting yeast which results in Lager beer and ale respectively. The yeast fraction is usually re-used several times (4-6 times) and can be taken from one fermentation tank to start the next (Ferreira et al. 2010). The excess or used yeast (spent yeast) is the second main waste from brewery industries (after apple pomace). The spent yeast is primarily sold as inexpensive animal feed after inactivation by heat or dried and used as a source of nitrogen (Ferreira et al. 2010). Yeast biomass is also known for its considerable concentrations of B-complex vitamins, nucleic acids and the biologically active form of chromium known as glucose tolerance factor. The use of yeast biomass, especially in its dried form, has long been known in food and flavour industries. For this purpose, yeast is usually undergoes autolysis reaction which is carried out under controlled heating in order to kill the yeast without deactivating its enzymes. The main purpose for autolysis is to release the intracellular content of the yeast to the medium using the self-destructing mechanism of the cells and also convert the complex structures of proteins, RNA and DNA to simpler amino acids and nucleotides or other useful monomers. Furthermore, recent studies report that spent yeast biomass can be employed as a bio-adsorbent to remove chemicals such as

dyes (J.-xia Yu et al. 2009), metal ions (L. Cui et al. 2010). Figure 2.14 shows how the valorisation of yeast biomass has changed during the time.

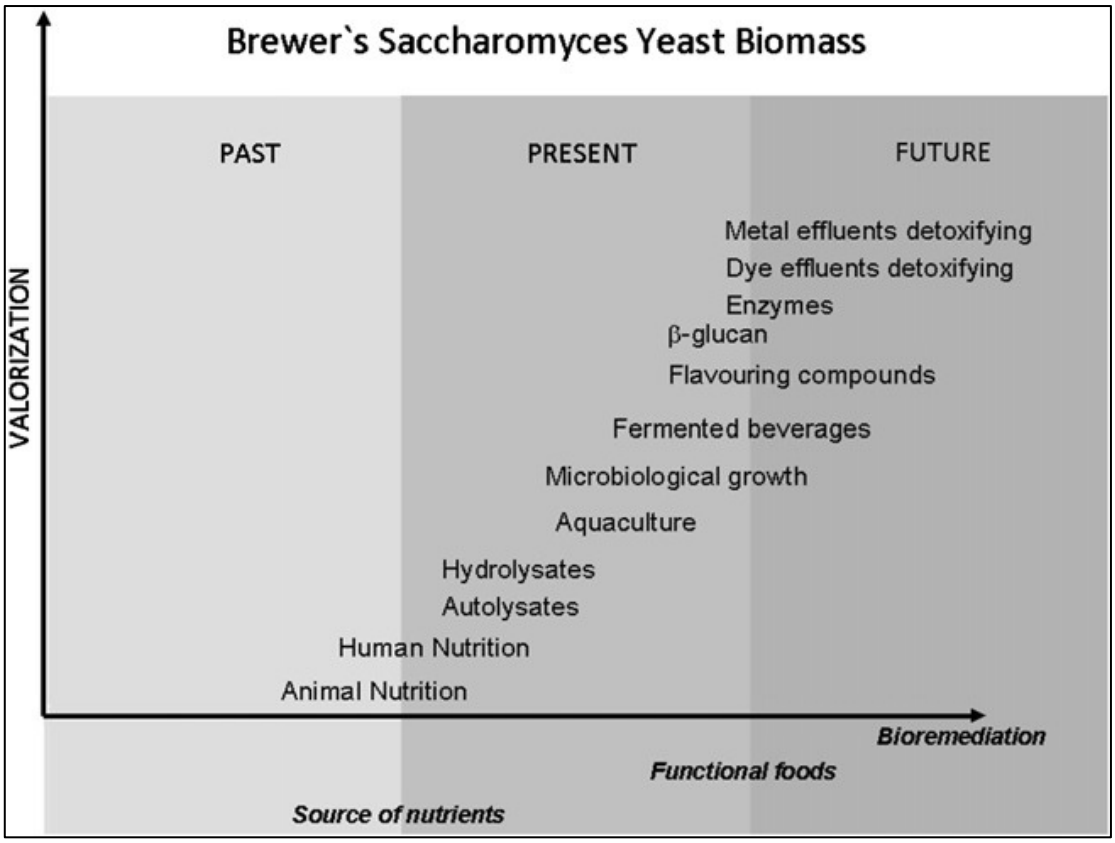


Figure 2.14 Schematic representation of the brewers yeast biomass valorisation at different times (Ferreira et al. 2010)

Cider manufacturers also deal with a similar problem as the two main wastes of these industries are apple pomace (apple residues after the extraction of apple juice) and spent yeast. Apple pomace, which is also a waste in apple juice production, represents up to 30% of the original fruit and consists of peel, core, seed, calyx, stem and soft tissue (Vendruscolo et al. 2008). Traditionally, apple pomace has been used in the production of pectin but recent studies suggest that it can also be used for other food-related products such as lactic acid and fibre-rich concentrates (Gullon et al. 2007), or as a substrate for other biotechnological applications to generate single cell protein, aroma compounds, ethanol, organic acid, enzymes and polysaccharides (Vendruscolo et al. 2008). In contrast to apple pomace, cider spent yeast has been addressed in a very limited studies for its further application. The traditional market for this waste is animal feed industries as well where its nutritional values captured and used in feed additive (dry brewey yeast costs £2-£4 per kg basaed on the price quoted by www.nettex-supplies.co.uk on Nov 2010) . However, this market is diminishing as novel formulations for animal feeds are becoming cheaper and more popular which limits the application of spent yeast. This has made cider manufacturers to investigate possible applications and markets for this slurry as alternative for the incineration or drying routes.

2.5.3 Hydrothermal conversion of yeast biomass

As discussed in section 2.4.3, hydrothermal treatment of biomass has been widely employed for the conversion of waste or raw materials to valuable compounds, especially in the past two decades. Subcritical water or hot-compressed water (HCW) may be used as both solvent and reactant in a range of different reactions which have previously been reviewed (Y. Yu et al. 2008). Water at elevated temperatures and pressures (but below its critical point) exhibit exciting physical and chemical properties such as low dielectric constant and higher diffusivity which can boost ionic reactions and is shown to be suitable for different

syntheses or degradation reactions (Y. Yu et al. 2008). This hydrolytic effect can offer an exciting route to transform or derive various compounds from biomass (Kruse & Dinjus 2007b; G. Brunner 2009).

In this projects, the studied biomass (cider spent lees or yeast) was comprised of yeast cells (*Saccharomyces bayanus*), apple juice and apple residues, plant debris, organic acids (lactic acid) and additives which are introduced during the fermentation step. However, the main constituent of the slurry was the yeast cells which are made of polysaccharide cell wall (β-glucan and mannoproteins are the main constituent of the yeast cell wall). When yeast cells start to disrupt and hydrolyse, their intracellular content will be released to the medium followed by further degradation of biopolymers (cell wall carbohydrate and proteins) to their monomers. The intracellular contents of the yeast cells are mainly comprised of proteins and amino acids, carbohydrates, nucleotides (DNA and RNA), nutrients such as vitamins and a small fraction of lipids. Due to the complex nature of the yeast extract which leads to the further problems in purifying individual components, yeast extracts are mostly used in their crude structure as additives in foods or for animal feed based on their nutritional properties. The hydrolysates form the hydrothermal treatment process are also a mixture of proteins and amino acids which have been shown to be a useful source of nutrients for culture mediums as reported by Lamoolphak *et al*. (Lamoolphak et al. 2007).

Proteins and sugars have long been known to undergo the well-known Maillard reaction in aqueous mediums at higher temperatures. This reaction has been studied extensively in its application in food, flavour and medical fields such as the caramelisation reaction, is known as a non-catalysed browning reactions. This reaction produces polymerised molecules known as melanoidins. As will be shown later, the reaction mixture of our experiments contain high concentration of proteins and monosaccharides, which is

expected to result in browning reactions (sugar oxidation and colour change) or Maillard reaction (colour change and formation of polymeric compounds). This reaction will be further investigated in the dry mass measurement section.

The extraction of glucan fraction present in the yeast cell wall has been of interest to other researchers when using fresh yeast (K. S. Kim & Yun 2006a) or brewery spent yeast (X. Y. Liu, Q. Wang, et al. 2008; Thanardkit et al. 2002) but on the other hand no work has been reported to the author knowledge on the use of cider spent yeast for such application.

It is believed that the extracts have antioxidant properties due to the presence of phenolic compounds in the extract. For this reason the Total Phenolic Concentration (TPC) of the extracts was measured as preliminary results suggested that the phenolic content of the extracts varies with the temperature and time of the reaction.

The extraction of phenolic compounds from natural sources and feedstocks have also been reported by (Conde et al. 2009; Cruz et al. 2004; R. Bitsch et al. 2000; S. K. Lee et al. 1998) because of their beneficial health effects.

2.5.4 Yeast autolysis for the production of yeast extract

Yeasts have been widely used in human culture to produce alcoholic beverages, leaven bread dough and at the same time for the production of yeast extract, which is widely used in the food products as an additive to enhance taste and flavour or as a protein and vitamin source for nutritional fortification (Tanguler & Erten 2008). Yeast extracts can be obtained by different methods such as hydrolysis, plasmolysis and the most frequently process which is autolysis. This maximizes the nutritional value of the yeast suspension, as they undergo a process during which the intracellular contents of the cells are released to the medium.

Yeast autolysis is a process carried out by activating the self destructing enzymes of the yeast cell to solubilise the cell wall. The term “autolysis” means self-destruction and it s a series of complex enzymatic reactions which start after the cell dies. Initially, the cell membrane disintegrates which permits the degradative enzymes attack the cell wall and to come in contact with cellular organelles and constituents to become soluble. These hydrolytic enzymes, particularly protease and nuclease, breakdown to insoluble macromolecules like proteins and nucleic acids to amino acids, nucleotides and their derivatives (Tanguler & Erten 2008). As a result of cell breakdown, the intracellular contents of the cells would also be released to the surrounding medium (Figure 2.15).

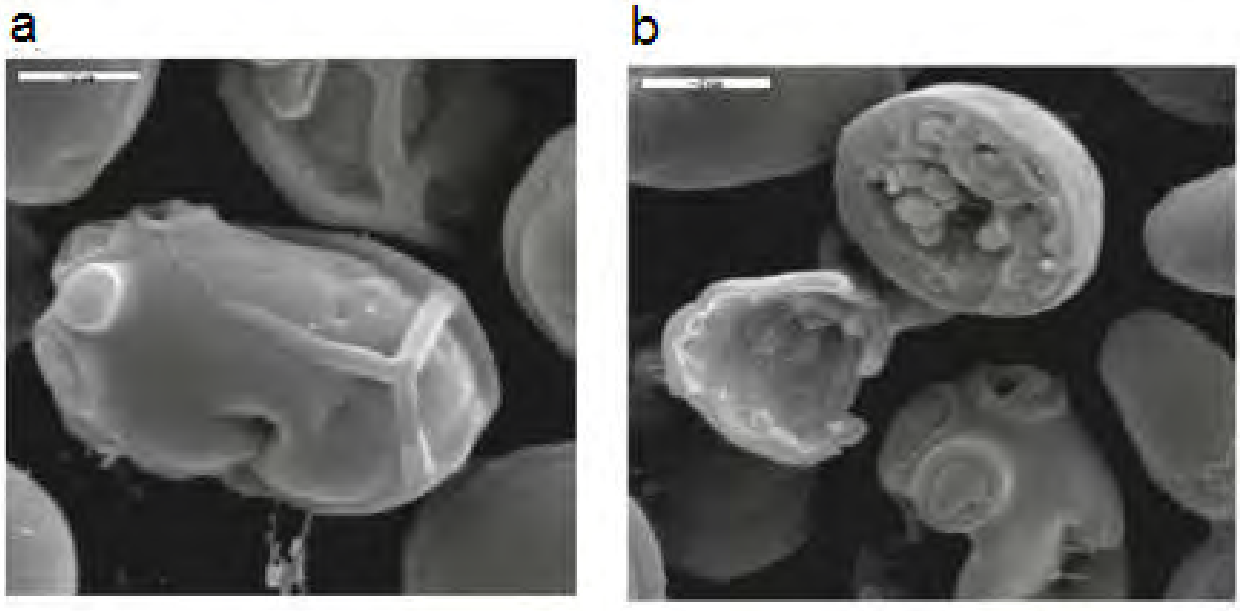


Figure 2.15 SEM images of yeast cells after 24 h of autolysis in a model wine system. A superficial ultra-structure of a yeast cell. B Image of fractured empty yeast cells which have lost most of their cytoplasmic content during the autolysis (Moreno-Arribas et al)

The autolysis is initiated by a controlled rise in temperature or osmotic shock which results in the cells dying without destructing or inactivating their enzymes. The main factors which influence the process and are decisive for an optimal autolysis process are temperature, controlled pH and duration of the autolysis (Sommer 1996).

2.5.4.1 Autolysis and extraction of valuable compounds

As a proven technique to release the confined intracellular compounds, autolysis has been specifically used to capture valuable compounds in the yeast cells. Due to the mild condition whereby enzymatic reactions digest the cell wall barrier, most of the target chemicals would be released with minimum modifications. In addition, these specific reactions can also be employed to modify the compounds in order to release them from bound structure or further modifications.

Autolysis has long been used for the extraction of cellular protein and is the basis for commercial production of yeast extracts (Tsang et al. 1979). Examples of using autolysis for other compounds can be as extraction of lipids which was studied by Pueyo and co-workers (Pueyo et al. 2000). They studied the release of different classes of lipids such as mono-, di-, and tri-acylglycerols, free fatty acids and sterol esters and their effect on the wine sensory attributes and foam characteristics. Amino acids and protein mixtures have also been a common product after cells being subjected to autolysis conditions (G.L. Huang 2010; Lamoolphak et al. 2007). As in Lamoolphak et al. work, autolysis was carried out to prepare a crude mixture of nutrients that can be used to fortify yeast medium cultures. Autolysis has also been suggested as an effective procedure for enzyme extraction in laboratory protocols as it was shown for the extraction of invertase by Illanes and Gorgollon (Illanes & Gorgollón 1986). The combination of autolysis and enzymatic treatment have also been shown to be an

effective method to facilitate the extraction of disodium inosine-5´-monophosphate (5´-GMP) from yeast extract (Sombutyanuchit et al. 2001). This compound, which is one of the main flavours and taste enhancers in the yeast extract with a large application in food industry, was shown to be released and extracted from autolyzed yeast by means of a crude extract from malt rootlets (which contain 5´-phosphodiesterase) (Sombutyanuchit et al. 2001).

While in most autolysis reactions, fresh baker or brewery yeast has been used for this purpose, some authors (Sombutyanuchit et al. 2001; Saksinchai et al. 2001; Jiang et al. 2009) have also suggested the use of spent yeast as the starting material for the autolysis which can also be regarded as a solution for large quantities of this biomass generated after fermentation.

2.5.5 Spent Cider Yeast (Lees)

2.5.5.1 Cider Process and Lees Production

During cider fermentation, the apple juice which is fortified by glucose syrups, is the main substrate that yeast cells exploit for their metabolism and formation of secondary product i.e. ethanol. The yeast is recycled and used several times during the process to start the next fermentation cycle while the excess yeast is removed and discarded from the process. A diagram of the cider fermentation step is demonstrated in Fig. 2.16, which shows the sources of cider spent yeast (lees) and how it is collected in the cider process. When collected, this excess yeast forms the second biggest by-product in brewery industries (Ferreira et al. 2010). Around 8,000 tonnes of *spent cider lees* are produced annually only in the UK by the leading cider manufacturer Scottish & Newcastle PLC. Multiples of this

volume is believed to be produced in beer manufacturing industries. The traditional market for this yeast waste has been animal feed industries to capture its exceptional nutritional values in animal diet. For this purpose, yeast cells which are suspended in very polluting slurry, needs to be dried and the dried powder is added to the animal feed. Also, beer spent yeast has been used to produce yeast extract (H. J. Chae et al. 2001; Tanguler & Erten 2008; Saksinchai et al. 2001), a highly nutritional extract which has been a good source of B-complex vitamins, amino acids, lipids, RNA and other minerals available in yeast cells. While this extract has been suggested to be employed in flavour industries (In et al. 2005) or as a microbiological culture ingredient (Saksinchai et al. 2001), no such investigation has been carried out for cider spent yeast. The current application for this by-product is limited to animal feed industry after being inactivated by heat. This method needs to compete with alternative protein sources like soybeans which manufacturers are becoming more interested (Saksinchai et al. 2001). Therefore, cider spent yeast has to be sold at a price which manufacturer may not even find enough to cover the cost of the drying process. Other common option is land disposal after being completely incinerated, which the remaining nutrients were not recovered and may result in toxic emissions sometimes may necessitate further waste/gas treatment (Lamoolphak et al. 2007). Hence, it is in the interest of manufacturers to seek alternative options to transform and upgrade these wastes to more valuable chemicals/mixtures or other uses.

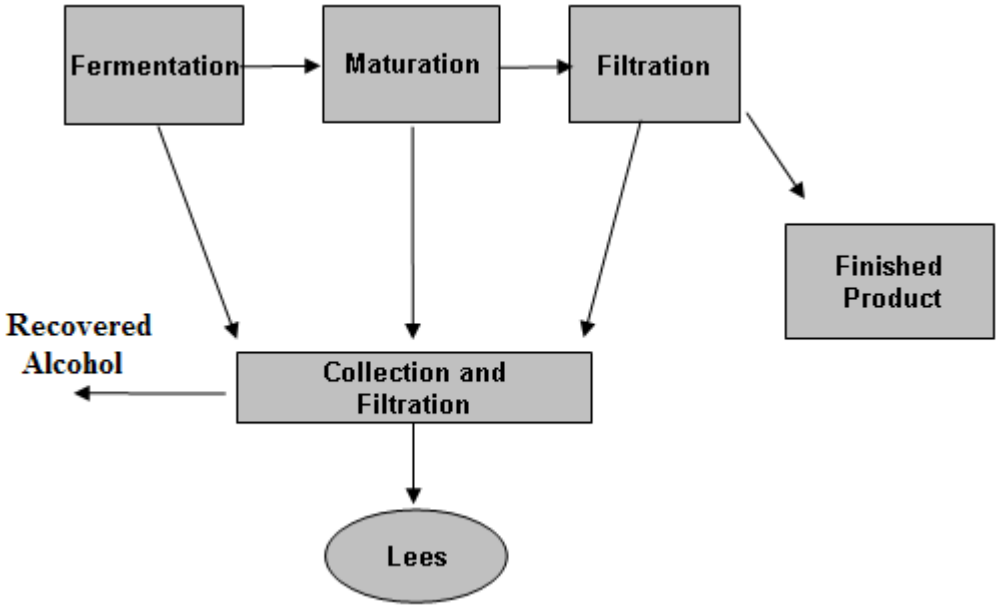


Figure 2.16 Process diagram of the cider manufacturing and collection of lees as waste (courtesy of Heineken UK (Bulmers)

Cider lees is mainly comprised of yeast cells, apple juice, apple and plants residues and other additives which were added in the upstream such as organic acids and nutrients for fermentation step.

Despite the similarity in the structure (and almost in the composition) of brewery spent yeast and cider spent yeast, the difference in the strain of the yeast and the type of process (which includes lactic acid fermentation in cider process) has a substantial effect on the utility of this waste for similar applications which is common for brewery yeast. As measured in this project, the pH of the spent yeast was in the range of 3.6 implying that it is acidic in contrast to brewery spent yeast which is typically neutral. Based on the lactic acid bacterial fermentation which occurs in the product, there is the possibility of the presence of bacterial residues in the cider lees. However, there might be potential applications for this waste in other products which requires the cells to be inactivated first with heat or other techniques resulting in a dry or semi-dry paste. Alternatively, the deactivation can be carried

out in a pressurized system where the water remains in the medium and temperature can rise to the sterilisation temperature (120 °C) or beyond levels.

The necessity of using heat deactivation for cider spent yeast highlights the potential of products which can be extracted after similar hydrothermal treatment. In addition, this emphasizes the importance of this study and introduces a new feedstock for existing hydrothermal conversion technologies. This suggest an opportunity to research and design a conversion platform based on subcritical water treatment and outline the possible products which can be derived from this biomass. Such a conversion process to upgrade microbial biomass has not been discussed in literature to the knowledge of the author. Therefore, the potential candidates which are discussed below are based only on background information about this biomass feedstock and include the components that are already present (intracellular and extracellular) and may be generated in the hydrothermal reactions.

2.5.5.2 Potential Products From Cider Lees

2.5.5.2.1 Proteins and Amino acids

The main study which has used subcritical water treatment to hydrolyse brewery spent yeast to useful products was carried out by Lamoolphak *et al*. (Lamoolphak et al. 2007). They studied the decomposition of yeast cells at temperatures between 100 and 250 °C and studied the hydrolysates in terms of protein and amino acid concentration. They showed that such conversion technologies can potentially be used to decompose proteinaceous waste such as spent brewer's yeast while recovering more useful products. The same results is believed to be valid for the cider spent yeast as it is believed that proteins and amino acids would be released from any yeast biomass under such conditions. They reported that the

protein and amino-acid fraction can be used as a source of nitrogen for other cultures mediums.

2.5.5.2.2 Carbohydrates (β-glucan) and related derivatives

Carbohydrates are the main component in biomass feedstock and are present in the form of polysaccharides i.e. cellulose, hemicellulose and starch (Cherubini 2010). A previous overview (section 2.4.3) which was provided earlier covered the recent advances in carbohydrate conversion and its potential derivatives. The composition of the carbohydrates in biomass, their concentration and physical properties show their feasibility for further exploitation.

Yeast cells are enclosed by a rigid β-glucan structure which is formed of β-1-3- and β-1-6-glycosidc bonds. Glucan is the general name for polysaccharides consisting only of D-glucose monomers such as cellulose, dextran and starch. The glucans are further specified by the type of the main glycosidic bonds in the polymer. Glycosidic bonds are formed when a carbohydrate is linked to an alcohol (which can be another carbohydrate) with the formation of one water molecule. The glycosidic bonds are distinguished into α- and β- bonds (Fig 2.17). An α-glycosidic bond for a D-glucose is situated below the plane of the sugar while the hydroxyl (or other substituent group) on the other carbon points above the plane (opposite configuration). A β-glycosidic bond is situated above that plane (the same configuration). The numbers (1-3 or 1-6) indicate which carbon atoms are linked. It is known that β-1-3-glucan is the major fraction of yeast cell wall and is therefore an interesting components to consider for added value products derived from yeast (Tonnies 2008). β-1-3-glucans are useful in different applications such as viscosity imparting agents, emulsifier, binders in tablets, food thickeners and also as carriers and coating agents in the pharmaceutical industry (Wheatcroft 1997). In a study by Thanardkit *et al*., β-1-3-glucan is

reported as an immuno-stimulating agent e.g. wound healing, antibacterial, anti-tumor, anti-flammatory and anti-viral activity (Thanardkit et al. 2002; Sandula et al. 1999).

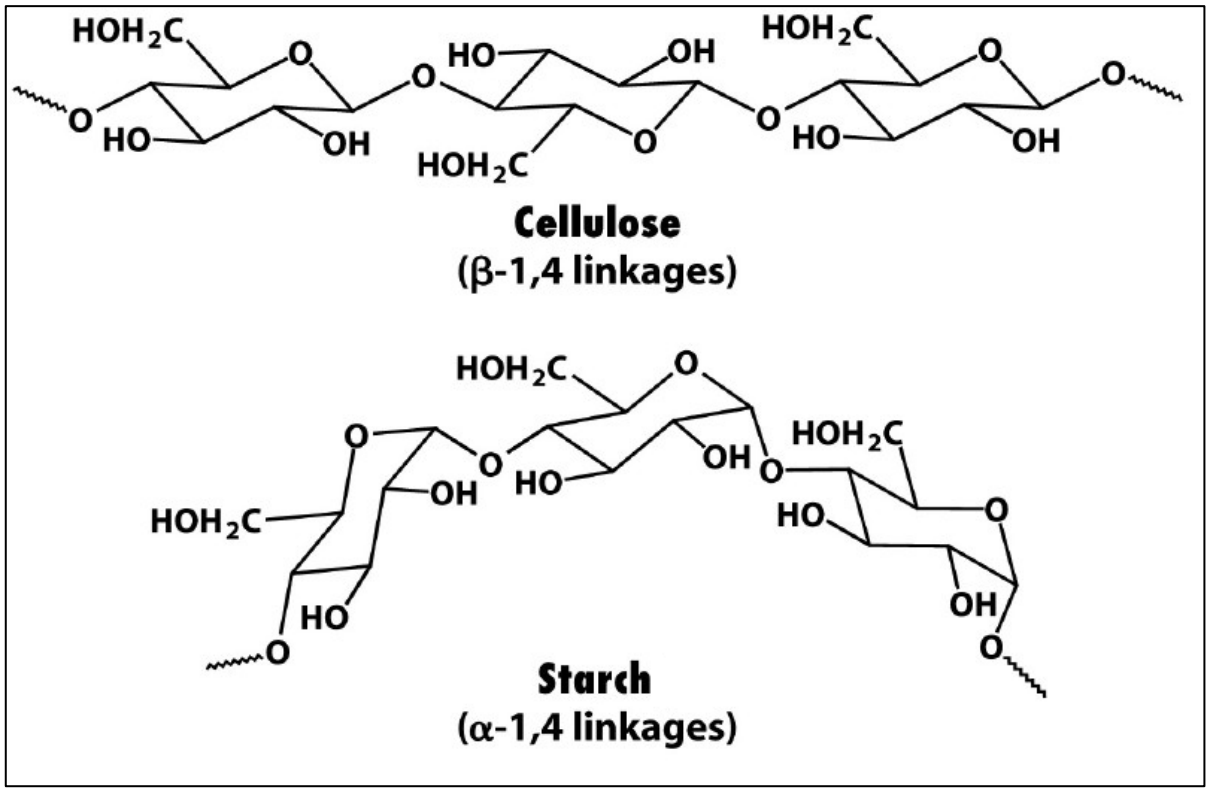


Figure 2.17 Structural difference between Cellulose and Starch and their glycosidic bonds (Ref: http://oregonstate.edu/instruction/bb450/fall2010/lecture/carbohydratesnotes.html)

β-1-3-glucan can also be extracted from cereals like oat and barley or other plant sources such as fungi. However, it has been suggested that the yeast sourced β-glucan is more effective in health and cosmetic products based on its specific composition (K. S. Kim & Yun 2006b). Most of the studies which focus on β-glucan from yeast have used freshly prepared yeast's biomass for their studies. Thanardkit *et al.*, however, reported that the β-glucan can also be extracted from spent brewer's yeast in higher yields using less extensive extraction procedures compared to commercial yeast-derived immune stimulants which is produced from more expensive baker's yeast (Thanardkit et al. 2002).

β-glucan in the yeast cell wall, however, can be considered as a source of sugar monomers which can be converted to its glucose and mannose subunits using a hydrolysis process such as hydrothermal treatment. Similar to other biomass feedstocks, this means a new window opportunity to derive versatile platform chemicals from the resulting sugar subunits. This idea was followed in this project and was discussed in detail in Chapter 5, where the formation of HMF from produced sugar monomers were discussed.

2.5.5.2.3 Vitamins and other Nutrients

Vitamins: An overview

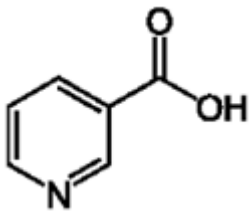
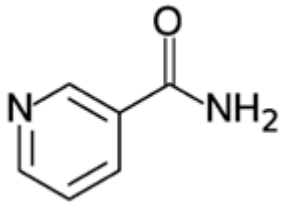
Vitamins are essential organic compounds which are for the most part sourced through a balanced diet. Vitamin deficiency can lead to severe symptoms but these are often easily remedied by administering the daily recommended dose of the vitamin (Ullmann 2007). Due to the detrimental effects of food processing on the nutritional properties of the food, it is often necessary to supplement food and feed products with vitamins to ensure a balance diet. Today most vitamins are chemically synthesised at industrial scale with the exception of vitamin B12 (cobalamin) which is produced via fermentation (Ullmann 2007). However there is an increasing interest to obtain vitamins from natural bio-based sources as a result vitamins A, D3, E and K are extracted from natural compounds.

Vitamin B3 (niacin, as the generic name for both nicotinic acid and its derivative nicotinamide) is one of the vital vitamins as its deficiency can lead to Pellagra disease. The chemical structure of nicotinic acids and nicotinamide are shown in Table 2.9. Nicotinic acid is mainly present in plants while nicotinamide is mostly available in animal products (Eggersdorfer et al. 2005). Nicotinic acid forms nonhygroscopic, colourless needles and is stable in air. It melts without decomposition at 236 °C under ambient pressure and starts to

sublime at 150 °C, with increasing sublimation rates above 220 °C. Nicotinamide is also a crystalline colourless compound that forms needles and has a melting point of 129-132 °C at ambient pressure. Niacin is needed for the synthesis of the coenzymes NAD^+ and $NADP^+$ which in turn catalyse redox reactions, e.g. in the synthesis and degradation of amino acids , fatty acids and carbohydrates. Over 350 enzymes require NAD^+ and $NADP^+$ as coenzyme (Shibata & Taguchi 2000).

Before the chemicals synthesis of vitamin B3 was successfully developed, there were many attempts to extract the vitamin from natural sources, such as yeast, wheat, liver etc. using hot water, HCl or NaOH (Teply et al. 1941). Yeast has long been known to be a good source of B vitamins particularly vitamin B3. In yeasts, vitamin B3 is mainly present in the form of nicotinic acid. The B vitamin contents of yeast are shown in Table 2.10.

Table 2. 9 Chemical structure and properties of nicotinic acid and nicotinamide

	nicotinic acid	**nicotinamide**
		
molecular weight [g/mol]	123.11	122.12
solubility in water [g/l] at:		
0°C	8.6	very soluble (~ 1000)
38 °C	24.7	-
100 °C	97.6	-
melting point (°C)	236-237	129-132*

* stable modification

The amount of vitamin B3 in yeast cells varies between 200 to 700 mg/kg dry mass based on the yeast strain and the culture condition (Tseng & Phillips 1982). Yeast can synthesize nicotinic acid enzymatically from 3-methylpyridine.

Table 2. 10 Concentration of Vitamin B's in yeast (Kirk-Othmer 2000)

Component	Baker's yeast (mg/kg dry mass)	Brewer's yeast (mg/kg dry mass)
Thiamine (B1)	90	91.7
Riboflavin (B2)	45	35
Niacin (B3)	-	448
Pantothenic acid (B5)	65	110
Pyridoxal group (B6)	40	43
Folic acid (B9)	15	10

The presence of vitamin B group in yeast has been reported from decades ago. Determination and extraction of these compounds from yeast have also been outlined in some studies, such as for vitamin B1 (thiamine) (Guha 1931), vitamin B5 (pantothenic acid) (Neilands et al. 1950) and vitamin B6 (pyridoxal phosphate) (Rubin et al. 1947). However, the extraction of vitamin from natural resources is diminished now as all of these chemicals (except vitamin B12 which is produced in a biotechnological process) are manufactured using synthetic chemistry (Kirk-Othmer 2000).

Despite the relatively high concentrations of vitamin B3 in yeast comparing to other vitamins, its extraction from yeast has never been discussed in detail. In the context of this project, which was studying the valuable compounds which may be derived from yeast after hydrothermal treatment; the release of this compound from yeast after this reaction was also investigated. This objective was further discussed in the chapter 4, where the release of

nicotinic acid and nicotinamide (amide of nicotinic acid which has also been classified as vitamin B3) from the cider spent yeast is demonstrated.

In the light of having a biomass which was consisted mostly of yeast cells, a set of experiments were designed to identify and possibly extract the vitamin B3 from the yeast cells using the subcritical water hydrolysis step which are dicussed in detail in chapter 4.

In addition to vitamins, there are other high value compounds present in the yeast which can be extracted such as sequalene (Bhattacharjee P & Singhal R. S 2010) and sterols (Quail & Kelly 1996). Phenolic compounds, in particular, are other potential fractions which can be recovered from cider lees as they are believed to being carried over from the upstream of the process and left in this waste after the separation of cider. Phenolic compounds and the possibility of their extraction from cider lees are discussed in chapter 6 because of their importance as natural antioxidants, a potential ingredient in cosmetic formulations and health supplements.

TLC analysis of Vitamins

As one of the fastest chromatography analysis techniques, TLC still plays a significant role in determination and qualitative and quantitative analysis of different compounds in biological and non-biological samples. The use of this technique has been well documented for many years and has observed extensive modifications such as the development of high performance thin layer chromatography (HPTLC) (Arup et al. 1993), two dimensional TLC (Zakaria et al. 1983), preparative TLC techniques (Loev & Goodman 1967) and ultra-thin layer chromatography techniques (Bezuidenhout & Brett 2008). TLC has been successfully used for the analysis of different vitamins compounds in biological samples, especially vitamin B group (Cimpoiu & Hosu 2007; Ponder et al. 2004).

Based on these results and the inherent properties of TLC techniques, as a fast, convenient with minimal effect on the sample, this method was employed for the analysis of the vitamin B3 in our extracts. The idea of using TLC technique for this purpose was based on the previous studies which employed for similar compounds (Sherma & Fried 2005; Cimpoiu & Hosu 2007; Ponder et al. 2004). Moreover, there were some advantages associated with the use of TLC plates as they offered less limitation on the size of the sample loading on the plate, offering less technical difficulty with the complexity of the sample matrix, no the need for prior pretreatment and possible use of only aqueous solvents. These advantages, which are not the case when an HPLC technique is used, was of great interest at that stage due to the extensive amount of samples need to be screened accordingly. Also, the silica which was the adsorbent on the plates was a cheap and practical material that offers economical and technical advantages could be useful in the light of further scaling up of the recovery process. Besides, an important aspect of this research was to use environmentally friendly techniques throughout the reaction and succeeding recovery steps by minimizing the use of organic solvents in the reaction (as a biphasic or neat medium) or in the extraction steps.

2.6 Phenolic Compounds

Natural phenolics have long been studied for their beneficial effects on human health and nutrition and their positive activity has been captured in numerous consumer products. As one of the major phytochemicals, phenolic compounds are believed to lead the positive health effects of vegetables and fruits mainly as an effective antioxidant. Hence, more research groups are becoming interested studying new natural phenolics from different substrates. These substrates can be fruits and related by-products such as apple (Escarpa & González 1999) and apple pomace (Schieber et al. 2003), berries (Cacace 2003), red grape

marc(Bonilla et al. 1999), grape seeds (C. Xu et al. 2010), olive oils (Montedoro et al. 1992), or non-fruit sources such as olive leaves (Japón-Luján & Luque de Castro 2006) and olive tree pruning (Conde et al. 2009).

In the natural samples, phenolics are either suspended in the liquid phase or are encompassed in a solid matrix like the seeds and leaves. Different techniques have been used for phenolic extraction such as polymeric adsorbents (Schieber et al. 2003), activated carbon (Q. Lu 2004), solvent extraction (R.M. Alonso-Salces et al. 2001) subcritical water (Rangsriwong et al. 2009; Ibañez et al. 2003; Baek et al. 2008) and supercritical carbon dioxide (Vatai et al. 2009). For the extraction of phenolics from solid matrixes, solvent extraction (supercritical fluids or organic solvents) have been traditionally used while novel technologies, such as microwave (Hong et al. 2001) or sonication (Kivilompolo & Hyotylainen 2009) are reported as effective while provide a more environmentally friendly process. These techniques are regarded as expensive approaches requiring costly equipments and are limited process scale which may not be applicable in these processes where larger volumes of substrates are used (Swami Handa et al. 2008). In contrast, using subcritical water extraction can potentially a platform with use of non hazardous chemicals and less advanced equipments. However, the capital costs for the process can still be high as the extraction must be carried out in heat resistant vessels (stainless steel, or hastelloy if at supercritical water conditions). The full advantages of the process should be studied after full technical and economical analysis of the process.

As cider spent yeast originates from cider process, phenolic compounds are expected to be present in the studied crude sample. They must have been carried over from the upstream of the process where apple juice (or apple juice concentrate) was introduced as the main raw material. Apple phenolics have been widely studied while is still in fruit or in related

products such as apple juice, cider and apple pomace. In case of apple pomace, which is another waste stream of the cider manufacturing, the phenolic composition have been studied elsewhere (Schieber et al. 2003; Vendruscolo et al. 2008; Gullon et al. 2007; Diñeiro García et al. 2009). However, no studies have been carried out on the phenolic composition of cider spent yeast to the knowledge of author. Studying the phenolics in this substrate is more important as it has been showed that subcritical water have been employed previously for their extraction from different substrates (as mentioned above). Hence, applying the high temperature and pressure condition to the aqueous substrate can enhance phenolic extraction or conversion form highly polymeric tannins. An overview of phenolic compounds, their composition and their health effects are provided below.

2.6.1 Plant phenolics: Chemistry and characterisation

Phenolic compounds (or polyphenols as they are mostly contain more than one phenol group in their structure) are secondary metabolites in plant kingdom which are present in variety of structures, starting from quite simple skeleton of phenolic acids, flavonols and catechin and extend to more condensed structures such as tannins with molecular weights in excess of 500 and up to 20,000 for proanthocyandins. Currently, more than 8,000 of these phenolic compounds have been identified and determined in plants, where they are synthesized. The major role of these compounds in plants is to protect them from environmental threats such as UV light, microorganisms, and chemical factors.

Phenolic compounds are divided into two major classes: Flavonoids, which are the major ones and include a range of different compounds and non-Flavonoids within which the phenolics acids are the most known ones.

2.6.1.1 Flavonoids

This group of phenolics are one of the most common and widely distributed types of plant phenolics and they usually occur as glycosides. As a group, it is believed that they are the most associated phytochemical class with antioxidant values in plants (Urquiaga & Leighton 2000). Their common structure is that of diphenylpropanes (C6-C3-C6) and consists of two aromatic rings linked through three carbons that usually form an oxygenated heterocycle (Proestos et al. 2008).

Fig 2.18 shows different subgroups of flavonoids and the main skeleton of flavonoids are listed in Fig 2.19 with an examples molecule in each group. Major difference between these subgroups is due to structural variations within their main structure which divides the flavonoids into several groups which are classified as below (Rice-Evans et al. 1997):

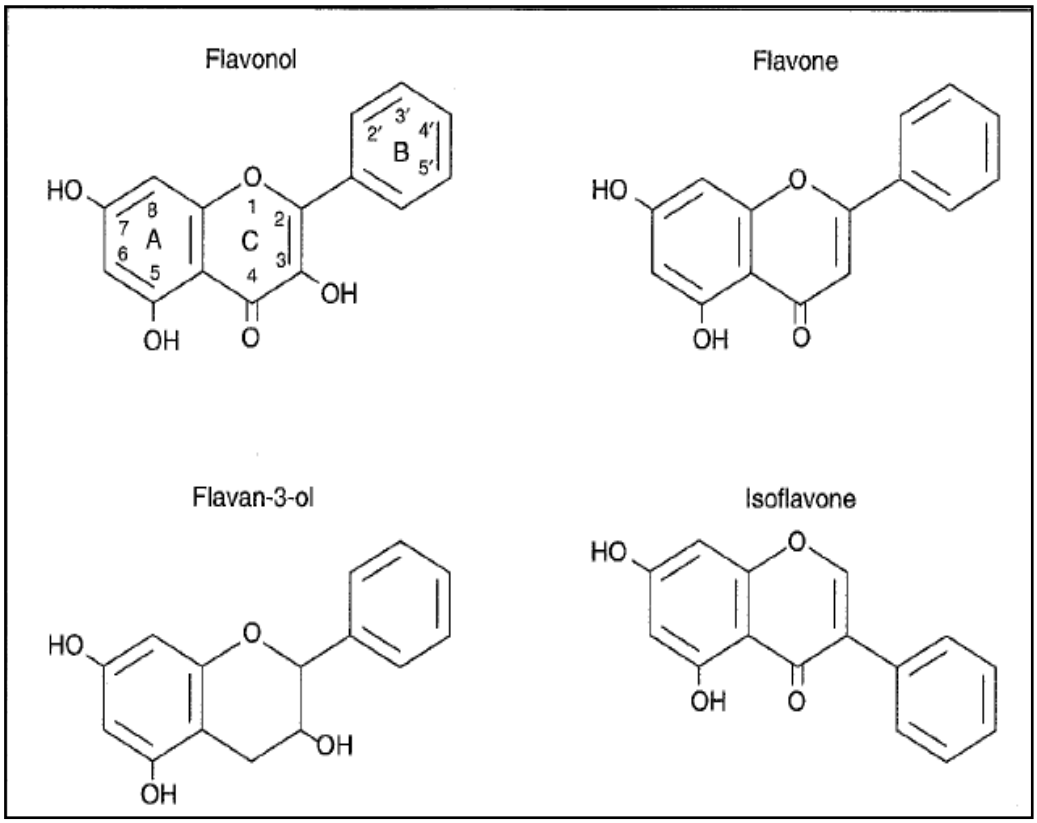


Figure 2.18 Structures of the flavonoids. The basic structure consists of the fused A and C rings, with the phenyl B ring attached through its 1' position to the 2-position of the C ring (numbered from the pyran oxygen) (Rice-Evans et al. 1997)

Flavonols, like quercetin and kaempferol with the 3-hydroxy pyran-4-one C ring. It is found in all plants with bright colours, such as berries and broccoli.

Flavanols (Flavan -3-ols) lacking the 2,3-double bond and the ketone bond. Mostly found in black and green tea, berries and dark chocolate. Single structures (monomers) are called catechins whereas multiple-unit structure (dimers, trimers or polymers) are called proanthocyanidins.

Isoflavones (like genistein and daidzein), in which the B ring is located in the 3 position on the C ring. They are mostly found in soy products.

Flavanones which are the characteristic phenolic in citrus fruits (oranges, grapefruit, lemons) examples are naringenin, silybin, and hesperidin.

Flavonals (anthocyanins) mainly in red, purple and blue fruits and vegetable (examples are cyanidins and malvidin).

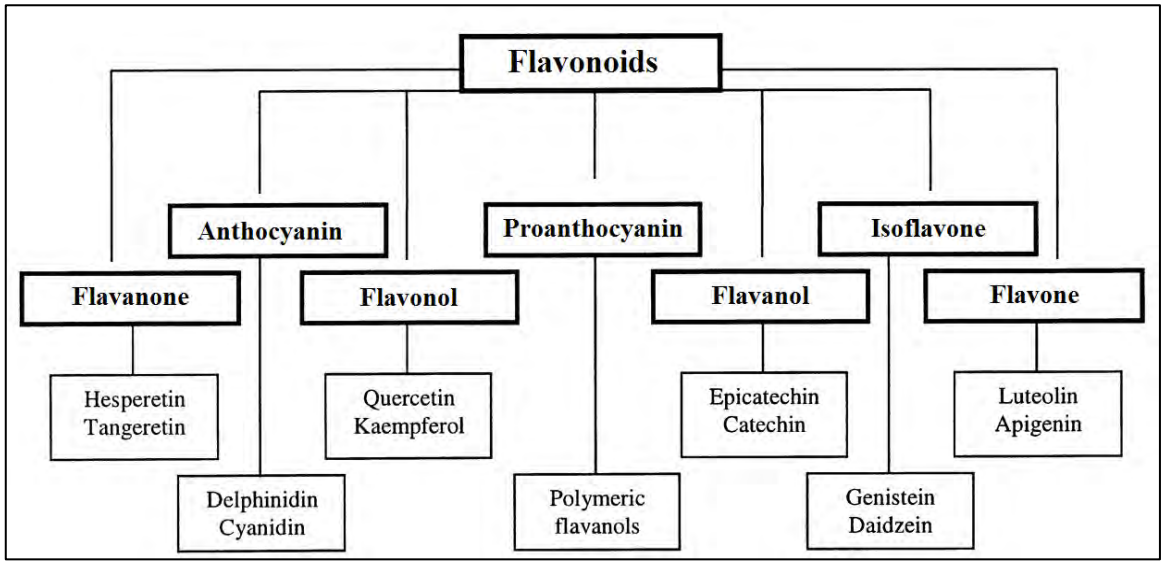


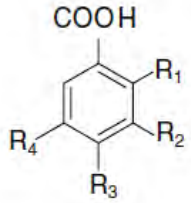
Figure 2.19 Flavonoids and their subgroups

2.6.1.2 Non-Flavonoids

Non-flavonoids are mostly consisting of phenolic acids (such as ellagic acid, tannic acid), hydroxycinnamic acid derivatives (such as caffeic acid, chlorogenic acid, ferulic acid coumaric acid, lignans and stilbenes such as resveratrol. Table 2.11 compares the molecular structure of two main groups of phenolic acids.

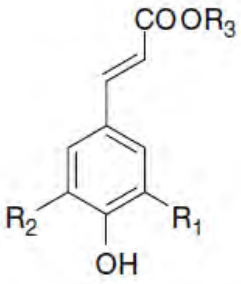
Table 2. 11 Structures of hydroxybenzoic acids (A) and hydroxycinnamic acids (B) (adapted (Moreno-Arribas & Polo 2009)

(A)



Hydroxybenzoic acids	R_1	R_2	R_3	R_4	MW
Gallic acid	H	OH	OH	OH	170
p-Hydroxybenzoic acid	H	H	OH	H	138
Salicylic acid	OH	H	H	H	138
Syringic acid	H	OCH_3	OH	OCH_3	198
Vanillic acid	H	OCH_3	OH	H	168

(B)



Hydroxycinnamic acids	R_1	R_2	R_3	MW
Caffeic acid	OH	H	H	180
Caftaric acid	OH	H	Tartaric acid	312
p-Coumaric acid	H	H	H	164
p-Coutaric acid	H	H	Tartaric acid	296
Ferulic acid	OCH_3	H	H	194

Nonflavonoids form the major part of phenolic composition of the wine and have a significant effect on the sensory parameters of it (Moreno-Arribas & Polo 2009).

2.6.2 Biological and Health Effects of Polyphenols

Phenolics, in general, have been proved to lead the beneficial health effects of vegetables and fruits and their contribution to the antioxidant capacity of the human diet is much larger than that of vitamins. A combination of free-radical scavenging and epidemiological studies have shown that there is an inverse relationship between the intake of flavonoids, in particular, and the incidence of certain diseases and malignancies (Rice-Evans et al. 1997). There are number of studies which study the effect of phenolics intake and their role on prevention or control of various diseases including different cancers such as breast cancer (Malin et al. 2003), skin cancer (Svobodová et al. 2003), colon cancer (Rajamanickam & Agarwal 2008) and other diseases such as type 2 diabetes, heart diseases, asthma (Knekt et al. 2002). A key factor in such studies, however, was to show how active these compounds are in the body or in other terms to investigate their bioavailability. This parameter show the ability of a certain compound to be adsorbed in the digestive tract and exert its activity in the blood plasma. In a major review by Monarch *et al*. which compared the results from 97 studies, the most well-adsorbed polyphenols were reported to be gallic and isoflavones followed by catechins, flavanones and quercetin glucoside (Manach et al. 2005).

2.6.3 Determination of Phenolics-Review of techniques and samples

The analysis of phenolic compounds can be carried out using different techniques which are described below. These techniques can be classified into six main groups, based on the type of the phenolics which introduced in section 2.6.1. Following, an overview of these methods are provided based on the related type of compound.

Total Phenolics, which is based on measuring all the available phenolic compounds. The most common method is the Folin-Ciocalteau assay which was developed specifically

for phenolics by Singleton and Rossi (Singleton & Rossi 1965) and employs the Folin-Ciocalteau reagent which originally was prepared for protein measurement. Other methods worth noting is the recently developed cyclic voltammetry that measures the redox potential of the phenolic compounds. In general, these non-specific assays suffer from the lack of differentiation of phenolics and tend to overestimate total phenol contents due to interference of reducing substances (Schieber et al. 2001).

Monomeric or individual phenolics: Which are measured mostly by reversed-phase high performance liquid chromatography (RP-HPLC) that separates different phenolic monomers and quantifies them with high accuracy. They also result in a characteristic UV spectrum due to their aromatic structure which is simple to measure as usually no derivatisation is required prior to UV spectroscopy.

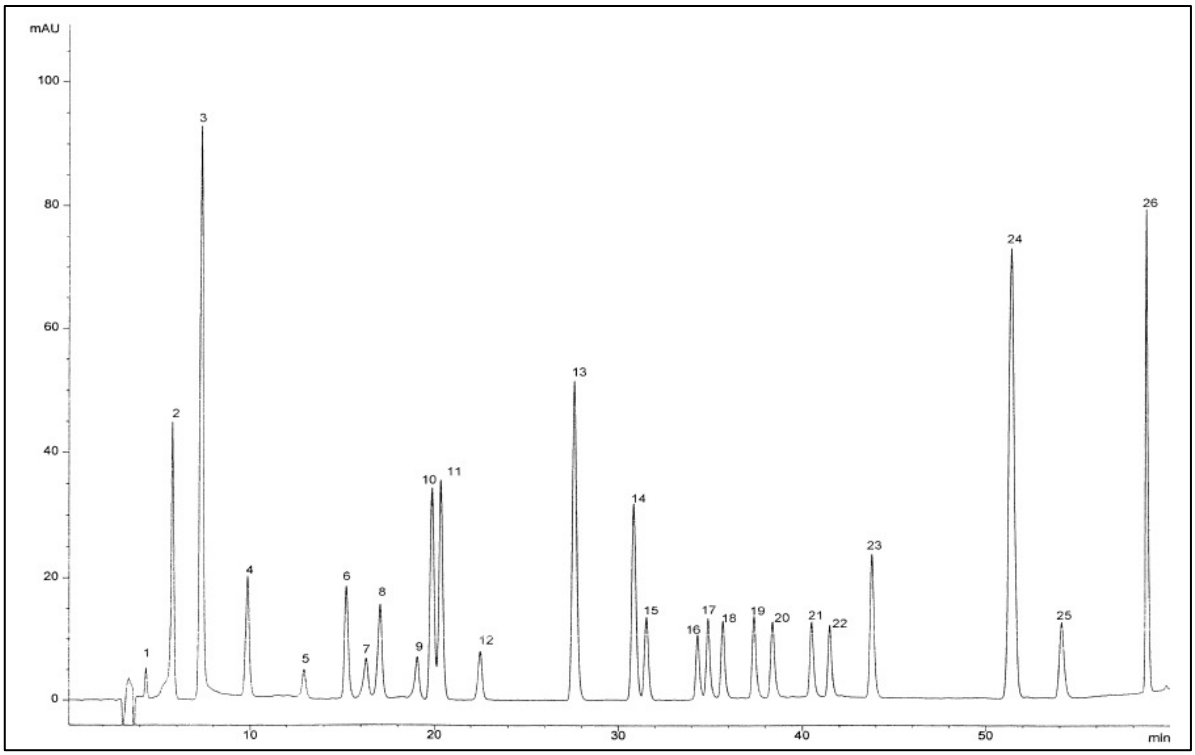


Figure 2.20 Separation of a standard mixture of phenolic compounds (and 5-hydroxymethyl furfural and cinnamic acid) by RP-HPLC (280 nm).

1=Arbutin, 2=gallic acid, 3=5-hydroxymethyl furfural, 4= protocatechuic acid, 5=procyanidin B1, 6=4-hydroxybenzoic. acid, 7=catechin, 8=chlorogenic acid, 9=procyanidin B2, 10=caffeic acid, 11=syringic acid, 12=epicatechin, 13=*p*-coumaric acid, 14=ferulic acid, 15=sinapic acid, 16=quercetin-3-rutinoside, 17=quercetin-3-galactoside, 18=quercetin-3-glucoside, 19=quercetin-3-xyloside, 20=quercetin-3-arabinoside, 21=quercetin-3-rhamnoside, 22=isorhamnetin-3-glucoside, 23=phloridzin, 24=cinnamic acid, 25=quercetin, 26= phloretin (Schieber et al. 2001).

A common HPLC setup for phenolics analysis consists of a reversed phase column followed by a single or multi wave UV detector or diode-array detectors (DAD) which provides more data points and generate UV spectrum for each peaks in chromatogram (Lazarus et al. 1999; B. Suárez et al. 2005; Schieber et al. 2001; Rizzo et al. 2006; Proestos et al. 2008; Q. Liu, Cai, et al. 2008; Mattila & Kumpulainen 2002). An optimized HPLC method is able to separate and identify several phenolic acids in a single injection as shown in fig 2.20 In order to have more accurate results that are accompanied by structural information of the chemicals, coupling a MS detector to the HPLC (LC-MS) or using GC-MS machine have been suggested in different studies (Lazarus et al. 1999; Mane et al. 2007; Rosa M. Alonso-Salces et al. 2004). However, using Gas chromatography-Mass Spectroscopy (GC-MS) requires the compounds to be volatile and thermo-tolerant. Derivatisation techniques are mostly required GC analysis of phenolic compounds because of their high boiling point (Zuo et al. 2002). Generally, LC-MS has been reported the most useful techniques for structural elucidation and confirmation of soluble and insoluble (highly polymeric) plant phenolics (R.M. Alonso-Salces et al. 2001).

However, the diversity and large distribution of these compounds leads to some difficulties, in terms of determination and separation of similar compounds that can be an analytical challenge. This is specially the case in apple phenolics while the simultaneous determination of quercetin glycosides and benzoic and cinnamic acid derivatives and dihydrochalcones are required which have similar structures (Schieber et al. 2001).

Flavanols, which normally involves a reaction with aldehydic reagents, followed by spectrophotometric measurement.

Proanthocyanidins, can be measured by cleaving the bonds between these compounds to form the subunits and then measuring the monomeric phenolics with HPLC. Alternatively,

they can be measured using techniques which separate molecules based on their size or molecular weight, such as size-exclusion chromatography (SEC).

Tannins, which can be measured by protein precipitation (UC Davis protein precipitation assay) which measures anthocyanins, tannins, small polymeric pigments and large polymeric pigments (Harbertson et al. 2002).

Polymeric phenols are measured normally with normal-phase high performance liquid chromatography (NP-HPLC) (A Yanagida et al. 2000) or SEC (Akio Yanagida et al. 2003).

2.6.4 Phenolics in the apple

Apple, as a rich source of polyphenols, has been the subject of different epidemiological studies and in vitro experimental researches that have addressed its contribution to human health and different diseases. They are an excellent source of numerous phenolic which is why it shows high nutritional values and significant antioxidant activity. The phenolic content and antioxidant activity of apple have been studied in several studies. It is been found that apples had the highest soluble free phenolics comparing to 10 other commonly consumed fruits (J. Sun et al. 2002). In a work by Khanizadeh *et al*., the antioxidant capacity of 20 fruits was measured using the oxygen radical absorbance capacity assay (ORAC), and apple was showed to be ranked 8 out of 20 (Khanizadeh *et al*., 2008). In another study by Boyer and Liu, it was reported that apples have the second highest level of antioxidant activity compared to many other commonly consumed fruits in the United States (Fig. 2.21) (Boyer & R. Liu 2004). Several authors measured total antioxidant capacity (TAC) in apple using total oxyradical scavenging capacity (TOSC) assay and showed higher TOSC in the peel of apple than in the flesh. Not only is the concentration of the total

phenolic compounds is higher in the peel than in the flesh, peel is a the only source of quercetin glycosides (Khanizadeh *et al*., 2008).

In vitro studies (K. W. Lee et al. 2003; Rezk et al. 2002) have suggested that phenolic compounds in apple show high antioxidant properties and there is a direct link between apple juice consumption and level of antioxidant in blood plasma (R. Bitsch et al. 2000). It has also been reported that this effect extends to the related products and by products of apple (apple juice, cider) and its by-products such as pomace (Diñeiro García et al. 2009; Schieber et al. 2003). Polyphenols, which are the more condensed structures comprised of phenolic acids and other small phenolic molecules, have great importance in the nutritional and commercial properties of food products due to their sensory properties such as colour and flavour (R.M.

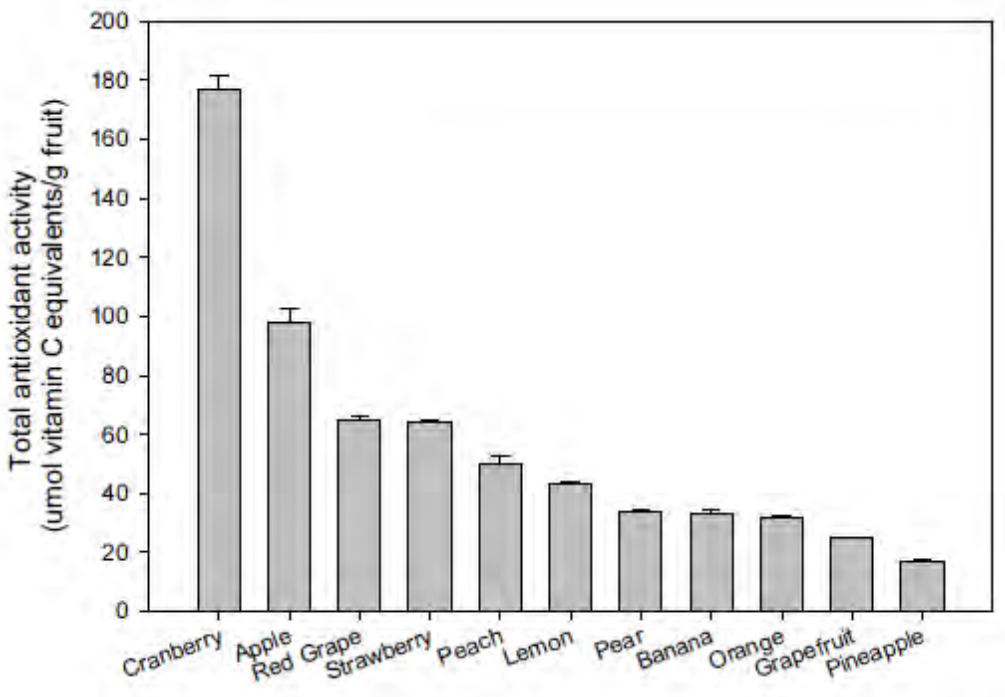


Figure 2.21 Antioxidant activity of various fruit extracts (Boyer & R. Liu 2004)

Alonso-Salces et al. 2001). This indicates the importance of polyphenolic composition of the used apple varieties in the quality of the produced cider. Cider taste is affected by procyanidins and their degree of polymerization as astringency increases by their degree of polymerization and bitterness follows the opposite trend (Rosa M. Alonso-Salces et al. 2004)

After averaging different apple varieties, the most concentrated phenolics in apple were indicated as quercetin-3-galactoside (13.2 mg/100g fruit, vitamin C, 12.8 mg/100 g fruit; procyanidin B, 9.35 mg/100 g fruit; chlorogenic acid, 9.02 mg/100 g fruit; epicatechin, 8.65 mg/100 g fruit, and phloretin glycosides, 5.59 mg/100 g fruit (K. W. Lee et al. 2003). Based on these epidemiological studies, it appears that apples may play a major role in reducing the risk of a wide variety of chronic disease and maintaining a healthy lifestyle in general (Boyer & R. Liu 2004).

2.6.5 Phenolics Interaction with the Yeast Cells

While yeast cells are believed to partly contribute their cellular ingredients (such as vitamins, amino acids and other nutrients) to the final extract, they have been reported to target suspended phenolic compounds by (mainly) physically adsorbing them on their outer cell wall in wine process. The yeast cell wall is made of different components with mannoproteins and oligopolysaccharides covering its outer surface. These mannoprotein are also bound to glucanose and chitin which makes a mixture of different polymers and sites with different levels of polarity, hydrophobicity and hydrophilicity each are potentially offer affinity for different molecules in wine and cider such as metabolic intermediates, pigments flavonoids (e.g. anthocyanidins) and volatile compounds (Morata et al. 2003). Another key factor is the high porosity of the wall leading to higher surface areas which favour the adsorption of different compounds. Studying the adsorption of procyanidins (which are more condensed phenolic structures) on the apple cell wall, Le Bourvellec at al. reported that the

polysaccharide plays the major role on this interaction and suggested that adsorption increases with ionic strength and decreases with increasing temperatures (Le Bourvellec et al. 2004). Based on these results, they suggested that the bonds which govern this interaction were weak bonds like hydrogen bonds and hydrophobic interaction.

Phenolic adsorption on yeast cells has been studied extensively in wine fermentation and was shown to have a significant effect on wine properties such as its colour, astringency and bitterness (J. M. Salmon 2006). In particular, anthocyanins are the main group of phenolics which their adsorption on yeast have been extensively studied due to their role on the wine colour (Morata et al. 2003; K. Medina et al. 2005; Morata et al. 2005). Similar to wine process, the concentration of phenolics can be affected throughout the cider making. In addition, it worth noting that there are other steps in this process such as enzymatic treatment, maceration and other physical clarification steps such as filtration, centrifugation and pressing (Nogueira et al. 2008).

Apple pulp and pomace have been shown to adsorb procyanidins due to their interaction with the cell-wall matrix (Catherine M. G. C. Renard et al. 2001). In addition to the discussed mechanisms, yeast cells have been reported to affect the phenolics concentration during cider fermentation (Nogueira et al. 2008). This interaction is attributed to the exposed oligopolysaccharides and mannoproteins which are exposed on the yeast cell wall (Morata et al. 2003). While the focus have been on the phenolic take-up, only few studies addressed the desorption of previously adsorbed phenolics from the spent yeast cells. Hence, it is still not very clear how this interaction works and what are the main controlling parameters influencing this. The release of phenolic compounds have been studied previously from adsorbents such as activated carbon (Salvador & Merchán 1996) and wine lees (Mazauric & J.-M. Salmon 2006), and it was of the special interest in this project to

investigate the possibility of using subcritical water to replace organic solvents. The ongoing research on subcritical water extraction suggests its use in parallel to traditional organic solvents for the extraction of natural compound offering a solvent-free process (Conde et al. 2009; Japón-Luján & Luque de Castro 2006). As the cider yeast cells have been in contact with a pool of numerous phenolic compounds, they are believed to have retained and adsorbed phenolic compounds based on the same principles as in the wine process.

Chapter 3

GENERAL PROPERTIES OF SUBCRITICAL WATER TREATED CIDER SPENT YEAST

3.1 Introduction

Yeast cells, which form the main structure of solid residues in the studied feedstock (cider lees) can be regarded as an alternative source of monosaccharides as well as other high-value compounds such as yeast nutrients or compounds which originate from apple. Cider lees was subjected to the hydrolysis experiments in a batch system and at high temperature and pressure (100-250 °C, 100 bar) up to 30 mins. The treated samples (hydrolysed yeast) were subjected to a series of analytical techniques in order to determine the major physiochemical characteristics of the extracts namely Total Organic Carbon (TOC), total protein content, total solids and total dry weight, UV-absorbance, Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM).

This chapter details the properties of the extracts in terms of protein content, total organic carbon, total phenolics content, antioxidant activity, solid and liquid phase dry

weights and overall structure of the cells using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The effect of temperature has been examined in order to ascertain the influence of conditions of hydrothermal process on the cider lees.

3.2 Materials and methods

3.2.1 Raw Material (Cider Lees)

20 litre of cider lees was collected from the Bulmer manufacturing plant in Hereford, UK on May 2009, was transferred and packaged on the same day and stored in 250 ml bottles at -20 °C freezer in the School of Chemical Engineering, University of Birmingham. This was to minimize any possible variability in the samples that may occur (pH and solid concentration) during the experiments. No significant change was observed in the stored samples after being stored in -20 °C for up to 6 months. All the experiments and measurements were carried out on the same samples during 6 months. Prior to each experiment or assay, samples were defrosted at room temperature to restore its original form.

3.2.2 Batch hydrolysis reactions

The hydrolysis experiments in this section were carried out in a high pressure bench-top magnetic-stirred batch reactor model 5500 from Parr Instruments Company, USA. Fig 3.1 shows the schematic diagram of the rig where the experiments were carried out. The reactor was constructed with 316 stainless steel and it was equipped with an electric heater and a magnetic stirrer. The temperature and the stirrer speed was set and controlled with a separate Control unit model 4838 from Parr Instruments Company, USA.

The main reactor features and fittings are listed below:

Total reactor volume = 300 ml

Reactor internal diameter (ID) = 55mm

Reactor inside height = 135mm

K-type thermocouple, with accuracy of ± 1 °C

Aschcroft pressure gauge with accuracy of ± 0.25 MPa

Pressure bursting disk

Sampling valves: Butterfly valve (stainless steel)

Stirrer control: a 4-blade turbine impeller, speed control from 0-1500 rpm

Stirred shaft height = 90mm; Stirred shaft diameter = 5.5mm

Pressure release valve: Butterfly valve (stainless steel)

3.2.3 Experimental method

Figure 3.1 shows a schematic diagram of the experimental rig used for the hydrolysis experiments in this chapter. Before each run, the reactor was washed and cleaned with distilled water to make sure no residue is left from previous experiment and was loaded with 200 ml sample (cider lees)

The reactor was closed by placing its head/lid on the top of it and fixing the two bolt clamps which were tightened with spanners allowing the reactor to be firmly closed. A PTFE o-ring fixed between the body and the lid of the reactor guaranteed that no leaks took place. All inlet and outlet ports in the reactor were placed on the head of the reactor. Nitrogen gas (laboratory grade, BOC, UK) was used to pressurise the reactor by opening valve (V-1). The pressure inside the reactor was maintained at 100 bar and recorded by using the pressure gauge (PI-1). The pressure was controlled during reaction by opening either valve (V-1) to increase the pressure or valve (V-2) to decrease it. Heating and stirring was then started.

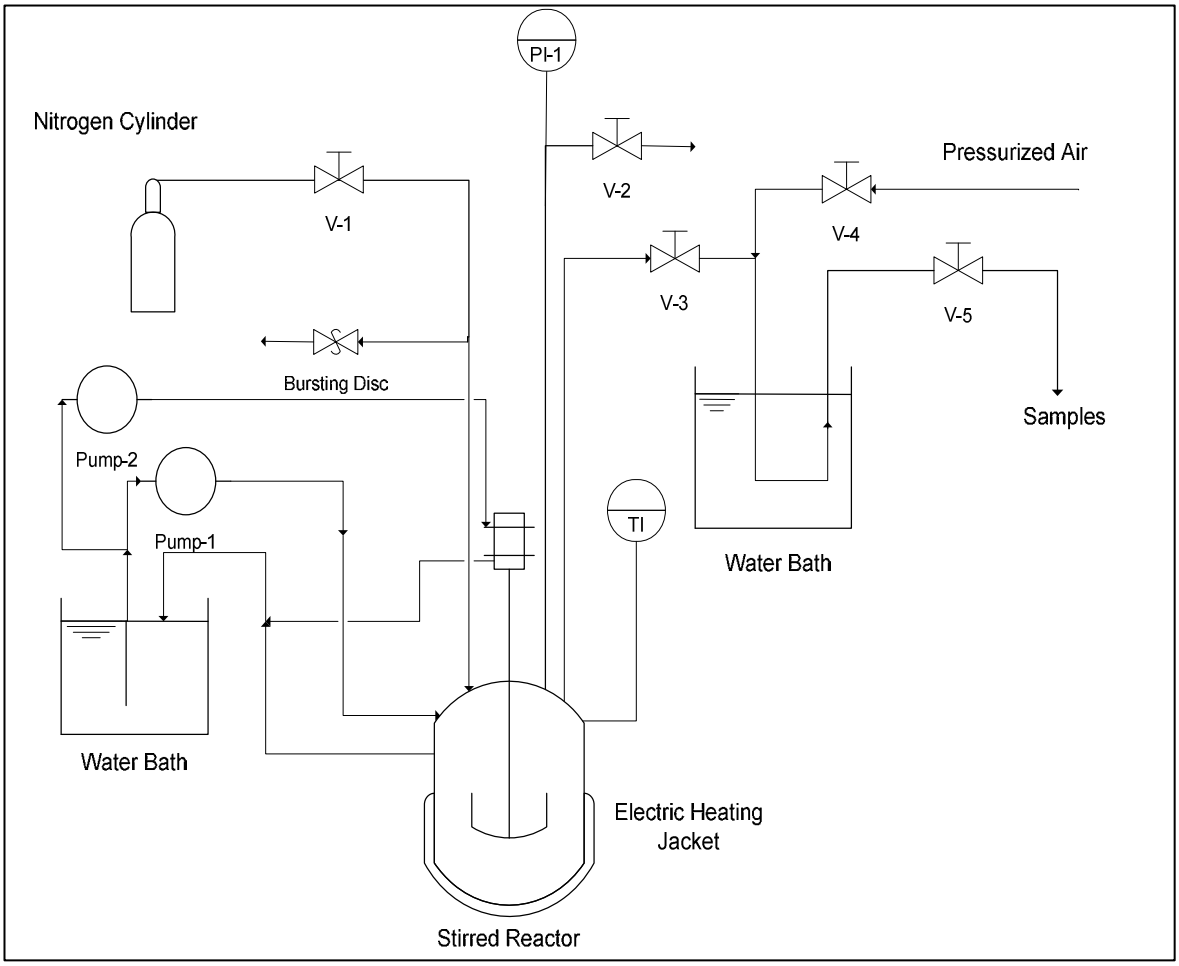


Figure 3. 1. Schematic diagram of the batch hydrolysis experiments

The reactor was heated to the desired temperature (ranging from 100 – 300 °C) by an external electric heating jacket. The temperature was measured by an iron-constantan thermocouple (K-type) which was connected to the heating control unit. The combined heating and stirrer speed controller was calibrated by the manufacturer to assure uniformity throughout the experiments. A 1/4 inch stainless steel inner tube cooling line was used to provide cooling to the reactor. This U-shaped tube was connected to two free ports on the head/lid of the reactor. Cooling was provided by pumping cold water from a cooling bath when required (P-1) (Peristaltic pump, Watson Marlow). The reaction time was measured with a digital stop watch as soon as temperature reached the set value.

Stirring was provided by a magnetic stirrer which was pre-installed on the reactor head by the manufacturer. Stirrer speed was set to 500 rpm for all the experiments. To prevent the magnetic stirrer head from being overheated, cooling water from the water bath was continuously circulated using a separate line connected to another peristaltic pump (P-2) (Peristaltic pump, Watson Marlow).

7 to 10 samples (7 ml each time) were taken from the reactor at regular intervals (varying from every 2 mins to every 5 mins depending on the operating temperature). To achieve this, first valve (V-3) was opened for a short time while valves (V-4 and V-5) were kept close. When the sampling line (volume 7 ml) containing the extracts was cooled down in the ice-cooled water bath, valve (V-5) was gradually opened and the contents were collected in a falcon tube. Samples were stored in freezer -20 °C after each sampling. To clean the line ready for the next sampling, pressurised air (from compressed air line already available in the pilot plant lab which was connected to the department's air compressor, Chemical Engineering Department, University of Birmingham) was introduced to the line by

opening the valve (V-4) in order to purge any remaining sample left in the sampling line through valve (V-5).

At the end of the experimental run after all the samples were taken, heating was stopped and the reactor was allowed to cool down by starting pump (P-1). Reactor was depressurised before opening the reactor head/lid by opening valve (V-2) and the reactor was fully washed with water. The main reactor cylinder which was removable was washed with copious amounts of water and dried with paper tissue after each run and before start the next experiment.

3.2.4 Total Organic Carbon (TOC) Analyser

To measure the total organic carbon dissolved in the liquid phase, samples were first centrifuged at 16000 rcf (rotational centrifuge force) with a bench-top micro-centrifuge (5418, eppendorf) for 10 min and the supernatant was used for TOC analysis. Three replicate samples were diluted 1:100 with distilled water each time prior to measurement by a 5050-A Shimadzu TOC analyser. The TOC was obtained by subtracting the IC (inorganic carbon) from the TC (total carbon). The TC was measured by a catalytic oxidation carried out at 953 K. The reaction takes place in a quartz combustion tube packed with platinum catalyst, which is contained in an oven that is maintained at reaction temperature. The oxidant material for the reaction is a high purity air, which is continuously saturated with water and introduced into the combustion tube. The air is also used as a carrier gas. When the sample is injected into the combustion vessel the carbon in the sample is converted to CO_2. The gas then carries the oxidation products into an IC reactor vessel, after which the products are cooled and dried. The gas sample is sent into a halogen scrubber and then to a cell where the CO_2 is quantified by a non-dispersive infrared (NDIR) gas analyzer based on the area of the signal produced by the sample.

The measurement of IC is performed by introducing the sample into the IC reactor vessel where the carrier gas flows as tiny bubbles through the IC reagent (phosphoric acid solutions at 20% wt). Only IC is decomposed to CO_2 which is then taken to the NDIR detector. The concentration of the IC in the solution is calculated following the same principle as the TC content.

3.2.5 Total Protein Measurement

To measure the concentration of suspended proteins in the solution of hydrolysed yeast samples, Coomassie Plus (Bradford) kit (23238, Pierce, IL) was used which is based on measuring the colour change after the reaction between the reagent and protein molecules (Bradford 1976). When Coomassie dye binds proteins in an acidic medium, an immediate shift in absorption maximum occurs from 465 nm to 595 nm which was measured using UV-Spectrophotometry technique. Bovine serum albumin was used as the standard for preparing calibration curve from a 2 mg/ml standard ampoule. Diluted albumin standard (BSA), which was provided by the protein kit, were prepared in concentration ranging from 2 mg/ml to 25 µg/ml. Samples were first centrifuged (Model 5418, eppendorf) at 10.000 g for 10 min to remove the solid particles and 1 ml of supernatant was collected and used for protein measurement. Supernatants were diluted to 1:50 using distilled water and 25 µl was taken from the diluted samples and loaded in 96-well plate. Three replicates were used at each time. Measurement was carried out using a 600 nm plate reader.

3.2.6 Dry weight measurements

Samples' total dry weight was measured by placing 1 ml of each sample in an aluminium weighing dish in a drying cabinet at 105 °C for a minimum of 24 h (24 h drying time was the minimum sufficient time to guarantee no change in the weight of the sample). Dry weight

was calculated by subtracting the weight of the dried dish from the weight of the non-dried dish. In addition, dry weight of the liquid phase was also measured by first removing the solid particles using a centrifuge (16000 rcf *, 10 min) and drying 500 µl of supernatant using the same technique. Results from this section were used to evaluate the effect of the temperature on solubilising the solid particles and its contribution to the total dry mass of sample.

Crude samples and the extracts were in the form of slurry and dry weight measurement includes both the dry weight of the solid particles and non-volatile compounds. To differentiate these two factors, samples were divided into the solid fraction and liquid fraction by centrifugation. Samples were collected at different times and a known amount was transferred to pre-weighted 1.5 ml eppendorf tubes. After centrifugation at 16000 rcf at 10 min, 500 µl of the supernatant was transferred to another pre-weighted tube.

All the tubes were dried in a drying cabinet at 105 °C for a minimum of 48 h and the dry weight of each fraction was calculated.

3.2.7 UV-spectrophotometry

To evaluate the UV spectrum of the extracts, a CECIL spectrophotometer (Model CE 7200) was used in the wavelength range of 200-600 nm. Samples had to be diluted from 1:10 to 1:100 in order to be in range with the calibrated measurement scale. 1 ml of final sample was used for each cuvette and the absorbance was measured by comparing it to distilled water used as the reference.

* Relative centrifugal force

3.2.8 SEM and TEM

To study the effect of the reaction condition on the surface of the cell walls, Scanning Electron Microscopy was carried out using by a PHILIPS SEM (XL30 ESEM FEG). Transmission Scanning Microscopy (TEM) was also used to study the changes in the intracellular structure of the cells before and after the yeast cells were subjected to different hydrolysis conditions. To prepare the samples for microscopic analysis, samples were added to a 2.5% glutaraldehyde solution and kept in the fridge for primary fixation. The samples were then placed on plates and they were dried with 70, 90 and 100% ethanol for 30 min each time. Plates were immersed in liquid CO_2 for 60 min at 81 bar and 31 °C for completely removing the water. The dried sample were mounted on electron microscope stubs, coated with 15 nm gold and examined under SEM. SEM and TEM measurement s were carried out in metallurgy and material department, University of Birmingham.

3.2.9 Total Phenolic Content (TPC)

Phenolic content of the extracts was measured using Folin-Ciocalteau assay developed by Singleton *et al*. (Singleton & Rossi 1965). This method is based on the Folin-Ciocalteau (FC) Reagent which is a mixture of hexavalent salts of Molybdenum and Tungsten and initially was used to measure the protein concentration due to the fact that the tyrosine which is present in proteins, give positive response to this test (Miniati 2007). Under alkaline conditions, phenols ionise completely and can be oxidised by the FC reagent. This causes a colour change from yellow to blue which can be measured with a spectrophotometer. Today, this reagent is widely used to measure the total polyphenolic content in foods, beverages and biological samples.

The Folin-Ciocalteau micro method was developed by Waterhouse and it was used for the samples as described below (Waterhouse, 2005).

FC reagent was purchased as 2N solution ready to use from Sigma (Sigma-Aldrich, UK). Sodium carbonate solution was prepared by dissolving 20 g of anhydrous sodium carbonate (99.99%, Sigma-Aldrich, UK) in 80 ml of distilled water and heated until boiling. After cooling, few crystals of sodium carbonate were added, stored for 24 h, filtered and water was added to complete 100 ml volume solution in a volumetric flask. Calibration samples were prepared by using solutions of 50, 100, 150, 250 and 500 mg/L of gallic acid (97% Sigma-Aldrich, UK). Standard curve was prepared using these samples and the results were reported in terms of Gallic Acid Equivalent (GAE). Samples were first centrifuged to remove the solid particles in 1.5 ml micro tubes at 16000 rcf for 10 min. In a separate cuvette 20 µl of sample was added to 1.58 ml of water and 100 µl of FC reagent totalling 1700 µl in volume. The mixture remained at room temperature for 5 minutes, and then 300 µl of the sodium carbonate solution was added to the mixture and shaken to mix. Samples were left at room temperature for 2 hr before the absorbance could be measured at 765 nm against the blank solution (20µl of distilled water). Calibration curve was prepared first which was used to calculate the unknown concentration of the samples.

3.2.10 Antioxidant activity

DPPH assay was used to measure the scavenging activity of the samples using the method developed by Lee *et al* (S. K. Lee et al. 1998) for microplate analysis. This assay is based on the reducing power of solution against 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical and measuring the resulting colour change with spectrophotometry. DPPH is purple in colour which is because of an odd electron in its structure and gives a strong absorption at 517 nm. When this electron is paired with hydrogen from antioxidant compounds DPPH is reduced, causing the colour to change from purple to yellow. The resulting change in colour is stoichiometric with respect to number of electrons captured.

3.3 Results and Discussion

The main objective of this section was to investigate the effect of reaction parameters (temperature, time and pressure) on the structure and composition of the cider spent yeast sample. Initial screening tests were carried out in order to verify if the selected reaction parameters already stated above had any affect on the selected properties of the extract such as TOC, TPC, Dry Weight, UV to name a few. Results of all the studied parameters showed that when different pressures of 3, 6 and 10 MPa were studied, they did not have an effect on the selected properties of the extract as opposed to temperature and time of the reaction. Thus, all the results are reported at different temperatures and times whilst the pressure was kept constant at 10 MPa. This is despite the fact that water properties would change at higher temperatures in terms of its polarity, ion product and density. These effects were believed to have no or minor effect contribution to the outcomes of the experiments. Results from each experiment are provided accordingly in the following sections.

3.3.1. General Properties of the crude sample

As discussed earlier, samples were mainly consisted of yeast cells which accounts for the major contribution to dry weight (solid concentration) of the slurries. Total dry weight of the crude samples was measured by drying 10 ml of the crude sample in an aluminium dish in a drying cabinet at 105°C for minimum of 24 h. The total dry weight (which is a sum of liquid known as the supernatant and solid dry weights) measured value was 14.5 ± 1 % (w/v) was slightly different (13-15 % w/v) for different samples which were collected throughout the year, so the same lot was used for all the experiments. This is because of the different blends of apple juice and products that the manufacturer makes throughout the year, which were used to make numerous brands of their cider. Also the dry weight of the supernatants was also measured by first centrifuging the sample for 10 min at 4000 rcf to separate the

yeast cells. 10 ml of the supernatants were dried at the same conditions and the dry weight was 3 ± 0.5 % (w/v). Based on these results, the total dry solids of the slurry were calculated to be 11.5% (w/v). The pH of the samples was 3.6 ± 0.4 and it was measured with a pH meter (Seveneasy®, METTLER TOLEDO). This acidic medium is a result of acidifying agents present in the upstream of the fermentation (mainly lactic acid).

Potato dextrose agar (PDA) cell culture medium was prepared by using PDA culture powder (Sigma Aldrich, UK) and distilled water and used to fill the dishes after sterilisation.

Figure 3. 2 Colonies of the yeast formed after incubation PDA culture plates with crude sample

Prepared agar plates were inoculated with crude samples in order to find out if the yeasts cells were still viable. This medium is optimised for yeast growth and it was used in the form of a premixed powder. Plates were incubated in 37 °C incubator for 48 h after being inoculated. The growth of the yeast colonies were observed in the inoculated plates (Fig. 3.2), which also confirmed under the optical microscope.

The total number of living yeast cells was not measured and the test was carried out as a qualitative analysis in order to determine if there is any living cell available in the samples. This does not necessarily means that the slurry is only comprised of only living yeast cells but it can be concluded that there is a population of yeast cells that may be in a vegetative form which is common after fermentation. This fact was of special interest in our work and has been fully discussed in the next chapter.

3.3.2 General characteristics of the extracts

The high pressure conditions in the rector prevented the formation of any vapour and thus the volume of the reactant was remained almost the same. Despite this, gaseous product formed during the reaction was due to the decomposition of the organic compounds which occurred at high temperature. The presence of the smoke was more considerable at higher temperatures (>175 °C) while the odour was also stronger. No gas phase analysis were carried out in this work but other studies suggested that major gases were produced from subcritical water treated biomass at similar process conditions were CO_2, CH_4, H_2 and CO (Feng et al. 2004) .

Colour and odour of the samples did not change much when treated to temperatures up to 125 °C. At higher temperatures the colour in the liquid fraction or supernatant started to

change slightly from light-brown as in the original crude sample to brown and the extracts started to lose the characteristic cider odour (Fig 3.3).

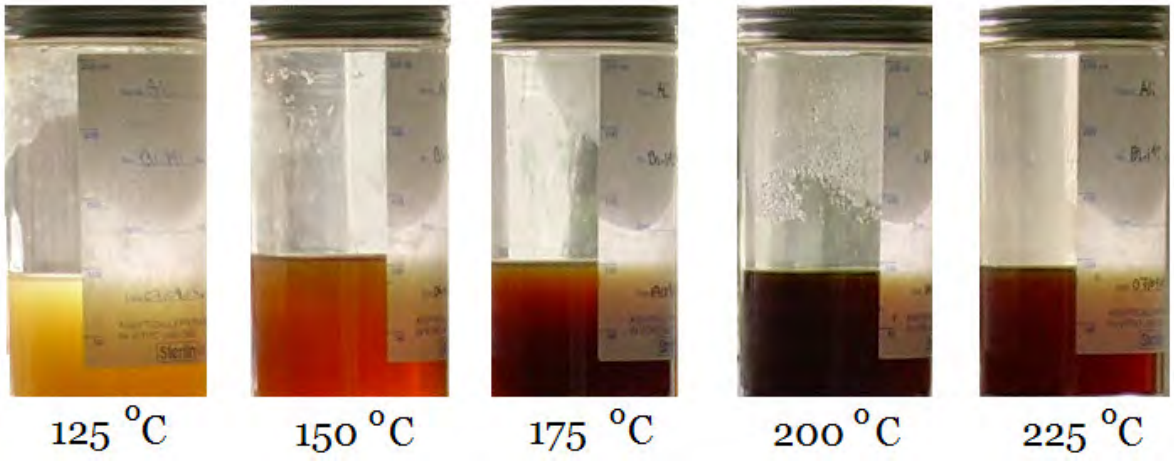


Figure 3. 3. Colour change in the supernatant of the samples at different temperatures. Reaction time was 30 minutes and the pressure was 60 bar for all the samples

This colour change was observed in both the solid residue and liquid fractions after these two were separated by centrifugation. Samples tend to became more viscous and sticky between 150 and 200°C but this changed back to a less viscous sample at higher temperatures (> 225 °C). At this stage, it is believed that the caramelisation of sugars (available and/or released fraction) occurred which was indicated by the distinctive odour of browning reaction in the extracts. When the samples were treated at 225 °C and higher temperatures, solid residues start to turn black and burning occurred. At these conditions, the supernatant also became dark red which is believed to be due to the considerable levels of hydrolysis and solubilisation of the organic compounds.

The pH of the samples was also measured after reaction at different temperatures and a slight change was observed. The pH of the crude sample was originally 3.7 and it changed to 4.2 at 250 °C after 30 mins. Although it is expected to see lower pH due to formation organic

acids in these conditions, it is believed that the degradation power of subcritical water had the major effect on pH by removing the existing organic acids and/or the ones that may had been generated.

Reaction temperature, however, had a remarkable effect on the dry weight and the solid concentration of the extracts which is discussed in the next section.

3.3.3 Solid concentration and sample dry weight

Yeast solids have been previously shown to hydrolyse at the same conditions used above due to the upgraded hydrolytic power of water in subcritical conditions (Lamoolphak et al. 2007). The main compounds structures which are subject to the hydrolysis are the carbohydrate backbone of the cell wall, highly polymerised proteins and other combinatorial structures. The main outcome of this reaction is solubilisation of solids in the liquid phase, formation of monomers (such as monosaccharides and amino acids) and also the formation

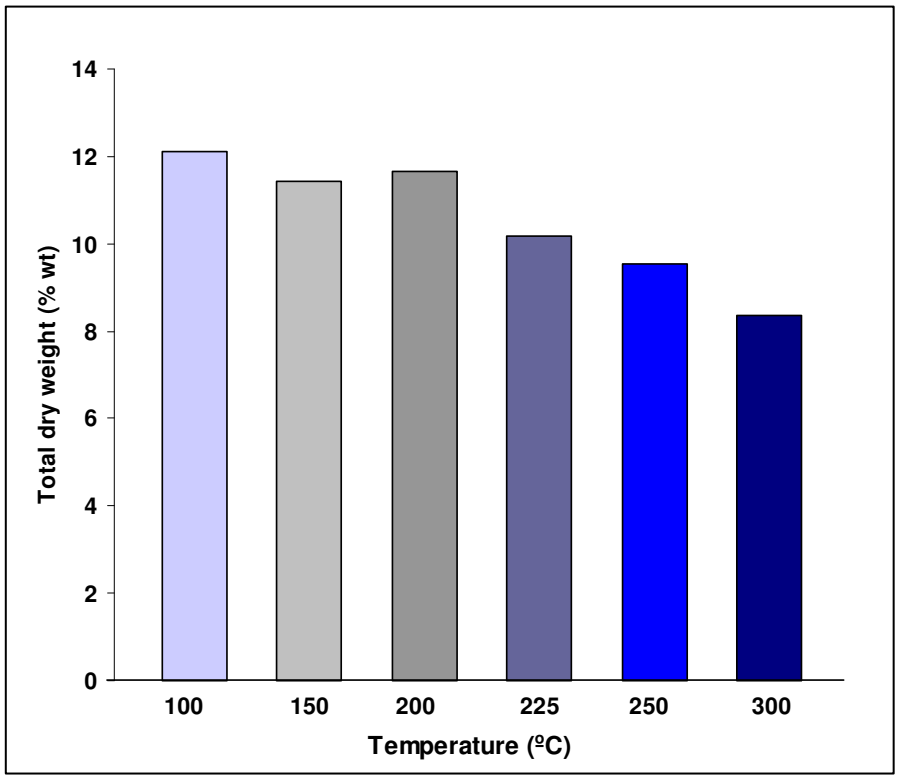


Figure 3. 4 Total dry weight of the extracts (solid and liquid) at diffferent temperatures after 30 mins at 100 bar

of increased levels of gaseous products at higher temperatures (> 250 °C). The latter would normally result in the drop of the total dry mass of the extracts at these conditions. This weight loss was also observed in our experiments as the total dry weight start to decrease at 225 °C and higher (Fig 3.4).

At higher end of the temperature range, up to 30% (at 300°C) of the sample was turned to gaseous products and left the reaction medium. Yeast cell solubilisation was further investigated by separating the solid and liquid/ supernatant fractions of the extracts and measuring the dry weight of each fraction. This indicated the degree of solid solubilisation and also how much of the solubilised compounds may have left the liquid fraction or phase, either due to the gas formation or dropping out of the liquid phase by centrifugation force after forming larger/heavier structures or aggregates at these conditions.

Solids and liquids/supernatants fractions were separated and dried following the method described in section 3.2.5 and the results are summarised in Fig 3.5. Increasing temperature had a significant effect on the solubilisation of the solids in the medium. This however was not observed at 100 °C and the solid and liquid fraction did not change significantly even after 30 min. At 150 °C, solids start to solubilise at higher rates especially at longer reaction times. Solid concentration decreased by more than 50% when the temperature increased to 200 °C. However, the same trend was not observed when the reaction time increased. At the start of the reaction (t=0, when the temperature reached the set temperature in the batch reactor), solid concentration was 40 mg/ml and increased gradually to 50 mg/ml. In addition, when the temperature increased to 225°C, solid weight was 60 mg/ml at t=0 and did not change significantly up to 30 mins. This is in contrast to the constant drop in total dry weight by increasing temperature which was more pronounced at temperatures higher than 200 °C. Some weight loss initially occurred during the heating-up

stage before time measurement starts, which are represented at t=0, the initial value of dry weight for substrate (120 mg/ml) can be used for comparison. In addition, Figure 3.6 also shows the normalized charts representing the values of solid/liquid ratio normalized in terms of the values at t=0 of each reaction set.

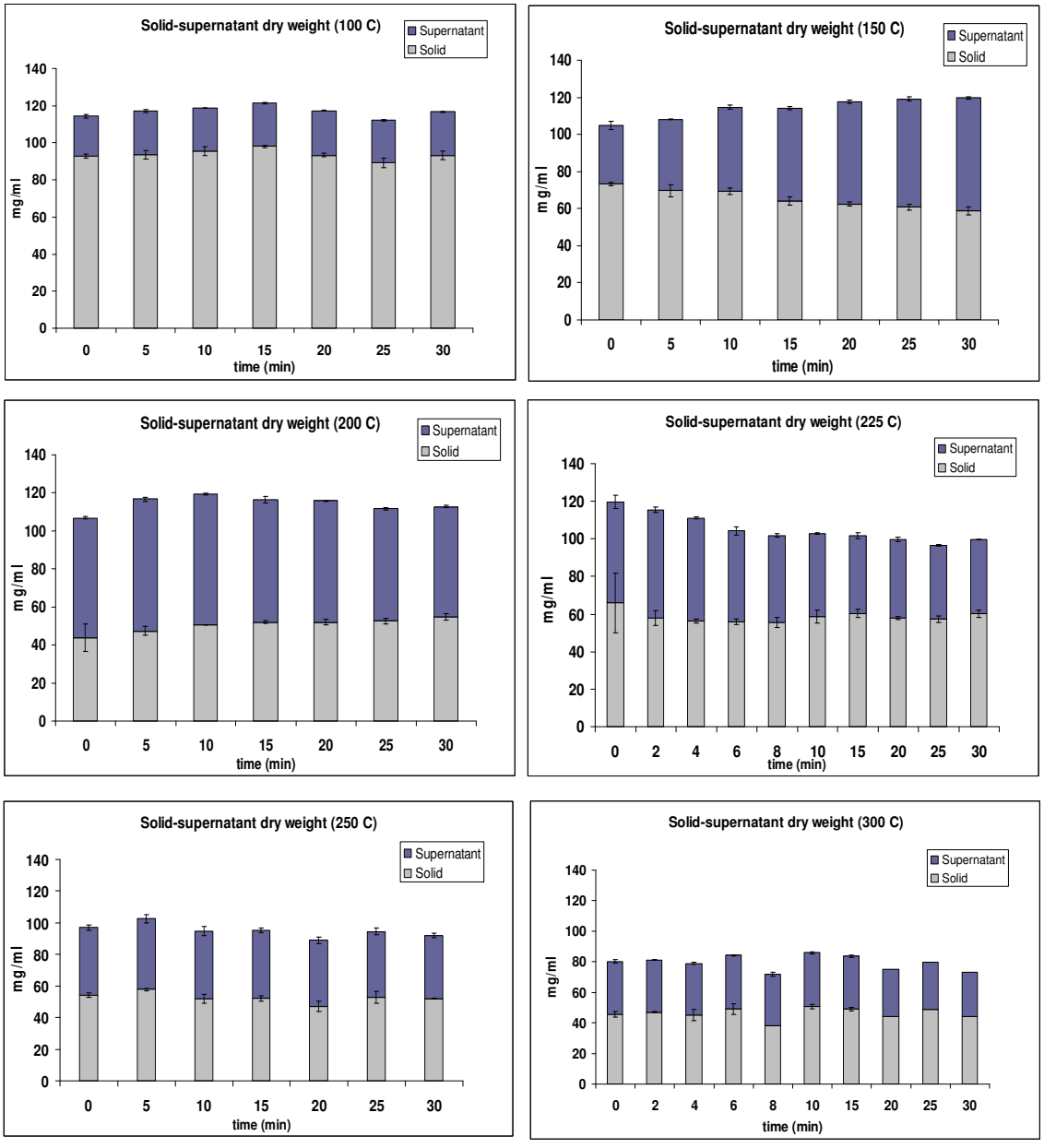


Figure 3. 5 Solid and liquid/supernatant dry weight after subcritical water hydrolysis at temperatures of 100-300 °C. Error bars represent standard error (n=3).

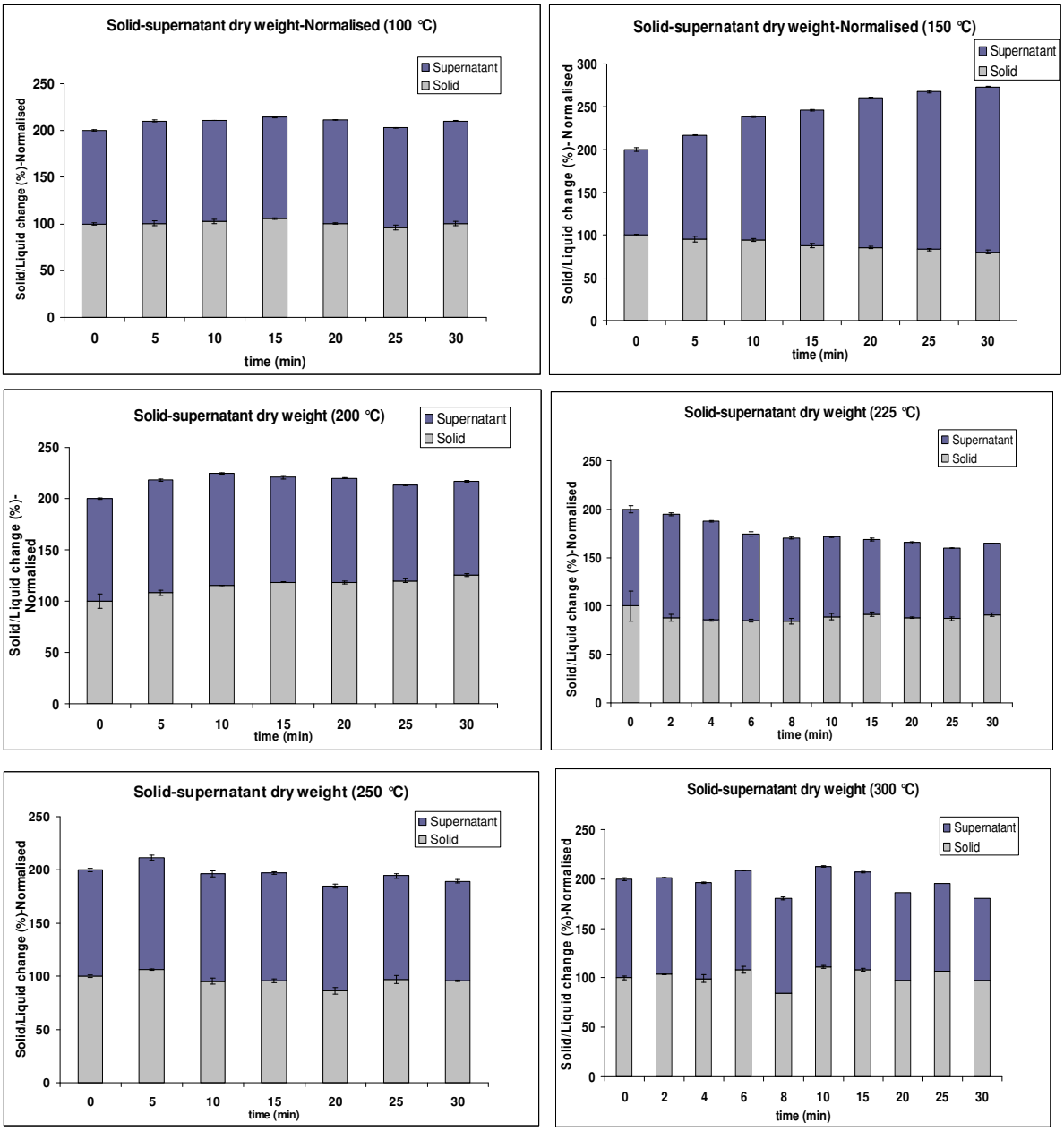


Figure 3. 6. Solid and liquid/supernatant dry weight after subcritical water hydrolysis at temperatures of 100-300 °C. Normalized to t=0 at each reaction temperature Error bars represent standard error (n=3).

While the decrease in total dry weight can be described by the gas formation, interesting results were observed when the dry weight of liquid phase and solid phase was compared. The dry weight of solid phase started to increase when the temperature increased from 200 to 225 °C. This slight increase in dry weight remained almost unchanged at 250 °C and decreased again at 300 °C. This increase in dry weight can be attributed to the formation of larger molecules in those conditions (225 and 250 °C) that had been formed and dropped out of the sample by centrifugation force. In this case, these compounds can contribute to higher levels of dry weight in these residues. A possible explanation can be the conjugation or polymerisation reaction that occurred between various components such as carbohydrates (sugars), proteins, and phenolics released from the cell or pre-existing in the medium. Formation of melanoidins, which are the aggregates of sugars and amino acids, can be attributed to this effect as described earlier in Chapter 2 and the introduction to this chapter. While the Maillard reaction is mostly studied at conditions of interest to food processing (-20 °C to 150 °C), it can also occur at higher reaction rates at temperatures as high as 250 °C (Peterson 2009). The darker brown colour of the extracts at higher temperatures (250 °C) can also be an indication of the occurrence of this reaction (Fig 3.3). The presence of phenolic compounds in the medium, and the similarity of their structure to the proteins, can influence the reaction resulting in the formation of further complex compounds which can be a subject for further investigation beyond this work. The dry weight of the liquid fraction/phase decreased at temperatures above 225 °C which is manly due to the gas formation or the already discussed polymerisation reactions.

3.3.4 SEM and TEM

Scanning Electron Microscopy (SEM) provided a better picture of the overall change on the cells' surface when they undergo the subcritical water treatment process. The size of

the cider yeast cells (*Saccharomyces bayanus*) in the crude sample was approximately 3-5 µm compared to the average size of the brewer yeast cells (*Saccharomyces cerevisae*) which is reported to be 10-15 µm. Fig 3.7 shows clusters of the untreated yeast cells with a spheroid structures and a smooth outer wall surface. Scar buds which are common in yeast cells can be seen in few of them.

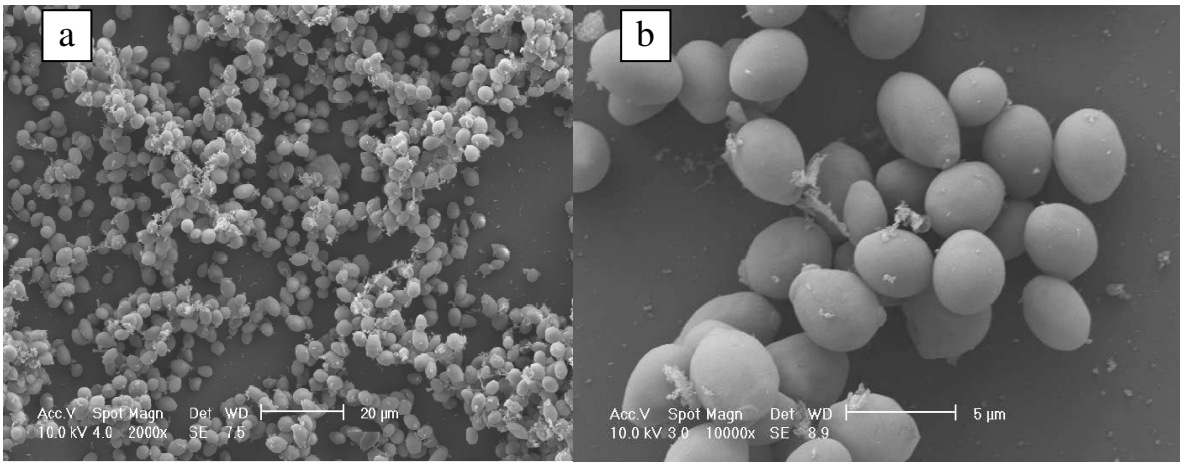


Figure 3. 7 Yeast cells (S. Bayanous) in the substrate samples at two different magnifications a: 2000X and b: 10,000 X

When the crude sample was subjected to the reaction, the outer surface of the cells experienced change and disfiguration. A selection of the SEM micrographs is shown in Fig 3.8 compares the treated cells at different temperatures. At lower temperatures (<140 °C), bubbles were formed on the cell wall which is possibly are the result of cell wall polymers release at these conditions. Also lipid content of the cell wall may have started to be release from the cell resulting in the bubble formations. At higher temperatures (140 - 160 °C), a fibrilliar structure emerged which lead to their agglomeration. This web-like structure could be either the mannoproteins or other polymers in the cell wall (i.e. β-glucan). At temperatures above 160 °C, more cell fragments can be observed and the cells seem to

decrease slightly in size. The integrity of the cells remained intact up to 200 °C and above this temperature the cells notably were destroyed and converted to irregularly shaped particles. Fig 3.9 shows the Transmission Electron Microscopy (TEM) micrographs of the yeast cell before and after treatment. The treated samples subject to temperatures of 160 °C or above shows the organelles and internal content of the cells being affected. While no visible effect was observed at temperatures up to 140 °C, it seems that the cells started to loose their intracellular contents at 160 °C which resulted in their size reduction. A change in the cell wall can be observed at temperature as low as 140 °C, where the cell wall line becomes less distinct. A web-like structure was observed with SEM at this same temperature. The cell wall started to completely disappear from the cell's main body, either solubilised or form individual aggregates with or without contribution of other components such as proteins and phenolics. This can explain the origin of the smaller particles that start to form at 180-200 °C adjacent to the cells (Figs 3.8 and 3.9).

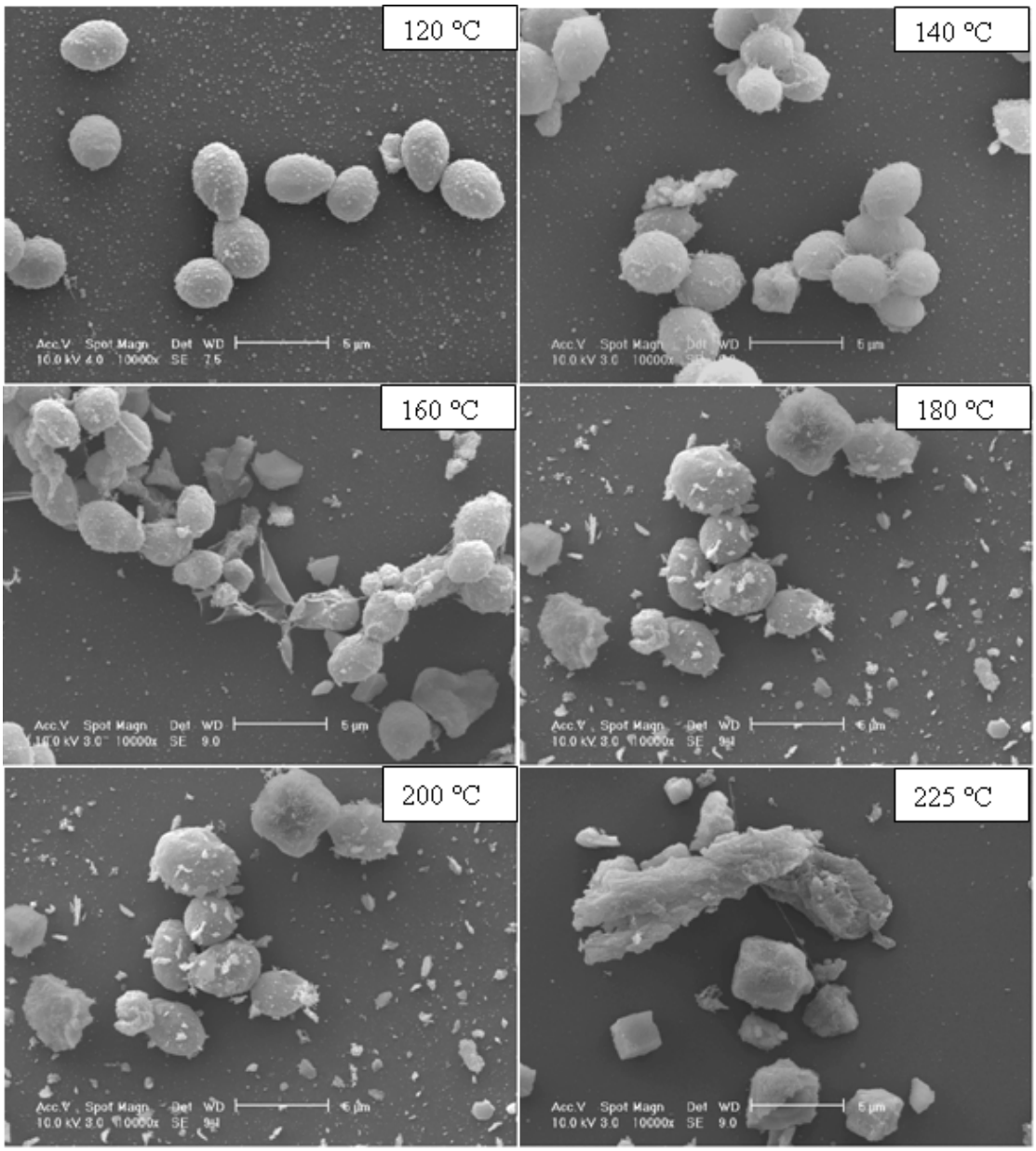


Figure 3. 8 TEM images of the yeast cells after being treated at different temperatures (X=5,000 for all the images except the crude sample image which is at X=20,000)

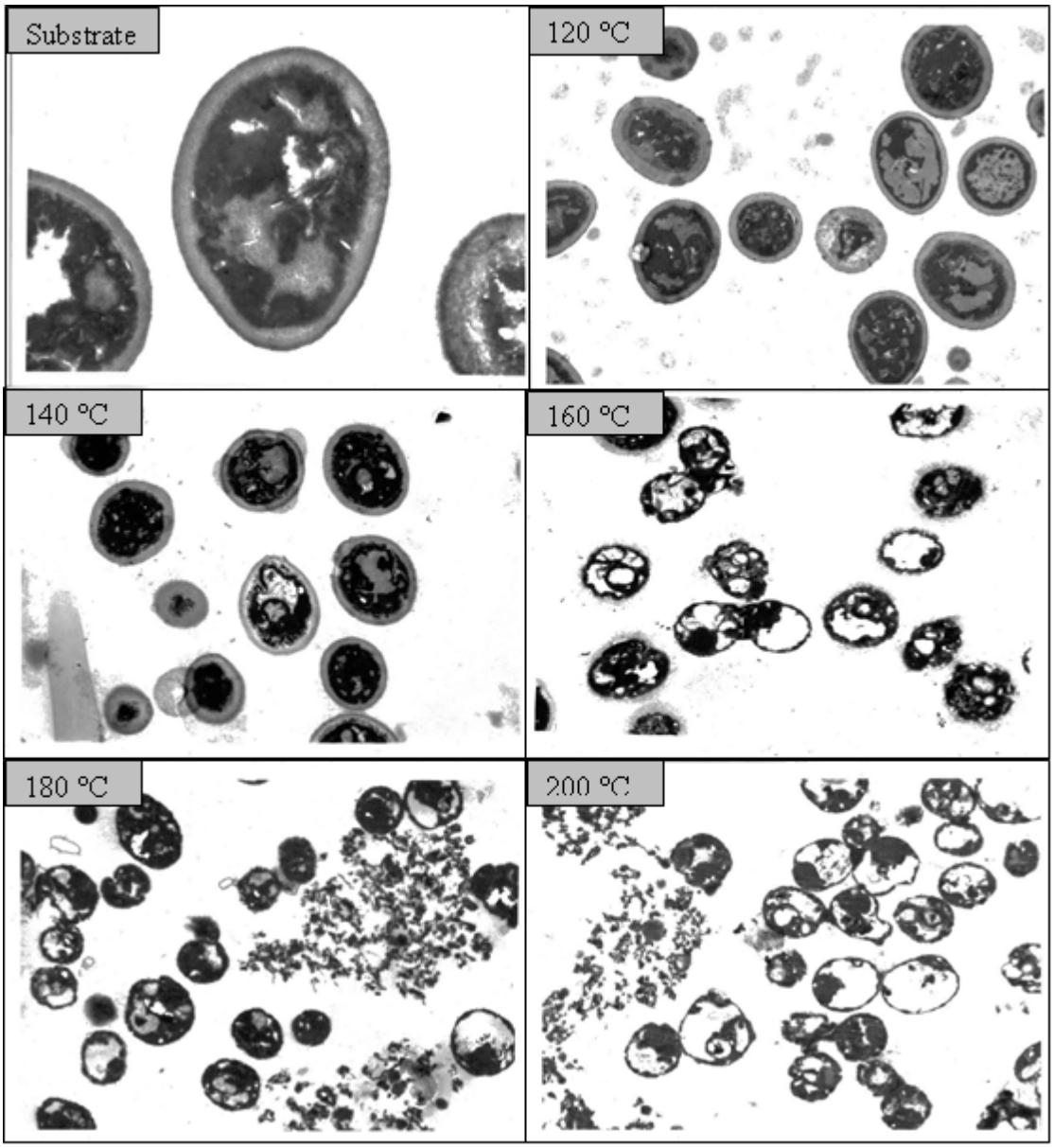


Figure 3. 9 SEM images of the yeast cells after being treated at different temperatures (X=10,000 for all the images)

3-3-5 UV-absorbance profile

Studying the UV profile of the sample and extract provided further information to characterise the structure and composition of the extracts. Figure 3.10 shows the absorbance of the extracts which were measured in the UV-spectrophotometry at the indicated wavelength range (220-500 nm). Samples were first centrifuged at 16000 rcf for 10 min prior to analysis and diluted in order to narrow the spectrum in the detection range of the spectrophotometer.

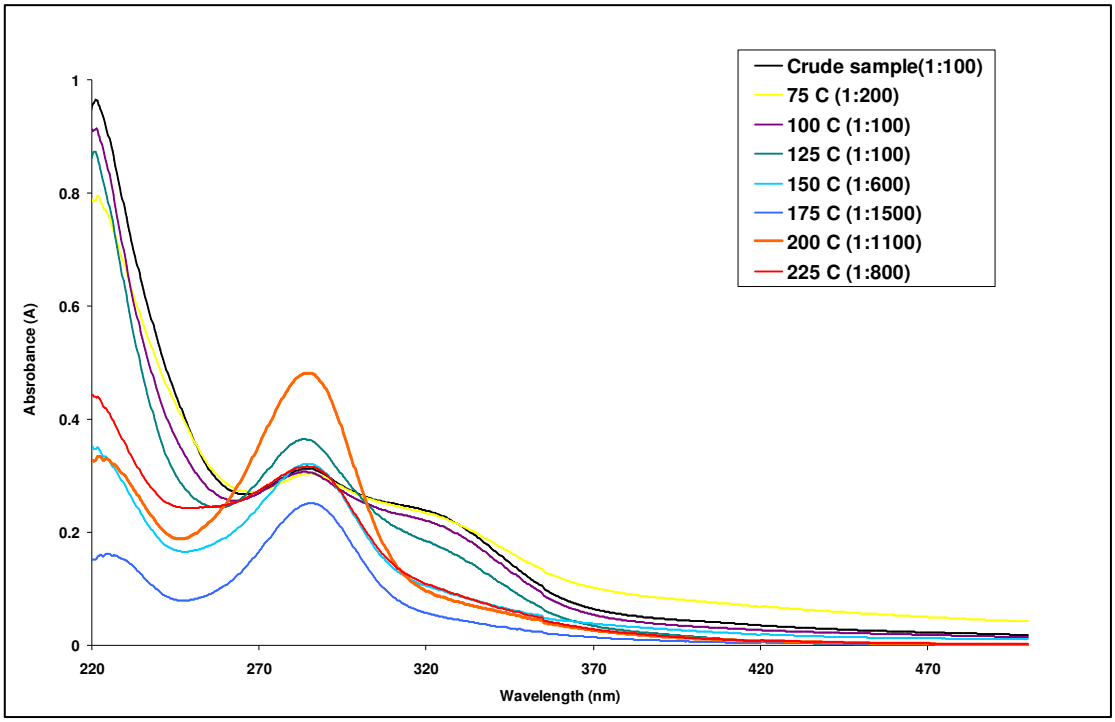


Figure 3. 10 UV-spectrum of the crude sample and the extracts at different temperatures (all at 10 min reaction time). The ratio for each entry is the dilution factor prior to analysis

The peaks shown at the 280 nm band range are representative of mainly amino acids, proteins and vitamin B3 in solution which are enhanced significantly in the samples at higher temperature. This is due to the release of yeast intracellular contents after to the cell breakage at higher temperatures freeing the protein and amino acids to the medium (further discussion

in protein measurement section). The concentration of the protein dropped at higher temperatures which are due to their decomposition at these conditions (> 200 °C). The peaks shown at the second band at higher wavelengths of 330 nm is believed to be represent the phenolic fraction in the extracts (such as chlorogenic acid and other hydroxycinnamic acid derivatives) originated from the apple. These compounds are the residues of the apple phenolics which had been introduced in the upstream of the cider process and have been carried out through fermentation by-product. The concentration of these compounds also increased at higher temperatures which is depicted in the same figure and are also discussed in detail in chapter 6. Fig 3.11 shows the comparison between the absorbance ratio of different samples at different temperatures and wavelengths after standardising dilution factors.

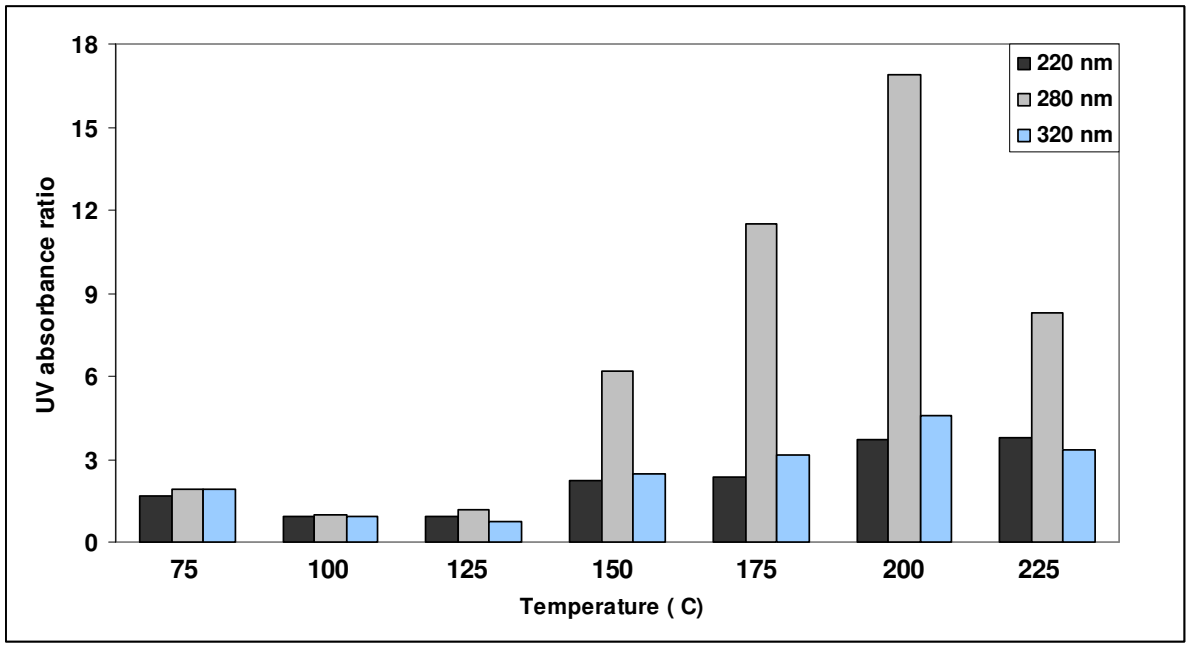


Figure 3. 11 UV absorbance changes in the hydrolysed samples relative to the crude sample at 3 wavelengths

3.3.6 Protein Concentration

Due to stronger hydrolytic conditions, yeast cells undergo the lysis and degrade as a result of the membrane and cell wall disruption. Cell wall hydrolysis will be further discussed in Chapter 5 where a description of cell wall structure, kinetics of its hydrolysis at the studied conditions and the intermediates produced will be discussed. Moreover, the destruction of the cell wall will further release the cellular contents to the medium. This will form a partly soluble hydrophilic fraction in the aqueous medium plus a small fraction of non-soluble lipids and hydrophobic compounds. The main fraction of the hydrophilic compounds is solubilised proteins and amino acids which normally represent the degree of cell disruption in biological application.

During autolysis or induced lysis processes, yeast cells are left at temperatures between 60-80 °C when cell lysis occurs which can take several days (Saksinchai et al. 2001). Fig 3.12 illustrates the protein concentration in the crude sample and in the extracts at different temperatures and reaction times. When hydrolysis experiments were carried at 100 °C lower concentration of proteins was observed in shorter reaction times (<20 mins) when compared to the crude sample.

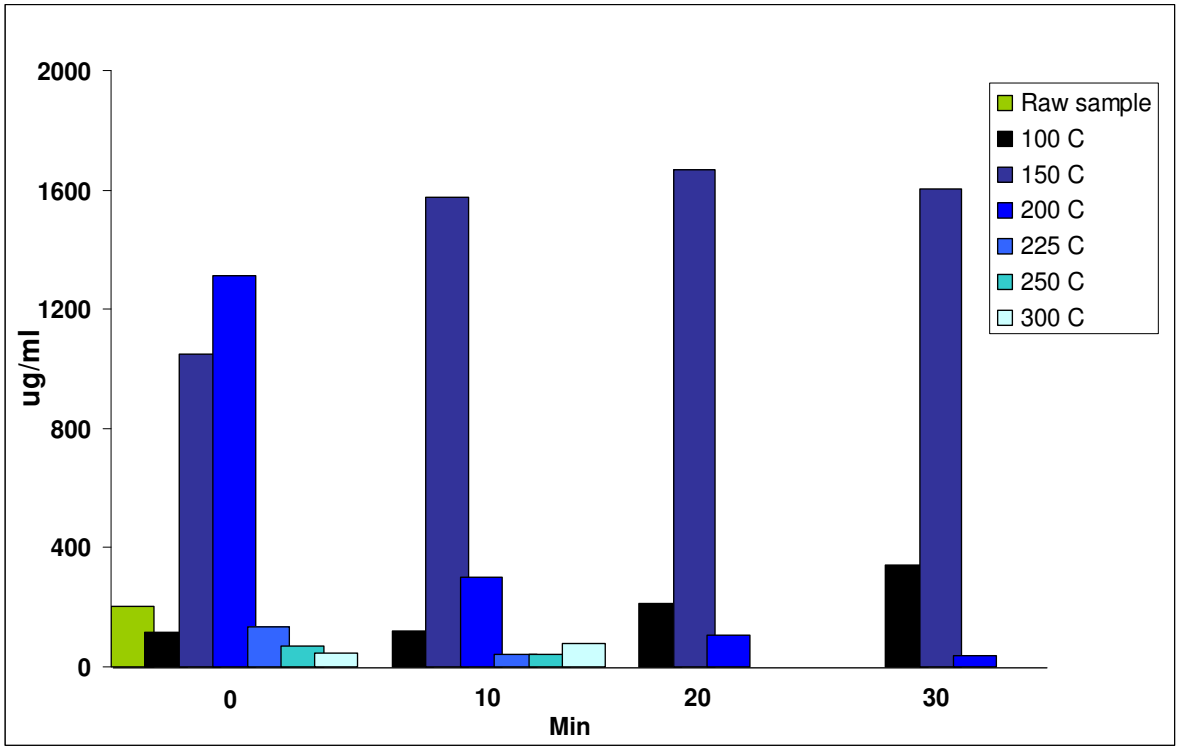


Figure 3. 12 Protein concentration in the crude sample and in the extracts at different temperatures and reaction times

This can be attributed to unknown reactions at these conditions which may result in formation of larger protein structure due to polymerisation or aggregation of protein-carbohydrate structures and subsequent precipitation (drop-out) of proteins in the centrifugation step prior to protein assay. When hydrolysis experiments were carried at 100 °C and longer reaction times, an increase of protein concentration was observed which is believed to be due to higher degrees of cell lysis and protein release. Increasing the reaction temperature to 150 °C resulted in a dramatic increase in the concentration of proteins which can be explained by the higher degree of cell disruption. This is in agreement with structural change of yeast cells (high cell disruption) at this range of temperatures as illustrated in Fig 3.8. The concentration of protein reached its maximum of 1.6 mg/ml at 150 °C and 20 mins reaction time. Also, the same trend was observed on the UV-absorbance of the extracts at

280 nm (Section 3.3.5), which represents the increasing concentration of protein molecules in the extract. Protein concentration dropped at higher temperatures (≥200 °C), which could be due to the high temperature condition and the resulting decomposition to smaller peptides with sizes below the detection limit of the assay. The detection level in Bradford assay was quoted by the manufacturer to be for proteins with MW > 3000 Da (Bradford Protein assay product description leaflet, Pierce ,UK)

3.3.7. Total Organic Carbon (TOC)

As temperature increases, more solid residues solubilises in liquid phase due to an increase hydrolytic power of subcritical water. This liquefaction mainly occurs in the yeast cell wall and in other solid residues (plant residues) originated from apple juice or other plant sources. This degree of liquefaction was measured and reported using TOC value of the samples and correlated with different reaction conditions as shown in Fig 3.13. As the final volume measurement of the samples after the reaction was not possible due to the limitation of the batch reaction and volume loss during depressurisation of the reactor, the values are reported as concentration rather than total value of TOC.

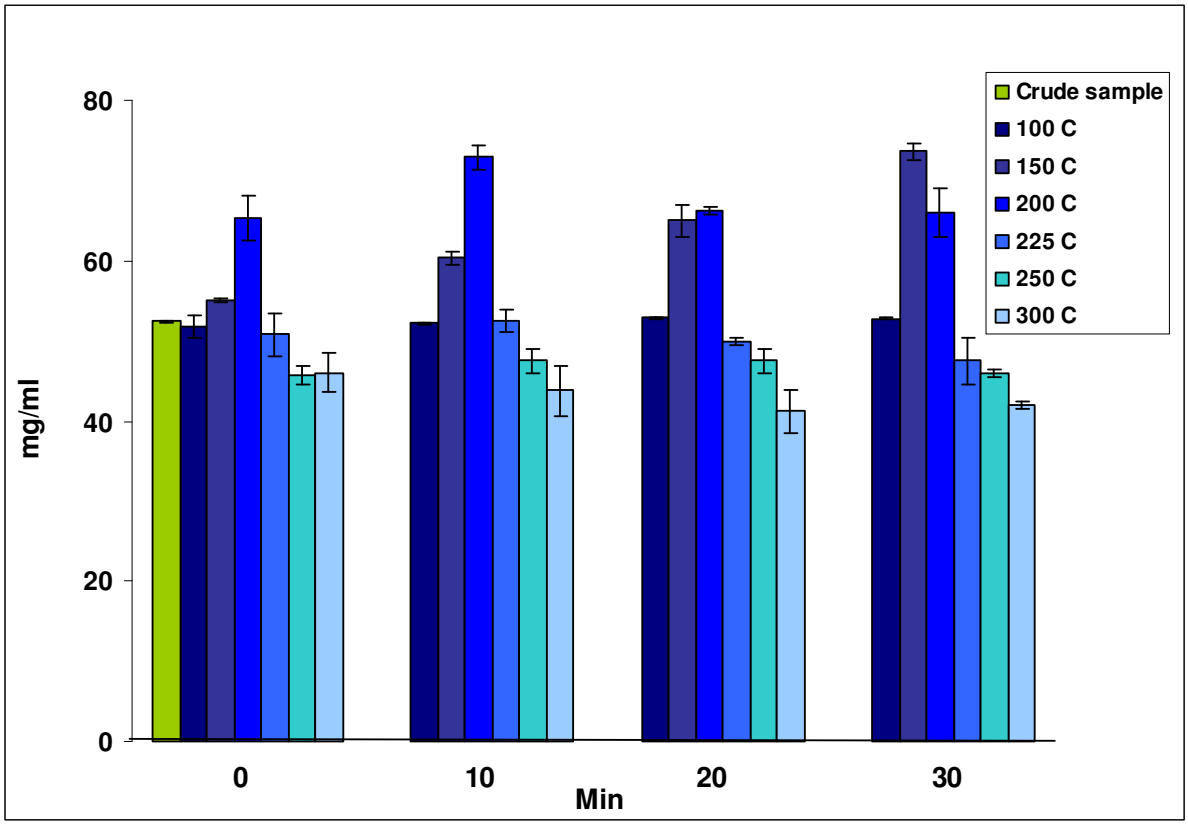


Figure 3. 13 Total Organic Carbon (TOC) of the liquid extracts at different temperatures and reaction time. Error bars represent standard error (n=3)

As the graph shows, the highest concentration of solubilised organic compounds was observed at 150 °C at 30 mins and not at the higher temperatures when a high degree of solubilisation is expected. This could be explained by the following:

1) At higher temperatures, solids solubilise faster but at the same time solubilised compounds start to form gaseous products which leave the liquid phase. This was observed at these conditions when the reactor depressurising valve was opened and a smoke was noticeable.

2) At higher temperature, a parallel polymerisation reaction occurs which is responsible for the formation of significantly larger molecules and their precipitation during

the centrifugation step. This was earlier discussed in section 3.3.3 in which the dry weight of solid and liquid fractions/phase were compared concluding that the dry weight of the solid phase (solid precipitate after centrifugation) increased at higher temperatures

3.3.8. Phenolic compounds

The presence of the significant band at the 330nm UV wavelengths corresponds to phenolic compounds therefore further work was carried out to investigate these findings. The Folin-Ciocalteau assay was able to identify and quantify the phenolic compounds in the extracts and the results are shown in Figure 3.14. Compared with the crude sample (Total Phenolic Content(TPC)=680-730 mg/ml), treated extracts showed much higher concentration of phenolic compounds. This value increased gradually from 100 °C (TPC = 1400 mg/ml) and reached its maximum at 200 °C (TPC = 3441 mg/ml) at 10 min reaction time. At the highest temperature of 250 °C the concentration decreased to as low as TPC = 2600 mg/ml at 30 mins reaction time but it was still significantly higher in than the crude sample.

While a significant fraction of phenolic compound was expected to be present in the crude extract, the increase in its concentration during hydrolysis at high temperature has not been reported so far. Therefore, this observation was further investigated and it was concluded that this increase was due to the desorption of phenolic compounds which were adsorbed on the yeast cell wall during the fermentation step. This observation was further assessed in a separate chapter (chapter 6) which supports the suggested hypothesis.

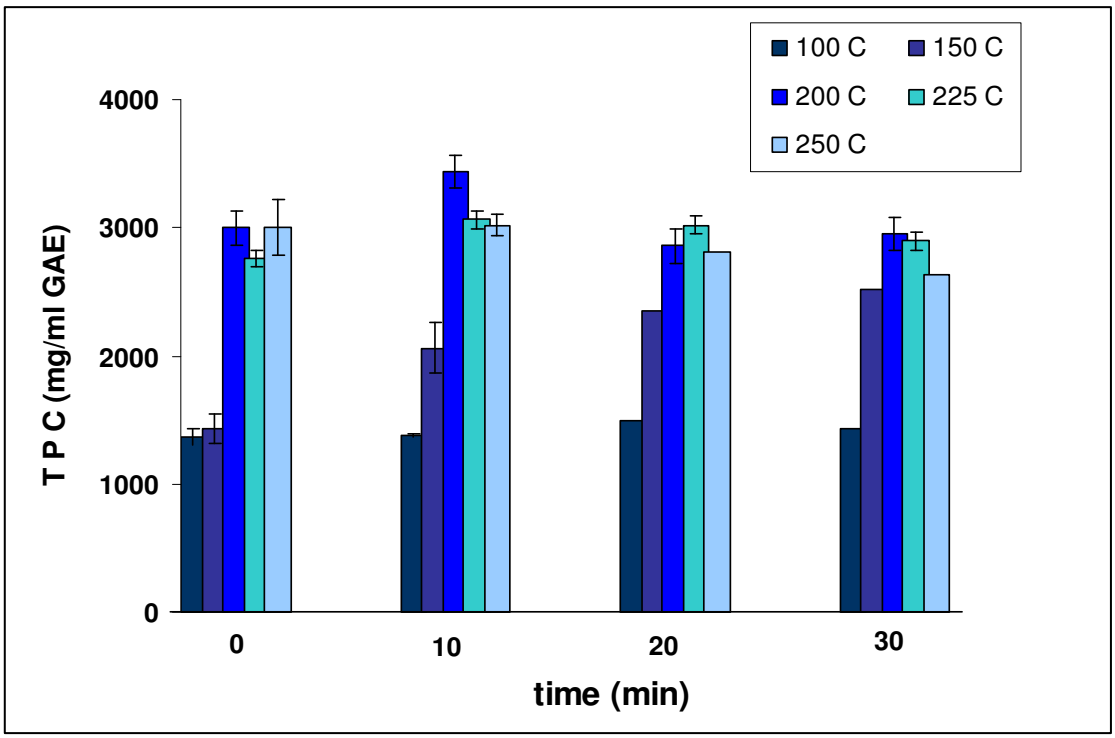


Figure 3. 14 Total phenolic content in the extracts at different temperatures and reaction times. Values are reported as Gallic Acid Equivalent (GAE). Error bars represent standard error (n=3)

3.3.9. Antioxidant properties of the extracts

Phenolic compounds which are available in the extracts are believed to influence the radical scavenging and antioxidant activity of the extracts which are directly associated with their presence (K. W. Lee et al. 2003). Figure 3.15 shows the results from DPPH assay which confirms that antioxidant activity was increased with reaction temperature. The results are reported as the equivalent antioxidant strength of ascorbic acid (AAE) in 100 ml and vary between 16 mg of AAE in the crude sample to 80 mg of AAE in the 225 °C samples.

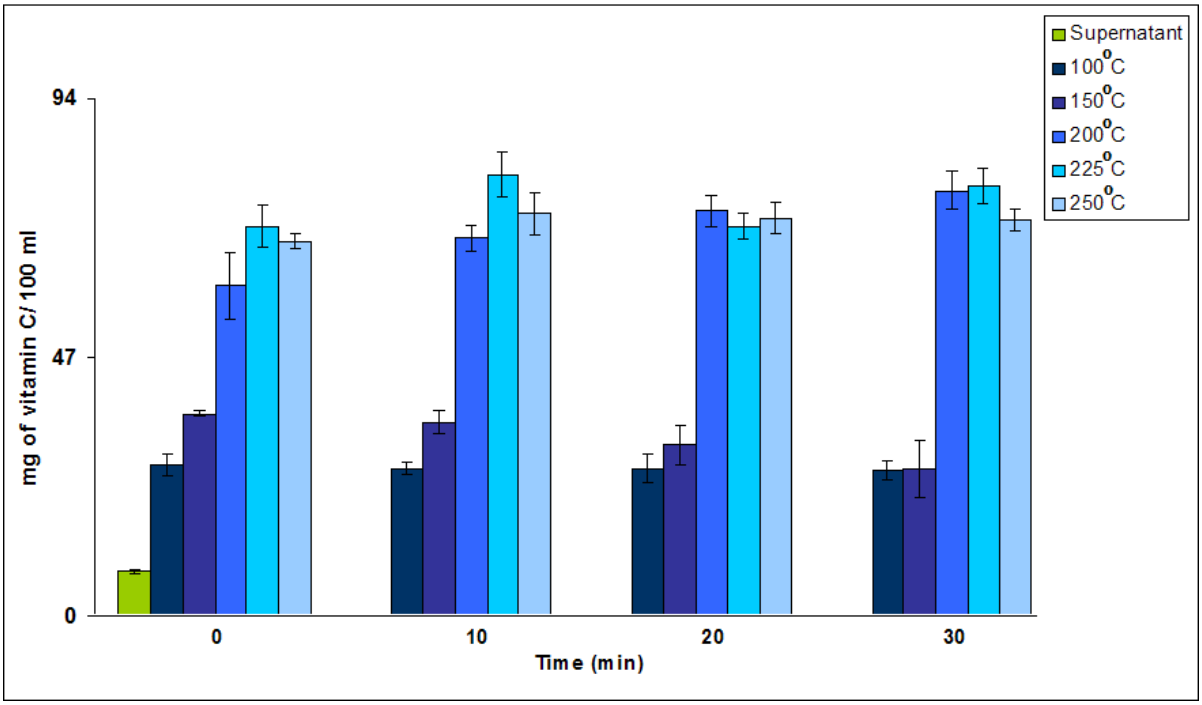


Figure 3. 15 Antioxidant power of the extracts at different temperatures and reaction times. Antioxidant values are presented as the equivalent of the vitamin C in 100 ml. Error bars represent standard error (n=3)

The significant increase occurred when the temperature changed from 150 °C to 200 °C resulting in AAE values of 75 mg after 30 min reaction time. At higher temperature, AAE remained almost constant and slightly decreased at 250 °C after more than 10 mins.

Comparing the antioxidant activity of the extracts with the phenolics concentration shows that both increase at higher reaction temperature. In particular, the extracts at 225 °C and 10 min reaction and the extracts at 200 °C and 10 min reaction time show the highest antioxidant activity and phenolic concentration respectively with both values decreasing as temperature increases.

3.4. Conclusion

The main objective of this chapter was to provide fundamental insights about the main substrate and how it was affected by being subjected to the subcritical water mediated hydrolysis in a stirred batch system. Significant colour, odour and structural change were observed in the sample after the reactions which correspond to the different chemical and physical change occurring under these conditions. When cells undergo such conditions, they start to lyse and gradually release their intracellular contents, such as proteins. This was followed by the change in their overall structure as it was shown by the SEM and TEM micrographs. At milder conditions, cells maintained their rigid structure while the cell wall started being fractionated and separated from the cell structure. At such conditions, intracellular contents of the cell were believed to remain encapsulated while modifications to the outer cell medium were expected. Consecutively, this resulted in higher degrees of cell solubilisation which was measured using dry weight and TOC analysis.

Further analysis of the heat treated extracts showed that proteins are released in the medium. By measuring protein concentration in different extracts showed that higher

concentration of protein had been released at 150 °C which corresponds to the higher degree of cell solubilisation. At higher temperatures and longer reaction times, protein molecules started to decompose which leads to the drop in protein concentration in the extracts. At the same time, phenolic content in the medium were enhanced which is expected to directly affect the antioxidant strength of the extracts. The idea of phenolics and where they originate were further developed and investigated in chapter 6 which discusses that these compounds originate from the yeast cell wall and they are release from the cell wall during hydrolytic conditions.

Using TOC analysis of the extracts and comparing its values with the solid and liquid dry weight of each extract lead to more support evidence that polymerisation reactions may occur at higher temperatures, which resulted in lower TOC values and higher solid weight at higher temperature samples.

Considering the overall properties of the extracts at different temperatures, samples tend to change dramatically into a mixture of proteins and phenolic compounds at temperatures below 200 °C. This rich slurry can present a potential product for different applications such as highly nutrient culture mediums or in products where specific activity of phenolic compounds is required such as their unique UV-absorbance or antioxidant activity.

Chapter 4

Evaluation of subcritical water mediated hydrolysis for extraction of Vitamin B3 from spent cider lees

4.1. Introduction

This chapter details the results from the experiments which were carried out to determine vitamin B3 in subcritical water treated extracts and the ones generated after the crude yeast cells were being subjected to autolysis reactions. Yeast is known as a source of vitamin B group in different concentrations. Hence, vitamin B3 was considered as a candidate chemical to investigate the efficacy of subcritical water extraction from yeast-based biomass. Thin layer chromatography (TLC) and HPLC analysis were used in combination for vitamin B3 determination in the samples.

4.2. Materials and Method

Silicagel glass plates where purchased from Whatman, UK (Partisil®), and HPLC grade water was from Sigma-Aldrich, UK. The vitamin standards (Thiamin-HCl (≥99%, TLC), Riboflavin (≥98%), Nicotinic acid (99.5%≥, HPLC), Nicotinamide (99.5%, HPLC), D-Pantothenic acid hemicalcium salt (98%≥, TLC), Pyridoxine (98%≥, HPLC), Folic acid (97%≥)) were also purchased from Sigma (Sigma-Aldrich, UK). Standard samples were prepared by dissolving 100 mg of each in 100 ml of Water:Methanol (1:1) in a volumetric

flask (100 ml). 1 ml of each standard was used for HPLC and 10 µl was used TLC analysis to determine the retention time.

4.2.1 Development of HPLC method for the analysis of Vitamin B3

Initially, HPLC analysis of nicotinic acid and nicotinamide was performed using a typical C-18 column (Luna C18, 250 × 4.6 mm, 5 µ, Phenomenex,USA) as recommended by the HPLC column manufacturer (Phenomenex Product Catalogue, 2007, UK). However, it did not show the required consistency and efficiency for the separation of polar compounds (nicotinic acid and all the compounds eluting early from the column), resulting in overlapping of peaks. This was mainly due to the nature of solubilised vitamin B3, especially nicotinic acid, which is a highly polar compound and were not retained in the column and elutes with other impurities and unretained compounds. The retention of highly polar compounds solubilised in an aqueous matrix with a reversed phase system, wherein a highly polar mobile phase is in contact with nonpolar stationary phase, can be challenging with normal reversed phased HPLC column. Vitamin B3 (nicotinic acid and nicotinamide) elute very quickly with C18 columns, overlapped by other non resolved peaks at the early time of elution. In order to obtain improved resolution for vitamin B3, a modified reversed phase column (Synergi-Fusion, 250 × 4.6 mm, 5 µ, Phenomenex, USA) was used after consulting with the manufacturer (Phenomenex, UK) to enhance the retention of polar compounds in the aqueous matrix. Results showed improved retention for nicotinic acid peak and resolution of early eluting peaks in Synergi-Fusion column comparing to Luna C18 column following the suggested HPLC method which was recommended by the manufacturer (Phenomenex Product Catalogue, 2007, UK) (Fig 4.1). The HPLC system was comprised of the high pressure quaternary HPLC pump, injector, online degasser, HPLC column equipped with a column guard (4 × 2.0 mm), column oven and a DAD detector. All the modules were

Agilent model 1100 except the DAD detector which was model 1200. Mobile phase was HPLC water (CHROMASOLV, Sigma Aldrich, UK) acidified with 0.1 % formic acid (HPLC grade, Sigma Aldrich, UK).

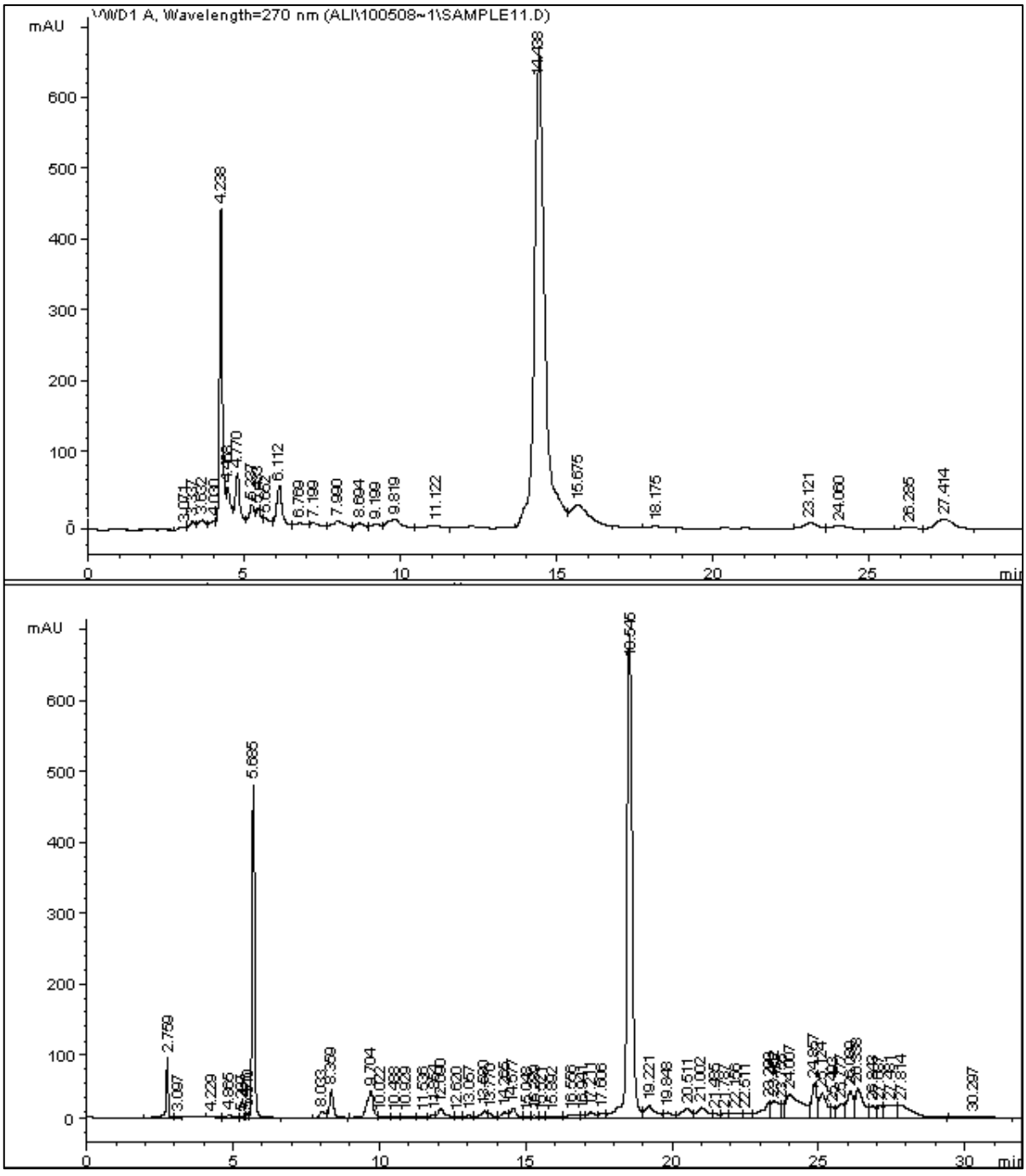


Figure 4. 1 . HPLC chromatogram of hydrolyzed sample (200 C, 20 min, 100 bar) eluted from Luna C18 (top) and Synergi-Fusion column (bottom). UV detector (270 nm)

HPLC flow rate was set to 1 ml/min and injection volume was 5 µl. The UV-wavelength of the detector was set to 270 nm and the spectrum was recorded for the wavelengths between 200-600 nm and used for further structural confirmation of the peaks in the chromatogram. To quantify the vitamin B3 with HPLC, external calibration technique was used and Figure 4.2 shows the calibration curves for the nicotinamide and Figure 4.3 shows the chromatogram of the standard vitamin B3 peaks.

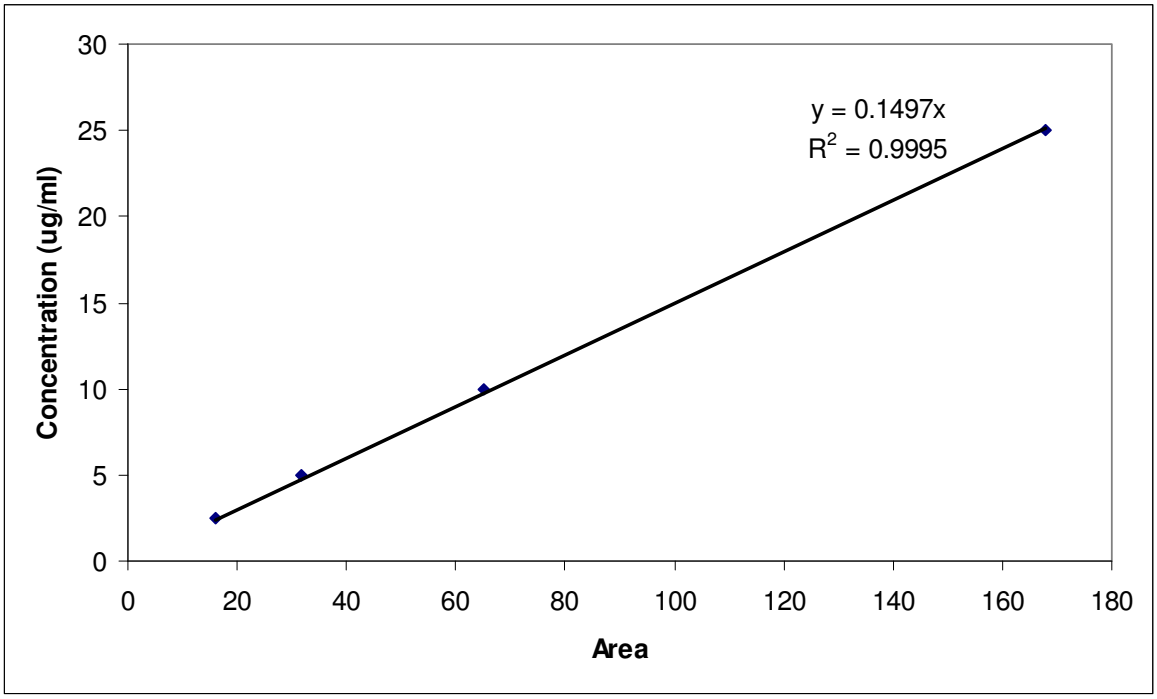


Figure 4. 2 Calibration curve for HPLC analysis of Nicotinamide

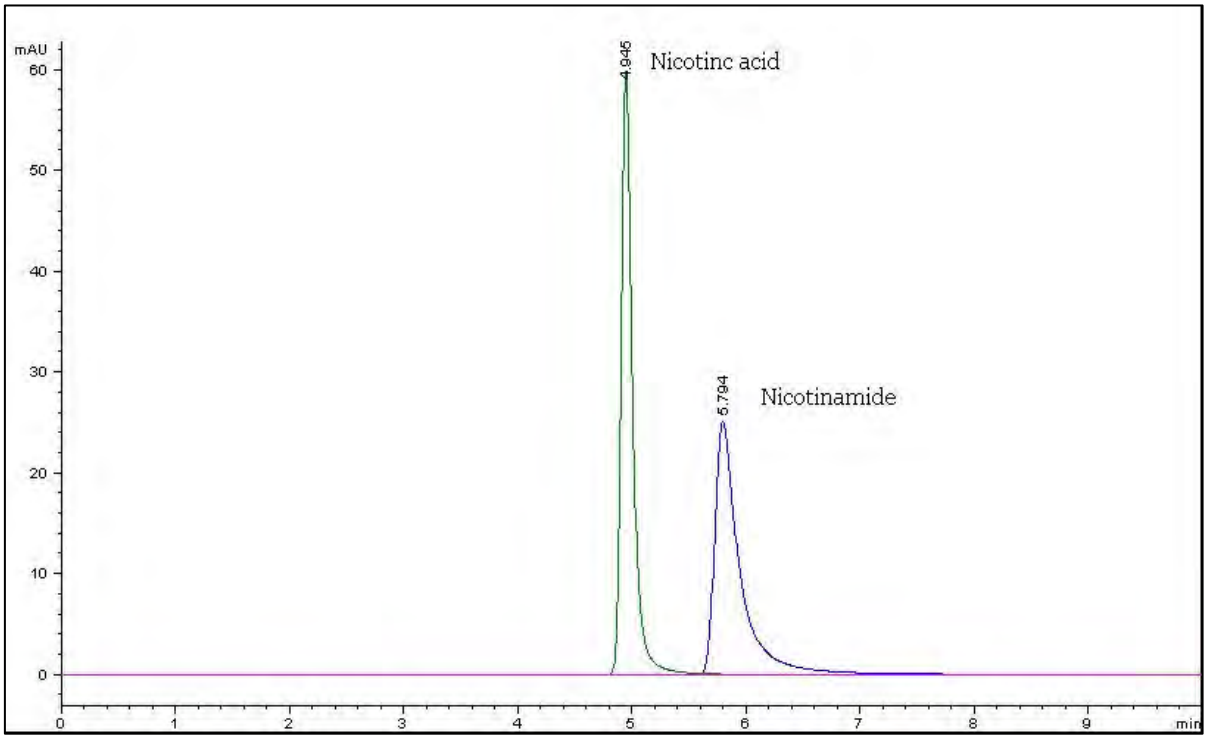


Figure 4. 3 Chromatogram of the standard vitamin B3 (Nicotinic acid and Nicotinamide)

4.2.2 Autolysis conditions

To minimise any uncontrolled and possible biological effects of storage on the yeast cells (change of cell viability, cell metabolism etc) and consequently variation to the autolysis reactions, all the autolysis experiments were carried out using fresh spent yeast which was collected from the Bulmer brewery site. Storing the samples (by freezing) was believed to have minimum effect on the chemical composition of the samples for the purpose of hydrolysis reactions. To remove the residues of the cider fermentation step and other unwanted soluble components, yeast cells were washed and debittered following the method which has been described by Tanguler et al. (Tanguler & Erten 2008). Briefly, cells were harvested from the original slurry by centrifugation at 4000 rpm and re-suspended in distilled water. The pH of the slurry was then adjusted to 9.0 by adding NaOH to the medium and constantly stirring for 30 min using a magnet bar. Yeast cells (suspended in cider lees) were

washed 3 times with distilled water using repetitive centrifugation and suspension in distilled water until the supernatant was clear. The solid concentration of the suspension was adjusted to the one for original sample 11 % (w/v). The extra suspensions which were needed for later experiments were stored at 4°C fridge. 200 ml of 3-times washed cells was used for each autolysis experiment. The pH of the slurry was adjusted to 5.5 with the addition of 1 ml of NaOH solution (2 M) as suggested by the above method. 3% salt (NaCl) was also added to the suspension to facilitate the autolysis through the osmosis difference of extra- and intracellular contents of the cell (hypertonic solution).

The autolysis experiment was carried out at a controlled temperature (45-55 °C) in the 300 ml stainless steel stirred reactor from Parr (Parr Instruments, USA). The specification of the vessel have been discussed and outlined previously in chapter 3. Stirring speed was set to 500 rpm and 200 ml of the prepared yeast suspension was placed in the reactor and reaction started after the vessel reached the set temperature.

To perform the autolysis with the spent cider lees, the same conventional method, which has been offered for yeast cells, was followed as no specific method was available for cider yeast to the knowledge of the author. Different conditions were applied to the starting slurry and the results were studied and compared to find out the optimum conditions which can be obtained with this type of the sample (Table 4.1). Screening tests showed that no autolysis occurs when the pH of the slurry remained unchanged at (3.6-4.0). Hence, the pH of the medium was increased to 5.5 by the addition of NaOH as described earlier. The parameter which used to measure the autolysis progress was the total protein concentration of the reactor content that were collected at different times of the reaction.

Table 4. 1 Conditions used for autolysis experiments

	Temp (°C)	**pH**	**Salt Conc. (% w/v)**	**Cells pretreatment**
Exp. 1	55	Original (4.1)	0	Washed 3 times
Exp. 2	55	5.5	3	Washed 3 times
Exp. 3	45	5.5	3	Washed 3 times

Sampling was done by taking 1.5 ml of sample by lifting the reactor head, heating up at 80 °C for 10 min in a bench-top oven to deactivate the enzymes and stop the reaction, stored at -20 °C freezer until before being analyzed for protein and vitamin concentration.

4.2.3 Protein analysis

As discussed previously, the amount of protein was used as an indication of the autolysis progress. Coomassie Plus (Bradford) kit (23238, Pierce, IL, US) (which was described in section 3.2.5) was used to quantify the amount of soluble protein in the medium.

4.2.4 Vitamin B3 determination in hydrolyzed extracts of yeast using TLC

As discussed earlier in 2.5.5.2.3, a TLC technique was developed based on the method used by Ponder et al ((Ponder et al. 2004) using silica plates and pure water as the mobile phase.

In addition to water based system, few experiments were carried out as trials to find out the elution behaviour of the sample when the mobile phase was changed to organic solvent. n-propanol was used as a candidate solvent and was used in similar conditions to water system. However, solvent front was completely unbalanced and irregular on the TLC

plate making the vitamin separation and Rf calculation impractical. As a result, experiments were followed in water system throughout the project.

All the experiments were carried out at room temperature in a glass TLC chamber. For the detection, plates were placed under the UV light at 250 nm. All the vitamins, except the vitamin B5 (Pantothenic acid) was detected without any derivatisation. To investigate the presence of vitamin B3 (which was expected to be the most concentrated vitamin in the extracts), the retention factor (Rf) of each standard was compared with the one for any corresponding spot on the plate loaded with real samples. Figure 4.3 shows the elution behaviour of vitamin B groups in the discussed system.

4.3 Results and Discussion

4.3.1 TLC analysis

Initially, the TLC method was checked by loading different volumes (2, 4, 6, 8 and 10 µl) of pure standards of the vitamins (5 mg/ml concentration prepared in HPLC grade water) on the silica plates. Plates were placed in the water for 60 minutes and detection was carried out under the UV light after the plates being dried at 50 °C drying cabinet for 1 hour (Fig 4.4).

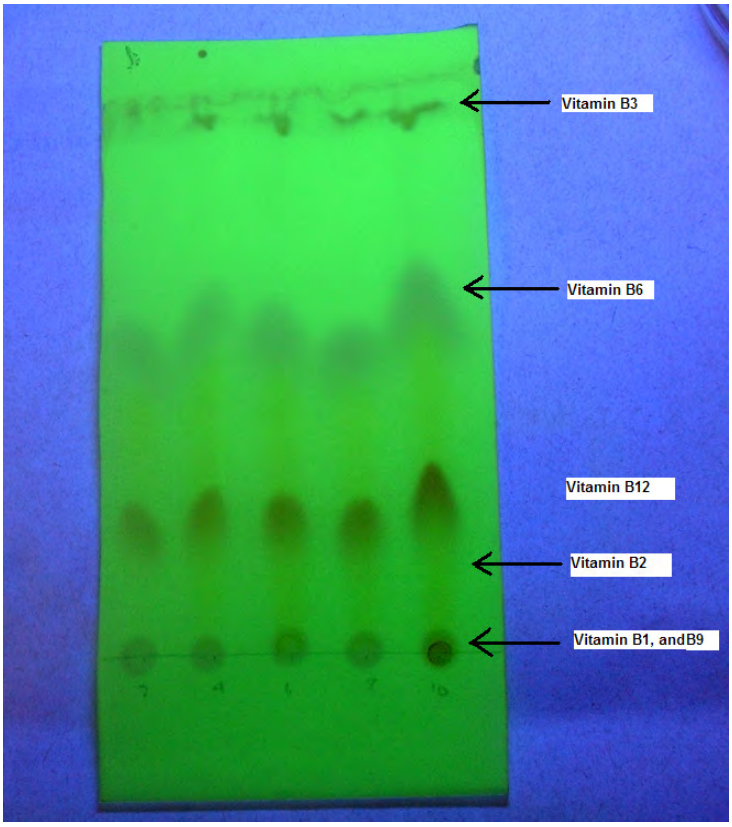


Figure 4. 4. Elution behaviour of the mixture of vitamin B group loaded at different volumes (from left 2, 4, 6, 8 and 10 µl of stock solution containing 1 mg/ml of each vitamin) using the silica-water TLC system . The Rf value of each vitamin is listed in table 4.

As Figure 4.4 shows, the vitamins separated at different rates which correspond to their Retention factor (Rf) in the system. Vitamin B1 and B9 stayed at the origin and did not move upward with water. Vitamin B5 (pantothenic acid) was later visualized by spraying the plate with ninhydrin reagent (Sigma-Aldrich, UK) and heating at 160 °C for 15 min. Nicotinic acid, on the other hand, moves very quickly with water front and obtained the Rf value of 0.89 in the system. The Rf values of each vitamin were measured and are shown in Table 4.2.

Table 4. 2 Values for retention factor (Rf) of vitamins on Silicagel plates and water as the mobile phase

Component	*Rf*
Thiamine-HCl (B1)	0.05
Riboflavin (B2)	0.4
Nicotinic acid (B3)	0.89
Pantothenic acid (B5)	0.49
Pyridoxine-HCl (B6)	0.52
Folic Acid (B9)	0
Cyanocobalamin (B12)	0.22

To investigate the presence of vitamins of B group, especially the vitamin B3 (nicotinic acid) in the hydrotreated yeast extracts, samples which were prepared at different temperature and different times (as discussed in chapter 3) were loaded on the same type plate and the results were compared with the plate for the vitamin standards. A selection of the resulting plates, which are based on loading 5 µl of extracts and running in water for 60 minutes, are shown in Figure 4.5. The extracts were the yeast slurry which were treated at different temperatures (140, 170, 200 and 230 °C) for 30 min and at 100 bar. Prior to the TLC analysis, extracts were centrifuged at 16000 rcf for 10 min to separate the solid particles and the loaded fraction is from the supernatants. In order to obtain higher amount of vitamin B3 on the plate, extracts were concentrated 5 times before loading on the TLC plate. To do this, 10 ml of each supernatant was placed in the rotary vapour (Rotavopr RE-111, Buchi, UK) and concentrated until 2 ml was left.

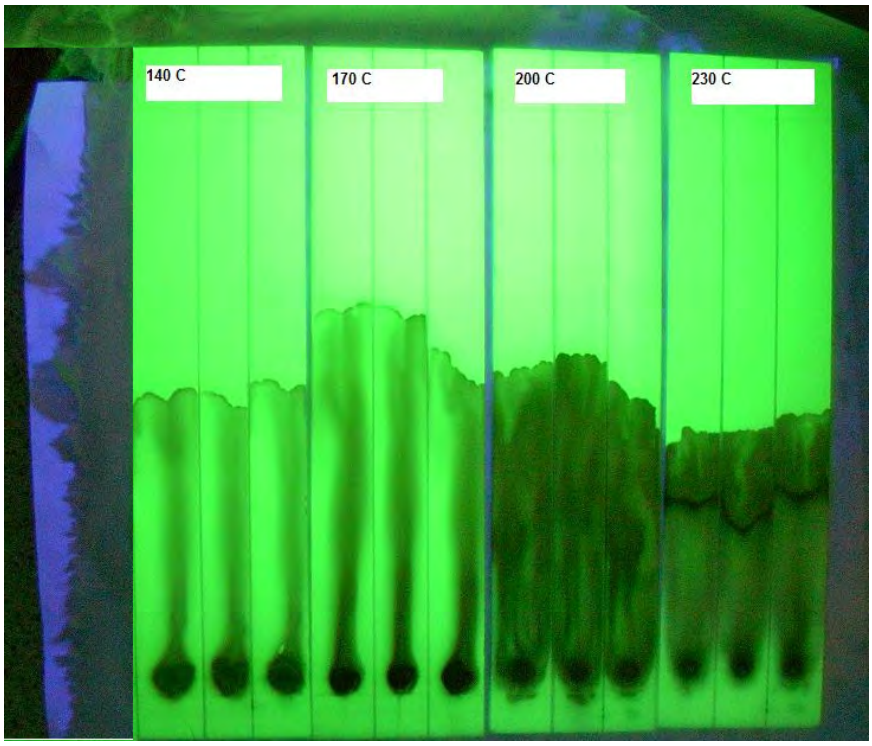


Figure 4. 5 Elution of the hydrothermal treated yeast samples in the silica-water TLC system. Each sample at the indicated temperature has been replicated 3 times on the plate.

The elution behaviour of the extracts in the silica-water system was very irregular and resulted in lanes covered with spots at different Rf's which is common in all the aqueous based silicagel TLC plates. Undoubtedly, the complexity of the samples, especially at higher temperatures, plays a significant role on such behaviour as the diffusion path for the mobile phase via the porous structure of the adsorbent is believed to be largely affected by the presence and blockage of hydrophobic and sticky components in the sample. Loading lower volumes of sample or using samples prepared at lower reaction residence time did not improve the resolution and elution of the sample, so made it impossible to identify the vitamin B3 spot on the plate.

Due to the complexity and irregularity of the sample elution on the plates and the difficulty of the identification of vitamin B3, optimization of the analysis was followed by first trying to resolve the issue with elution on TLC plates. To address this, other solvents such as n-propanol, ethyl acetate and ethanol were used but none of them could provide a clean separation between different spots, resulting in significant streaking of the sample throughout the plate.

4.3.2 HPLC analysis of Vitamin B3

To investigate the accuracy and functionality of the TLC system for separating the vitamin B3 from the extracts, HPLC was used along the TLC to analyze the spots on the plate. This was carried out by running HPLC on the separated spots from the TLC plate, which was believed to be associated with the Rf of nicotinic acid (Rf= 0.82). First, the corresponding spot/area of silica was scrapped from the glass plate with a spatula and separated silica was washed in a microfuge tube with 0.5-1 ml HPLC water to elute any components that had been adsorbed on the silica. After shaking for 1 min, solid silica particles were separated from the liquid with centrifugation at 16000 rcf for 5 mins and the supernatant was used for the RP-HPLC analysis. The accuracy of this method was first determined by following the same procedure with a spot which was corresponding to a plate loaded with pure nicotinic acid. 5 replicates were prepared and it was shown that 95% ±2 of the nicotinic acid can be quantified with the HPLC using this technique (Fig 4.6).

To develop the HPLC method for the nicotinic acid and nicotinamide, standards of each chemical were injected to the HPLC column to find out its retention in the Luna-C18 column (Rt=4.1 min). This retention time was compared later with the any corresponding

peaks from the hydrolysed samples prepared following the procedure explained above (Fig 4.7) shows a typical chromatogram of the extract prepared following the procedure explained above. The sample for this analysis was treated at 200 °C after 30 minutes at 100 bar and ran on TLC plate for 1 hours before being scrapped of the plate. Despite initial results, which made us believe that the remarkable peak at 4.2 minutes corresponds to nicotinic acid (Fig 4.7), further investigation of the extracts with a modified HPLC method (a new HPLC column Synergi-Fusion, 250 × 4.6 mm, 5 µ, Phenomenex, USA) showed zero concentration of this compound in these samples. The new method (described in 4.2.1) was carried with a spiking experiment which samples were spiked with nicotinic acid standard (1 µg). The developed HPLC method suggested that the vitamin B3 did not exist in the extracts under

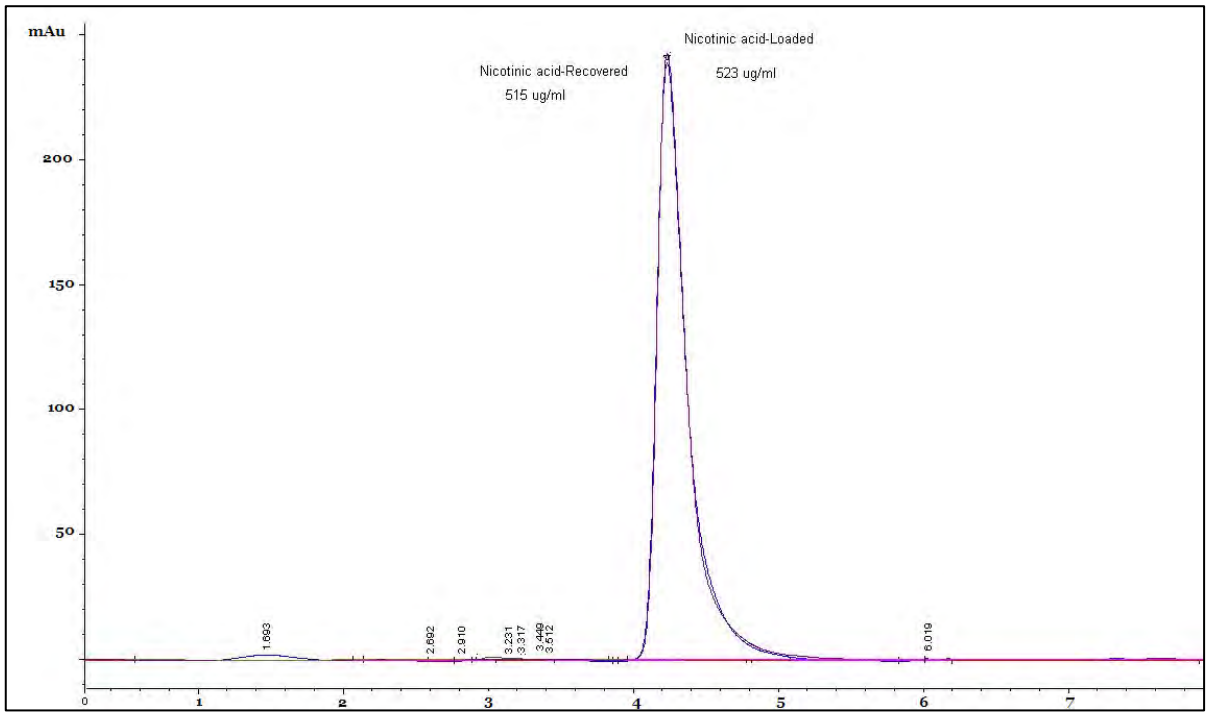


Figure 4. 6. Comparison between peaks of Nicotinic acid in Lunca C18 column before and after loading on the TLC plate showing 95 % recovery (Red line is standard nicotinic acid solution and blue line is nicotinic acid efter elution and recovery from TLC plate), Rt for nicotinic acid is 4.1 min

the studied conditions. Unless otherwise stated, all the other samples were directly injected to the HPLC system without being pre-loaded on the TLC.

Although yeasts are believed to have a very low levels of nicotinamide, TLC plates were examined for this compounds using the HPLC and no peak was detected for nicotinamide as well.

Following the negative results from vitamin B3 analysis, the project was carried out at a faster pace towards finding out a replacement component and also considering the possibility of alternative reactions and components. On this part, the focus was to first identify the other major peaks which were eluting in our HPLC system and were showing change at different process conditions. During the process of peak identification, which was the combination of taking fraction of HPLC or semi-preparative chromatography, GC-MS or direct injection to Electrospray Mass Spectroscopy, identification of other major peaks in the

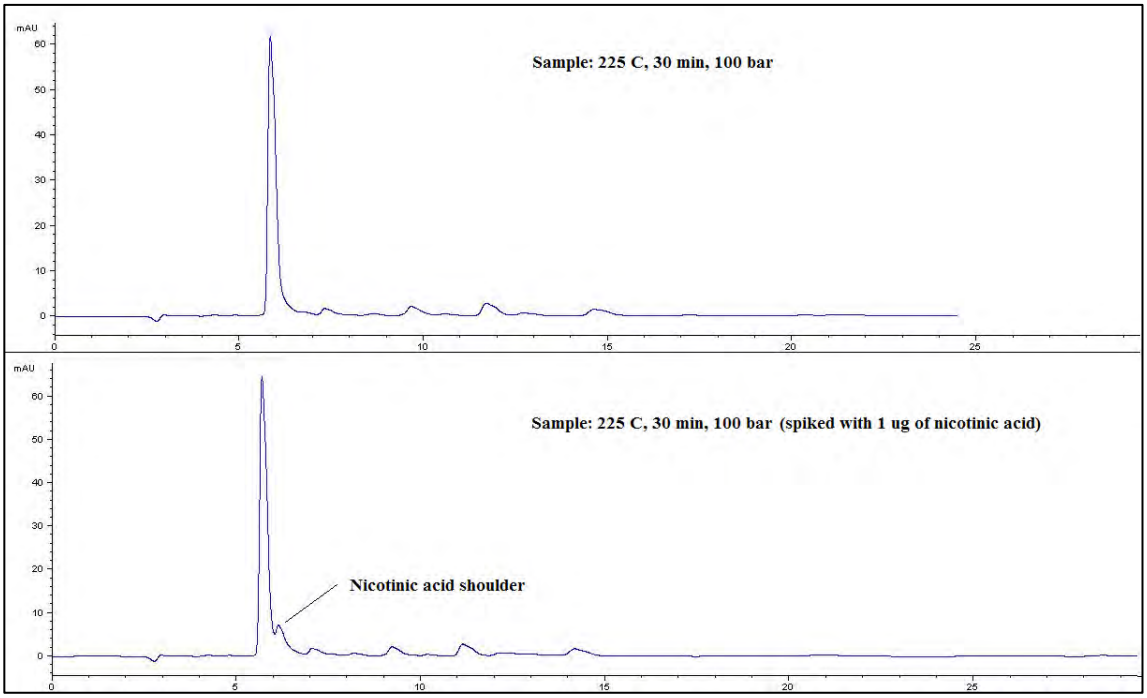


Figure 4. 7 Chromatogram of the hydrolysed sample and the same sample spiked with nicotinic acid

chromatogram, which was achieved with extensive analytical work with Mass Spectroscopy showed the presence of 5-hydroxymethylfufural in the extracts which was discussed in detail in chapter 5 of this thesis. At the same time, it was of our special interest to find out an explanation for the absence of vitamin B3 in our extracts. This lead to a complimentary study that autolysis experiment was carried out and was shown to be able to generate the vitamin B3 compounds. The autolysis experiments are discussed in detail in the following section.

Based on the results on this section, TLC analysis was not able to show the nicotinic acid in the generated extracts. Though it was later showed that vitamin B3 was not available in the extracts in its pure form, still there was some problems using this technique for the purpose that was followed in the project. This was mainly due to the nature of the extracts, which were a very complex mixture of different chemicals that may not separate efficiently on the TLC plate. It can also be attributed to the combination of silica-water in the TLC system, as this system may not work properly in perfect TLC operation as was hoped initially. An explanation for this could be the polarity of both mobile phase and the stationary phase which does not provide a selective adsorption for different compounds in the mobile phase.

4.3.3 Autolysis of the yeast biomass

In the present work, cider spent yeast was subjected to autolysis conditions and total amount of released protein was monitored constantly. The extracts were analyzed to investigate the release of vitamin B3 (nicotinic acid and nicotinamide) which was shown to be released following the autolysis. This, ultimately, would explain the absence of these compounds in the hydrothermal treated samples as they are believed to be in a more complex or bound form. Autolyzed samples did not show significant change in their structure and colour except some degrees of foaming which was observed in the reactor after 12 hours

which can be due to the release of foaming agents from yeast. As discussed earlier, the outcome of autolysis reaction was known by measuring the released protein in the medium. Consequently, the samples with highest concentration of released protein were expected to show highest concentration of released of vitamin B3.

4.3.3.1 Total Protein Concentration

Figure 4.8 shows the total protein concentration at different time intervals measured with Coomassie (Plus) Bradford assay. The initial concentration of protein in the washed samples was around 11 µg/ml which increased to 140 µg/ml after 16 hours in the Experiment No. 2. The difference between the concentration of protein for different experiments shows the effect of temperature and the salt addition. The concentration of the protein almost remained constant after 12 hours and dropped after 18 hours which can be due to further hydrolysis of the released proteins.

The level of protein concentration (~0.14 mg/ml or 1 µg/mg of dry yeast) was significantly lower from ones which were observed in other studies (20-40 mg/ml (Illanes & Gorgollón 1986) or 95 µg/mg of dry yeast (Lamoolphak et al. 2007)). This can be due to the unfavourable autolysis conditions which did not promote higher degrees of autolysis in the studied suspension. This is expectable as the spent yeast in this study is originated from a different fermentation and yeast strain to these studies. Furthermore, different storage periods and condition for the spent yeast in the manufacturer site may change the yeast cells ability to undergo autolytic conditions later and lead to different behaviours and outcomes during and after autolysis.

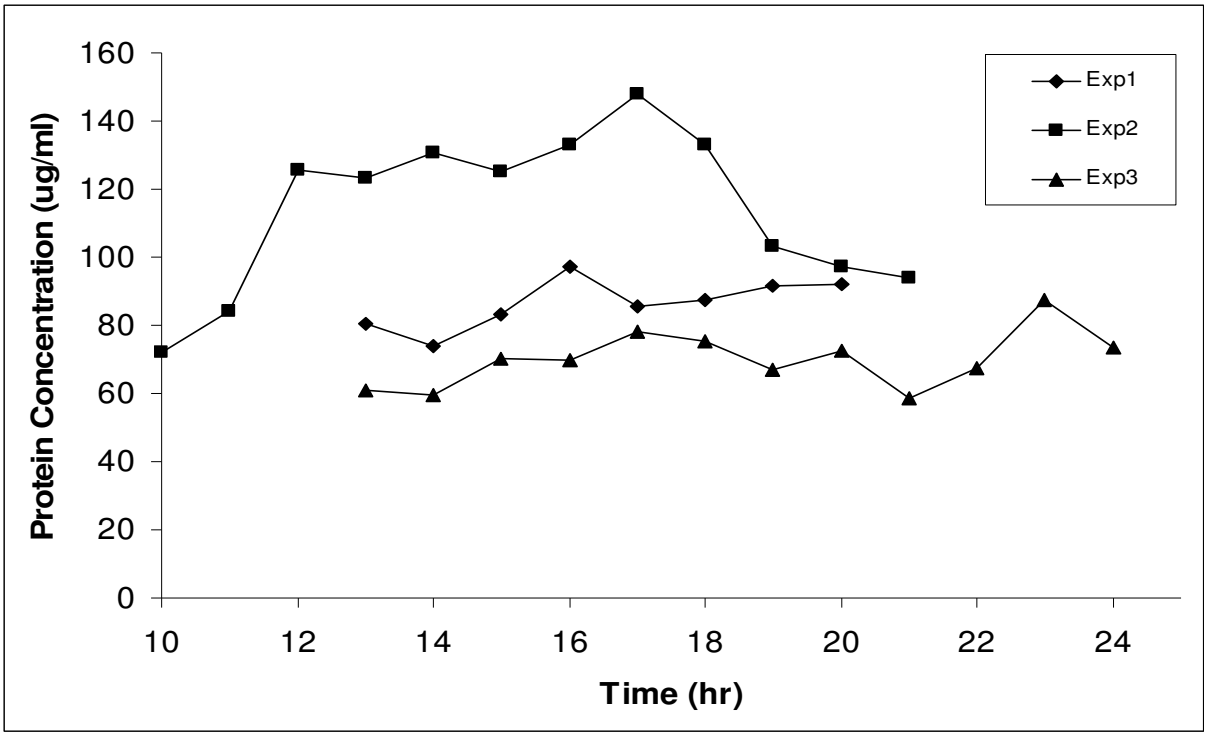


Figure 4. 8. Total protein concentration in different autolyzed samples

4.3.3.2 Vitamin B3 determination in autolyzed samples

The optimized HPLC method (4.3.2) was used to identify and measure the release of nicotinic acid and nicotinamide in the autolyzed extracts. HPLC chromatogram for one of the autolyzed samples (Exp2, t=22 h) is shown in Fig 4.9 and has been compared to the chromatogram of a control sample (untreated crude sample stirred at room temperature for 22 h). The appearance of multiple peaks at different retention times is an indication that several compounds are available in the autolyzed sample which is believed to be due to autolytic conditions and some of them may also originated from the can be released from the yeast cells in such conditions. Considering the retention times of nicotinic acid (Rt= 4.9 min) and nicotinamide (Rt= 5.7 min), earlier elution times in the chromatogram was of special interest in this assay.

In order to provide a better picture of the HPLC peaks which correspond to the vitamin B3 retention times, the associated parts are magnified and are shown in Figures 4.10, 4.11 and 4.12. These graphs would also facilitate the identification of these compounds in the extracts. Figure 4.10 shows the part of the chromatograms which corresponds to the nicotinic acid peak in the autolyzed sample. A little shoulder in this sample was observed at time 4.10 min which is believed to correspond to nicotinic acid. For further confirmation, Diode Array Detector (DAD) generated the UV-spectrum of the peak which was compared to the one for nicotinic acid standard (The reference spectrum for nicotinic acid was prepared earlier by running the nicotinic acid standard and storing its spectrum in the library of DAD). As can be seen in Fig 4,10, there is almost 87% degree of correspondence (calculated based on the purity analysis software in the HPLC software, ChemStation, Agilent, UK) between the observed peak and nicotinic acid which is presented in the small scaling table in the chromatogram in the same figure.

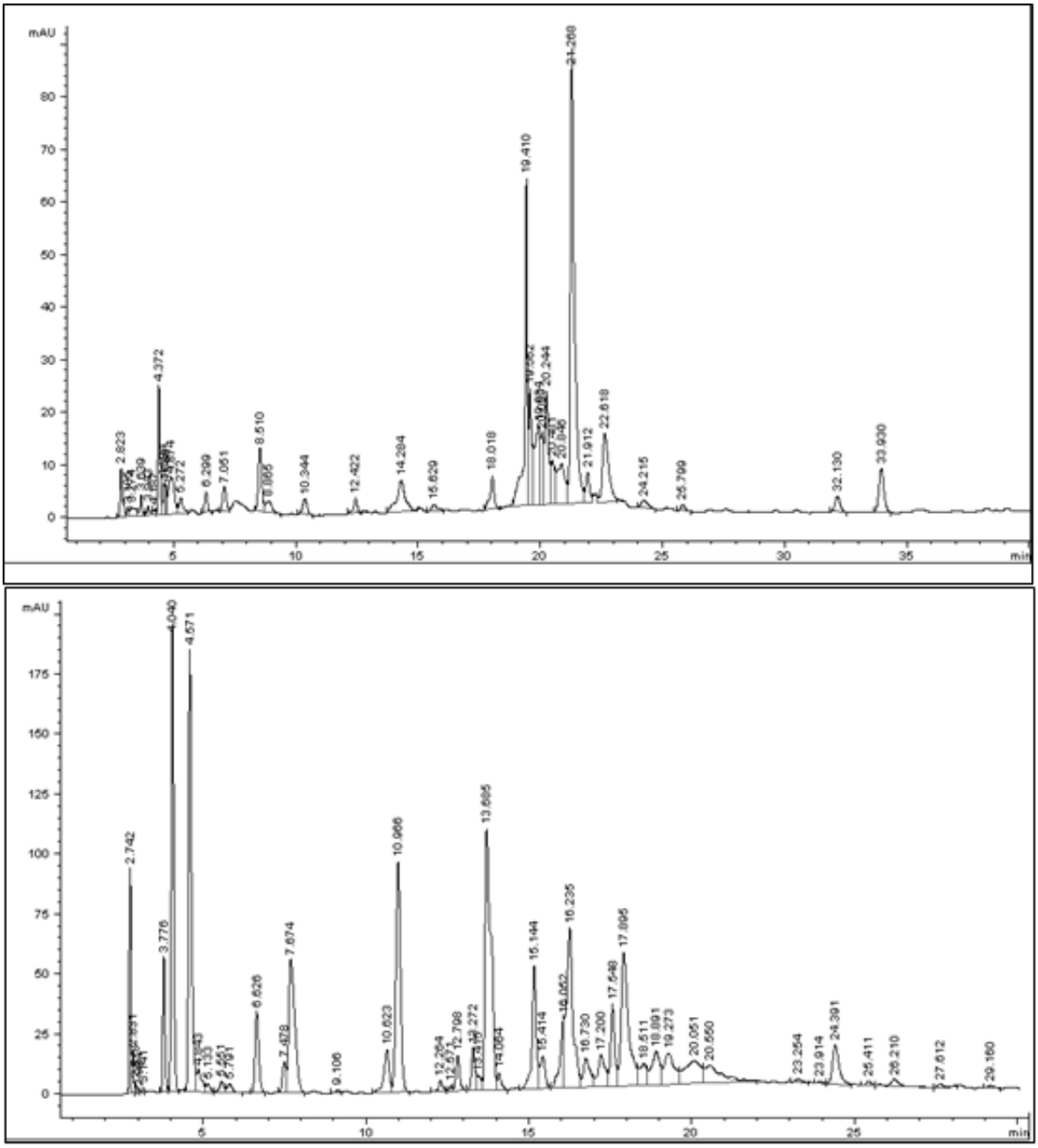


Figure 4. 9 HPLC chromatogram of control sample (top) and autolyzed sample (bottom) after 22 hour of stirring at room temperature (control) and 55 °C (autolyzed sample-Exp2)

This peak was not detected in the original sample, control sample or other samples autolyzed at different conditions when the same analytical technique was used for all of them.

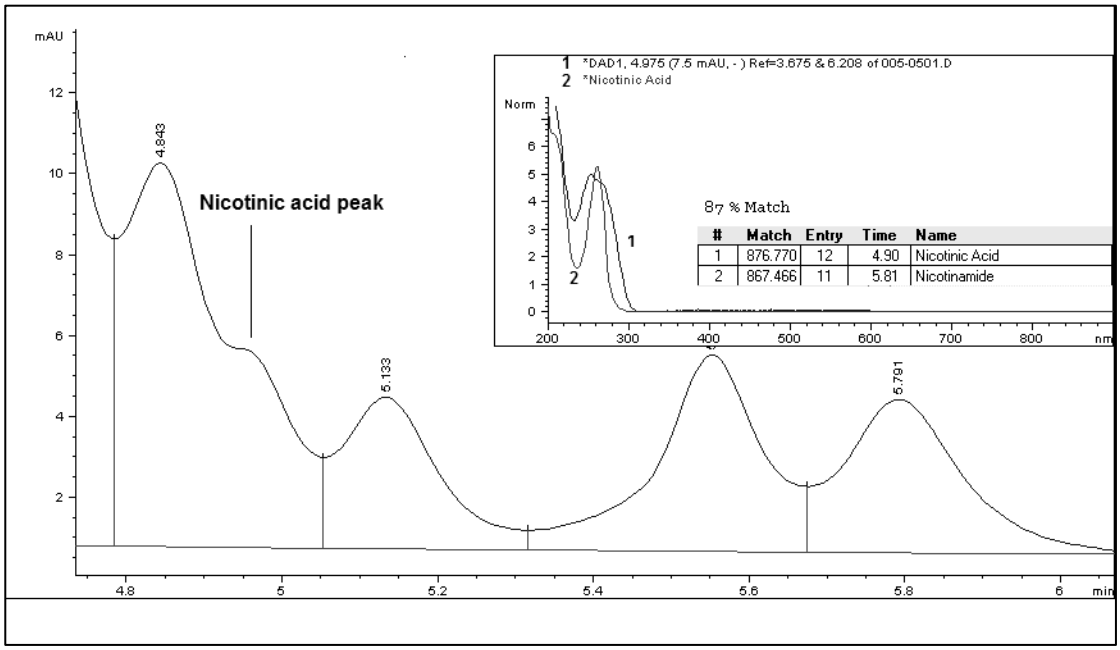


Figure 4. 10 Nicotinic acid peak at 4.95 min and the UV spectrum of the observed peak (No. 1) compared to the one for standard nicotinic acid (No.2) on top right of the figure

An overlay of separate chromatograms at different autolysis times is shown in Figure 4.11. The size of this shoulder peak, which is directly related to the concentration of nicotinic acid, did not change significantly but only a slight increase at longer times which may correspond to an increase in its availability at longer autolysis time. There is a slight change in retention time of the peak which can be attributed to the slight variation of HPLC condition between each analysis. The peak UV spectrum was confirmed for each individual sample and same confirmation was observed. Due to the small size of the peak and its overlap with neighbouring peaks, it was impossible to measure its area accurately and report its concentration in the sample.

Nicotinamide elutes one minute after the nicotinic acid in the used HPLC system (Rt=5.8 min) and its UV spectrum is very similar to the one for nicotinic acid due to their similar chemical structure. While checking the autolyzed samples, a new peak was detected at nicotinamide retention time and further examination of the UV spectrum showed an acceptable match with the one for nicotinamide. Similarly, the magnified chromatogram of the autolyzed sample (at 22 h) is shown in Figure 4.12 accompanied by its UV spectrum which presents the availability of the nicotinamide in the autolyzed sample.

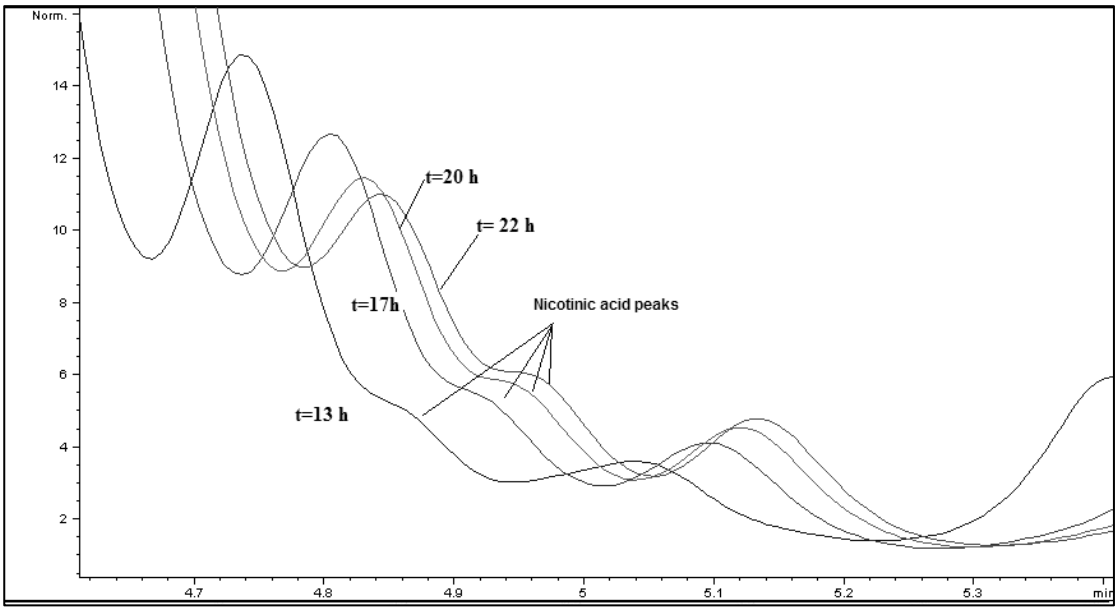


Figure 4. 11 Nicotinic acid peak for the autolyzed sample at different times during autolysis reaction

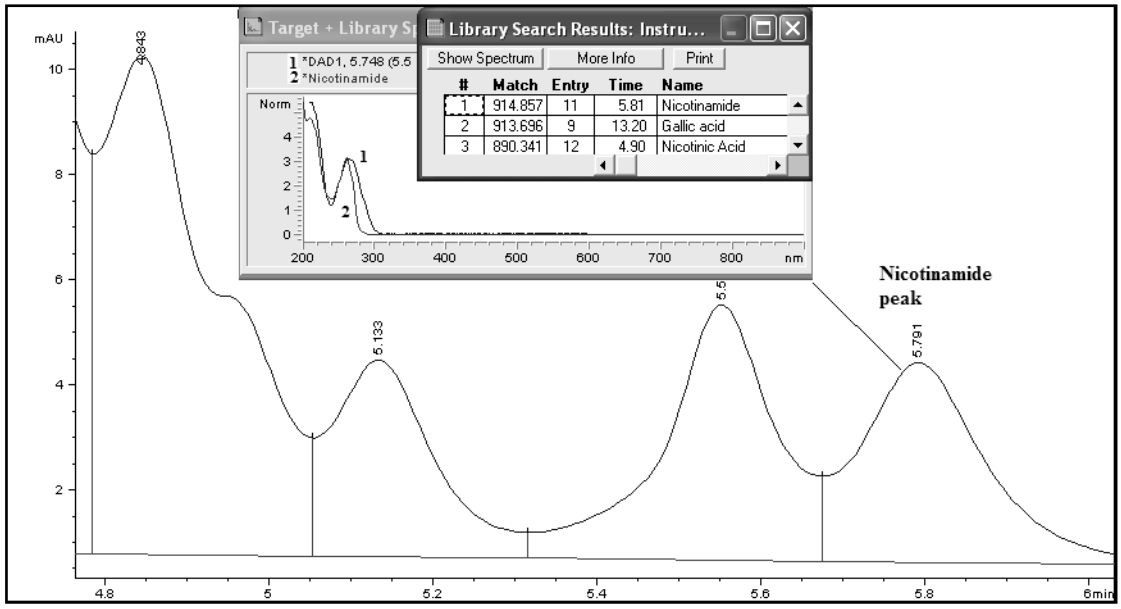


Figure 4. 12 Nicotinamide peak at 5.8 min and the UV spectrum of the observed peak (No. 1) compared to the one for standard nicotinic acid (No.2) on the inlayed window

Based on the external calibration curve which was prepared previously for nicotinamide, nicotinamide was quantified in the samples and its concentration was 4.89µg/ml in the extracts (Exp No.2, t=22 h). This concentration was almost constant in all the samples at 14 hours and more of autolysis (Table 4.3). Considering that there is about 110 mg/ml of dry cell in the samples, the vitamin B3 concentration (based on only nicotinamide concentration) was about 41 µg/g of dry yeast.

Table 4. 3 Nicotinamide concentration in autolyzed sample at different times during the autolysis

Time after start of autolysis (hr)	Concentration (µg/ml)
13	4.2
17	4.5
20	4.9
22	4.8

The total concentration of vitamin B3 *Saccharomyces bayanus* (the yeast strain available in the samples) has not been reported anywhere to the knowledge of the author, but it is suggested that its concentration is between 300-1000 µg/g of dry yeast in brewer's yeast (Halsz & Lsztity 1991). This value is much higher than the levels that were obtained in this study which can be due to several reasons such as different condition of the starting cells, different yeast strains or employing other autolysis procedures.

4.4 Conclusion

The main objective of this chapter was to show the results of the study to investigate the possibility of using hydrothermal reactions for the extractions of vitamin B3 from the cider lees. However, the developed HPLC analysis confirmed that no vitamin B3 is present in the hydrothermal extracts, at least in its free form. However, it was possible to show small levels of this compound being produced from the cider spent yeast after subjecting the cells to autolysis. This suggests that vitamin B3 is available in these yeasts but not in high levels or in their free form to be extracted.

The complimentary autolysis study helped to find an explanation why no vitamin B3 was detected in the hydrothermal treated samples and second, suggests that the presence of vitamin B3 depends on the involving cellular enzymatic reactions. These reactions can be

promoted to the release of vitamin B3 during autolysis similar to what happens in the production of yeast extract in industry. It is believed that these specific reactions would be triggered during autolysis while they would be stopped or hindered at high temperature and pressure conditions during the hydrothermal treatment. Ceasing the activity of these enzymes would result in the loss of vitamin B3 in the hydrothermal extracts as observed in our study.

Nicotinic acid can also be available in a modified form of its derivatives such as Nicotinamide or bound (active) form such as Nicotinamide adenine dinucleotide (NAD^+) or its reduced form (NADH). Hence, as a recommendation for further studies, other techniques such as microbiological assay can be used for the determination of vitamin B3 as they can estimate the presence of nicotinic acid (or its derivatives) regardless of their form (microbiological assays were not used due to the limited time and resources available for this purpose). In contrast, HPLC techniques (with no mass spectroscopy detector) are mostly limited to analysis of standard and known forms of vitamin B3.

Chapter 5

HMF synthesis through subcritical water treatment of cider spent biomass

5.1 Introduction

Using food crops for biorefinery plants can negatively affect the food supply chain and increase the price and depletion of such raw materials resources. This highlights the importance of using any other feedstock or waste that can be employed for such applications which offer similar milder structures. The matter of using alternative feedstock or stick to abundant feedstock with higher processing costs can only be discussed if all the potentials of new process is known. Hence, it is important to check the viability of the new feedstocks as it can offer similar productivity and feasibility compared to the typical resources. Moreover, the chemistry of the feedstocks should be thoroughly studied to identify all the possible streams and products that can be used to offer more revenues.

In this chapter, the hydrolysis of cider spent yeast (lees) was carried out in subcritical water in the conditions which were discussed in previous chapters. The main objectives of this chapter was to show first) how an alternative β-glucan source undergoes under a non-catalytic, complex and non-purified medium; second) to determine the kinetics and progress

of a selected reaction pathway (HMF synthesis) and compare the results with other feedstocks. Cider spent yeast slurry was showed to demand milder hydrothermal conditions during its structural depolymerisation and its hydrolysis to monosaccharides. The applicability of subcritical water was further examined by addressing the HMF formation, as one of the top-based candidate compounds originating from the released glucose.

In addition, background analytical information, which discussed earlier proved the formation of significant amount of HMF in the extracts. Complimentary analysis of both liquid and solid fraction also highlighted the concentration of monosaccharides in the hydrothermal treated extracts and the solid residues. The results from these assays offered the chance to better study the hydrolysis of β-glucan in the cell wall to monosaccharides (glucose and mannose) and secondary reaction of glucose to the HMF.

Kinetics of the reaction were studied and modelled to evaluate the effect of temperature on different reaction steps. No additive, catalyst or pretreatment steps were employed throughout these experiments. The exploitation of unpurified biomass instead of pure model monomers or polymers will also assist in filling the gap in the literature on the HMF obtained from different feedstocks. The outcome of this work can be of special interest for biofuel industries (ethanol or any related process which comprised of a fermentation step) as they need to handle increasing amounts of this by-product as a direct outcome of growing interest in alternative fuels.

5.2 Materials and Methods

5.2.1. Raw material

Samples were collected form the cider manufacturer site (Bulmer, Herefordshire, UK) from a collection vessel which stores the lees after alcohol removal using a combination of

filtration and distillation. The impact of alcohol removal was not determined as it was part of the process which was carried out in the upstream of the process. It is mainly comprised of excess and unwanted spent yeast generated during and after the fermentation. Samples were transferred to the laboratory and were packaged in 250 ml bottles and were stored in -20 °C freezer. For each single experiment, a bottle of frozen sample was removed from the freezer, the contents were thawed at room temperature and then used for the experiments. Yeast cells, which ere the main solid fraction in the sample, are enclosed by a rigid structure of mainly helical beta-glucan chains which form its surrounding cell wall and may account for up to 30% of cell dry mass (F. M. Klis 1994). The main constituents of the cell wall are β-1-3-glucan, β-1-6-glucan, mannoproteins, chitins and a small part of lipids (Kollár et al. 1997). In contrast to cellulose, β-glucan chains in yeast, are formed of β-1-3 glycosidic bonds (that are branched by β-1-6 bonds) which make them more flexible and susceptible to hydrolyze due to the lower amount of inter-chain bonds when compared with cellulose (Stephen 1995). In addition to the glucan chains, a smaller fraction of mannose is present in mannoprotein structure which can be released in hydrolytic conditions. Mannose has also been shown to convert to 5-HMF via a similar pathway as glucose (Binder et al. 2010). The raw material was considered as a mixture of glucose and mannose polysaccharides and the concentration of each monomer was determined accurately at each step for estimating conversion, yield and kinetics of the reaction.

Total solid concentration in the slurry was 12% (w/v) in which the yeast cell concentration was about 10% (w/v)in the slurry. Yeast cell concentration was measured after filtering the slurry with filter paper (Whatman® grade No.1) and subsequent washing of the retentate with distilled water and drying the filter paper with retentate. The pH of the sample

was 3.8 (±0.2) and no significant change in this value was observed after hydrothermal treatment over different conditions.

5.2.2. Hydrolysis Experiments

Batch hydrolysis reactions were carried out in a stainless steel tube reactor (L=215 mm, id = 4.6 ± 0.1 mm) placed in a fan assisted oven. The reactor was equipped with a K-type thermocouple only to monitor the tube internal temperature. The temperature of the oven was set and measured with oven controller. This configuration was different from the one in the stirred high pressure vessel which was used in the other parts of this research to prepare the extracts. The main reason was the special importance of heating up throughout the kinetics study. As there was long delay during the initial heating-up stage (induction time) in the 300 ml Parr reactor (Fig 5.1) which restricts the accurate measurements of reactants conversion and the effect of time and temperature. For reactions such as biomass hydrolysis or hexose sugar dehydration, reactions may start well ahead reaching the set temperature when the set temperature is above 225 °C. The range of studied temperature was between 175°C and 275 °C. For each experiment, 3 ml of sample was injected into the reactor (50% of reactor volume left empty) and the tube was placed in the oven. The reaction time started (t_0) when the temperature in the tube reached the set temperature. Heating up time was measured and was between 300 and 360 sec for all the experiments. The tube was cooled with immersing in cold water to quench the reaction and the sample was removed from the tube for analysis. Separate experiments were carried out for each individual time-temperature point.

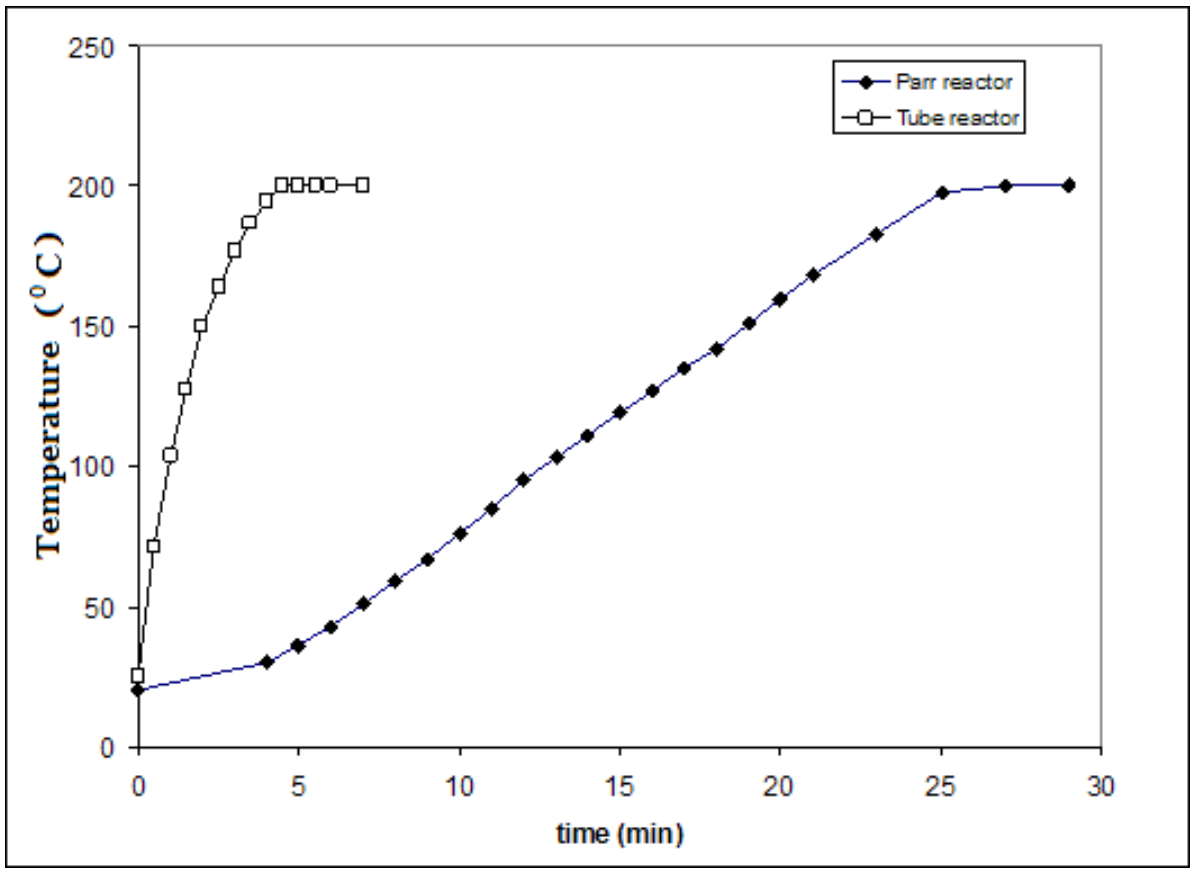


Figure 5. 1 Heating up profile for the 300 ml Parr reactor containing 250 ml sample and the tubular reactor containing 3 ml sample. Temperature was set to 200 C in both reactors

To determine the conversion of glucose and HMF, 20 mM solution of each compound was prepared by adding the pure component to the supernatant of the slurry (after the solids had been previously removed by centrifuged) instead of distilled water in order to include any effect that the crude mixture may have on each compound. For mannose, the same conversion rates as glucose was used based on their very similar molecular structure and reaction scheme sulphur (Binder et al. 2010)

5.2.3. Carbohydrate analysis of the yeast cell

To measure the concentration of the monosaccharides in the yeast cells, the method developed by Dallies *et al*. was used with some modifications (Dallies et al. 1998). It is consisted of a hydrolysis step using a combination of sulphuric acid and high temperature to completely hydrolyse the polysaccharide chain followed by HPLC analysis of released monosaccharides (discussed in detail below). This method was used to measure the amount of cell wall polysaccharides (based on the moles of glucose) at time t=0 ($C_{CW,\ 0}$ as used in reaction kinetics in section 5.3.3) and throughout the reaction at different times (C_{CW}) to determine the polysaccharide hydrolysis.

5.2.3.1 Preparing solid samples for carbohydrate analysis

Solid residues were collected after each experiment by centrifuging the extract at 3500 g for 10 minutes (Beckman Centrifuge). Supernatants were used for the liquid fraction analysis by HPLC. The pellets were re-suspended in distilled water and washed three times until the supernatant became clear and dried in a drying cabinet for minimum 24 hrs at 105 °C. Dry solids were ground with pestle and mortar, meshed and monosaccharides were quantified by carbohydrate assay.

5.2.3.2 Hydrolysis with concentrated Sulphuric acid

At the first step, 175 µl of 72% sulphuric acid (Sigma Aldrich, UK) was added to the dried cell residue and the mixture was left at room temperature for 3 h. The slurry was diluted to 2 ml with distilled water and heated to 100 °C for 4 h. After cooling, samples were neutralized with concentrated $Ba(OH)_2$ solution. The supernatant was analyzed for

monosaccharides after overnight refrigeration at 4°C and centrifugation to remove the precipitate.

5.2.4 HPLC analysis

To quantify the amount of studied chemicals, an Agilent 1100 HPLC system (Agilent, USA) was used which was comprised of an auto-sampler, high pressure quaternary pump, online degasser, column oven and RI detector (1200, Agilent, USA). A 5 micron REZEX-ROA (300 × 4.6 mm) column (Phenomenex®, USA) equipped with a column guard with the same stationary phase (4×2.0 mm, Phenomenex, UK). The mobile phase was 0.5% formic acid solution prepared of HPLC water (Sigma Aldrich, UK) which introduced to the column at 0.5 ml/minutes flow rate. A separate REZEX-RCA column (Phenomenex®, USA) with similar specification and in the same conditions was used to quantify mannose. The method was based on the recommendation of the column manufacturer (Phenomenex, UK). For quantification, calibration curves were prepared by injecting pure standards of each compound as described in 4.2.1.

5.3 Results and Discussion

5.3.1 Determination of HMF peak with Mass Spectroscopy

The main objective of studying HMF reaction was because of its potential wide application as a bio-based chemical to replace petroleum derived chemicals. However, learning about the origin of this compounds in the hydrolysates of this study, which was initially an unknown peak which was observed in the HPLC chromatograms, was only possible by carrying out additional analytical techniques and dedicating significant time of this project. A brief overview is provided below which discusses different steps taken to identify HMF.

Fig 5.2 shows the HPLC chromatogram of a sample prepared at 200 °C and 100 bar after 30 minutes at the early stages of the project before finding out about the presence (and synthesis) of HMF in the hydrolysates. The chromatograms (prepared using the method described in 4.2.1) at these temperatures (200 °C and higher) were dominated by two major peaks at 5.6 and 18.5 minutes. These two peaks showed a changing profile, which indicated their accumulation at certain temperatures of the subcritical water hydrolysis reaction. A combination of HPLC and Mass Spectroscopy techniques were used to identify these peaks, but it was only possible to determine the second peak which was the more significant one. In order to identify the unknown peaks in the absence of an HPLC-MS system in the lab (no other LC-MS system was located off site during the limited time available for this part of project), the method which was initially developed for the HPLC was transferred to the semi-prep HPLC unit (Summit HPLC systems, Dionex). Higher volume of sample was injected (100 µl comparing to 5 µl in analytical HPLC) with higher flow rates (10 ml/minutes comparing to 1 ml/min) of the same mobile phase in order to maximize the concentration of compound and obtain sufficient material for further analysis.

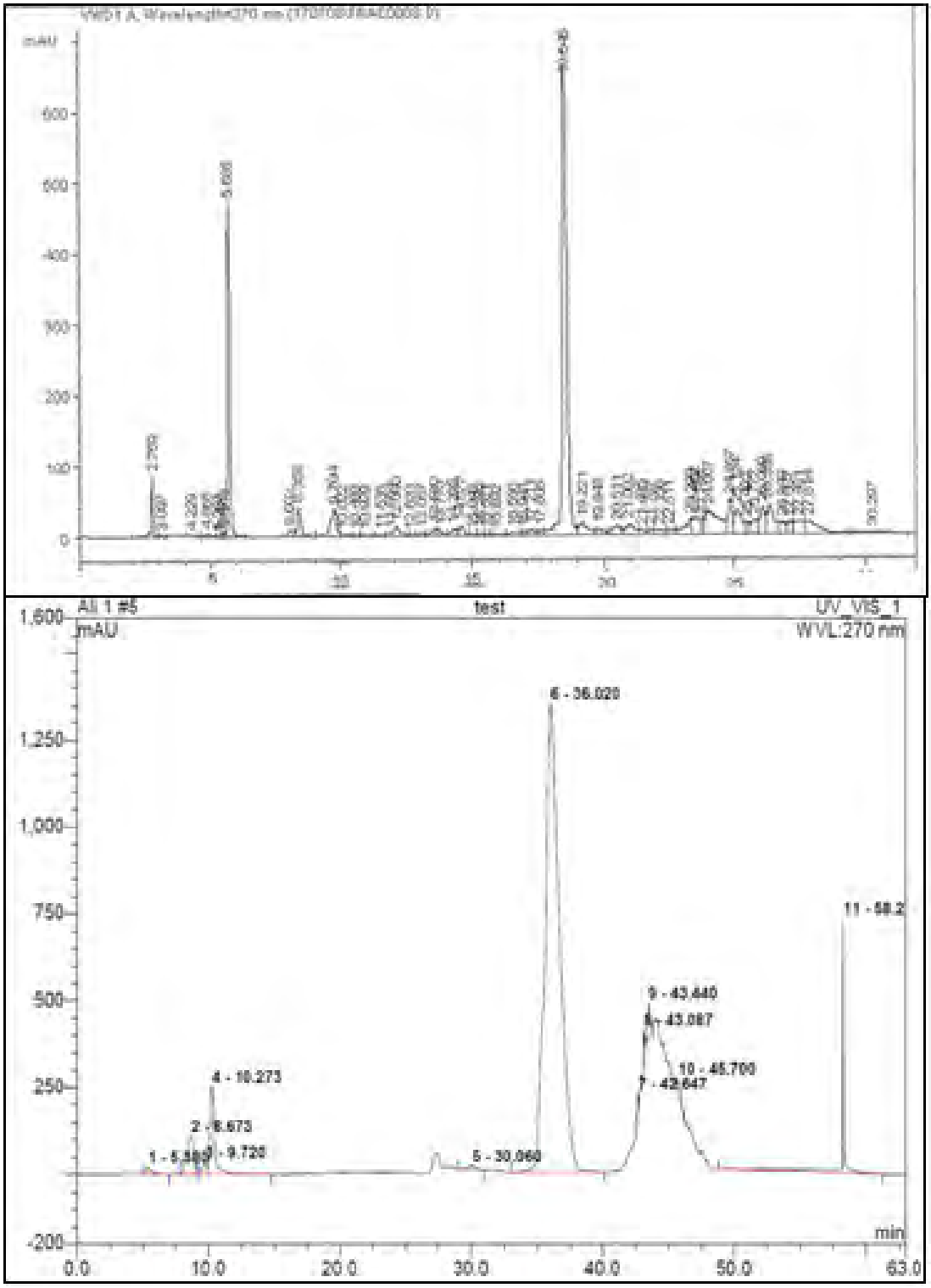


Figure 5. 2 Chromatogram of hydrolysed sample analyzed with analytical HPLC (top) and injected to semi-prep column for fractionation (bottom). The peak of interest was eluting at 18.5 in HPLC and 36 in semi-prep system

The collected fraction (peak at 36 minutes in bottom chromatogram in figure 5.2) was concentrated with rotary vapour 5 times, confirmed with HPLC and was directly injected to Electrospray ionization mass spectrometry (ESI-MS) (Micromass, Manchester, UK) and MassLynx data acquisition (Fig 5.3). The HMF structure was confirmed in the resulting spectrum (Fig 5.3.b, the peak at 126.2).

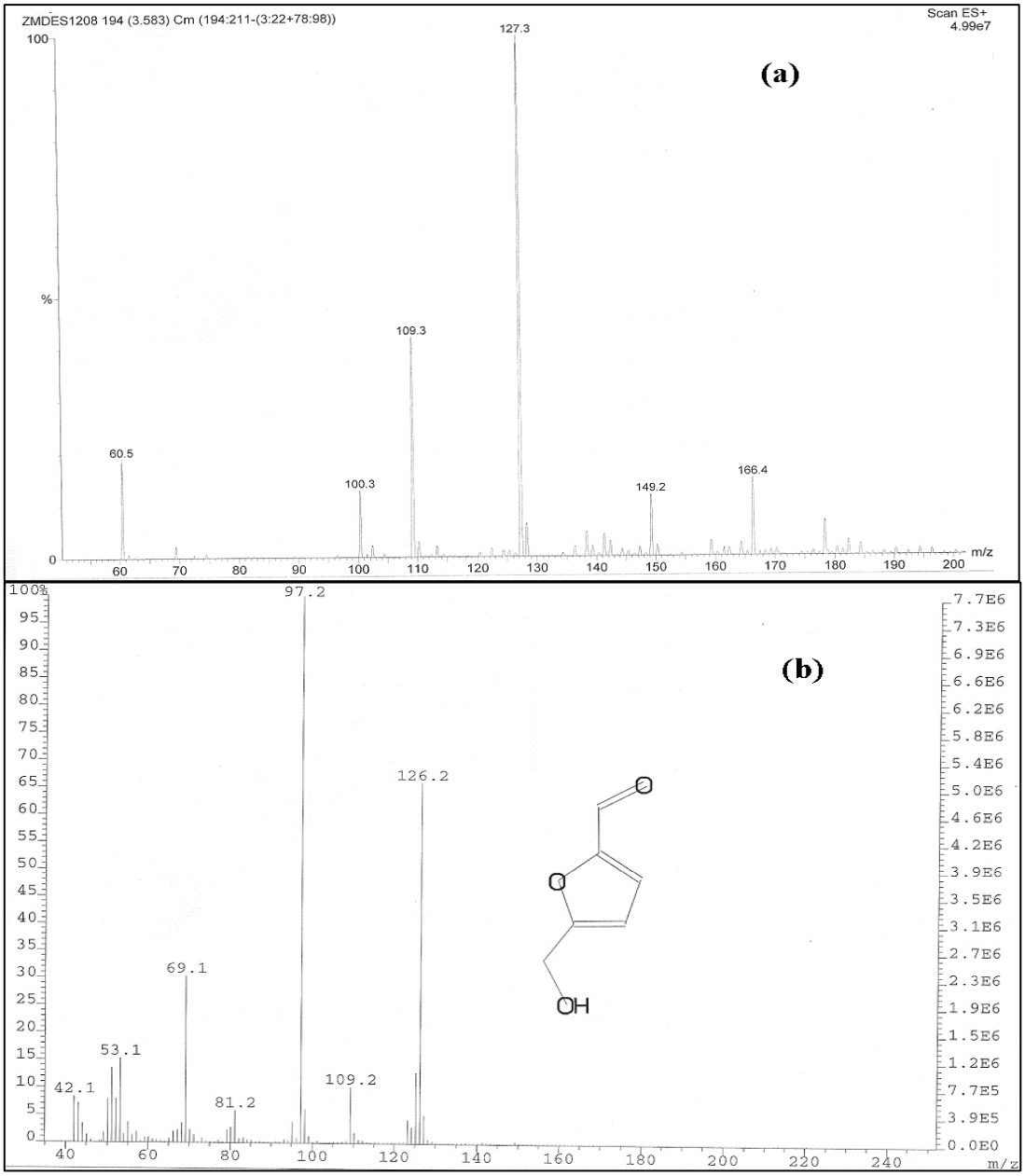


Figure 5. 3 Mass spectrum for the collected peak from HPLC in quadropole positive ion mass spectroscopy (a) with 127.3 as HMF (and a H) and the structure and fragments of the HMF molecule (b)

5.3.2. Cell wall decomposition in subcritical water

The cider yeast cell wall is composed of β-glucan (60%), mannoproteins (37-40 %) and small fraction of chitin (<3%). Our objective was to investigate the efficacy of subcritical water mediated hydrolysis of the yeast cell wall in order to provide a substrate for HMF production downstream. Most previous works have concentrated on model systems for example fructose, glucose or cellulose but our complex system which is acidic offers the opportunity to evaluate the potential of one step conversion of β-glucan to HMF. This aligns with previous works (Chheda et al. 2007; Claude Moreau et al. 1996) which highlighted the effect of acidic catalysis for the synthesis of HMF.

The concentration of polysaccharides and peptidoglycan were determined indirectly by measuring the amount of glucose and mannose. Kinetic model was developed assuming both the glucose and mannose were the only substrates for HMF production. The potential substrate availability was derived from measuring the amount of glucose and mannose in the soluble fraction from the supernatant and the solid residue.

The subcritical water mediated hydrolysis of the yeast biomass was studied at temperatures between 175 °C and 250 °C at 10 MPa and the conversion of biomass under these conditions is shown in Fig 5.4. Biomass (β-glucan) conversion was measured by comparing the amount of glucose (as a measure of β-glucan) left in the solid residue and what was available in the untreated yeast biomass (substrate). The decomposition of the cell wall appears to start at 175 °C (based on the solid analysis) and under these conditions more than 50% of the solid biomass was hydrolysed after 30 minutes.

The hydrolysis of biomass continued to increase with temperature up to 250 °C, where full hydrolysis of the cell wall was achieved after 5 minutes as no further glucose or mannose was detected in the solid residues. Our findings can be compared to other works in which

cellulose reached to complete conversion at temperatures equal or higher than 250°C in non-catalyzed conditions (Jing & X. Lu 2008; Sasaki et al. 1998). At higher temperatures (≥225 °C), the reaction is very fast and may have not been represented very well with the used reactor because of the initial heating up stage resulting in some discrepancy between experimental points and fitted lines.

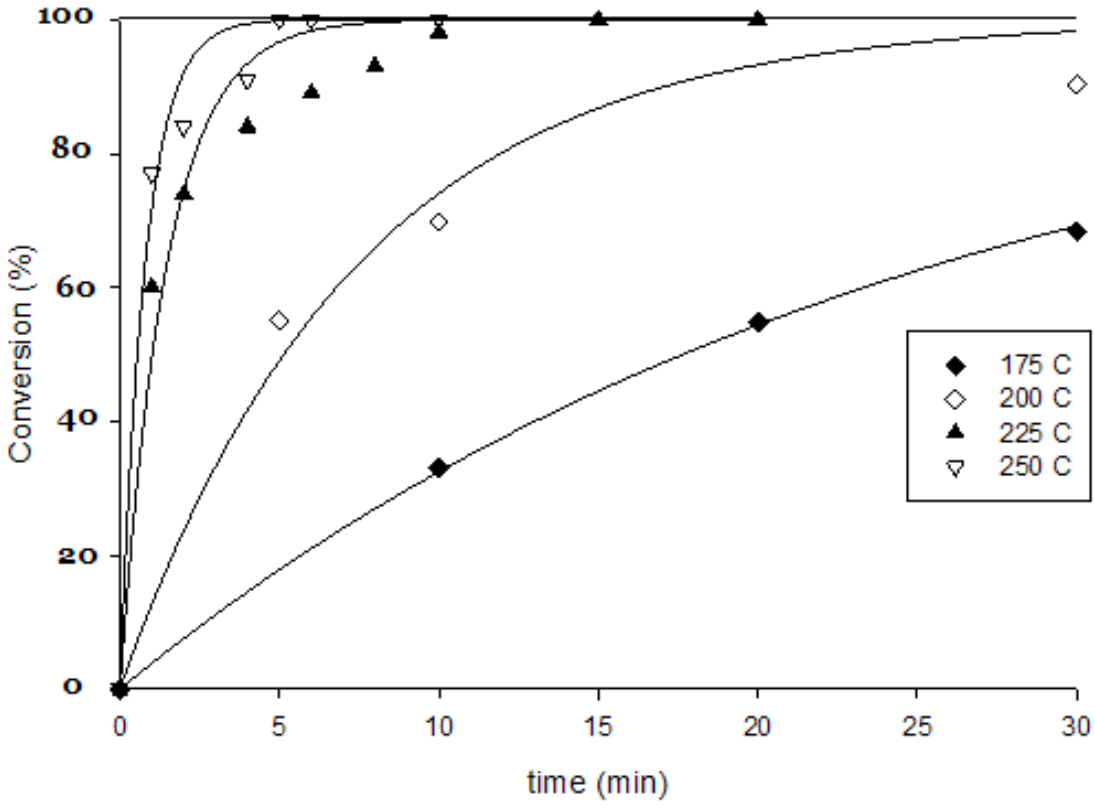


Figure 5. 4 Effect of temperature and time on the hydrolysis of β-glucan of the yeast cell wall, experimental data and kinetic model (solid lines)

The extracts from the experiments were analyzed using HPLC to resolve both carbohydrates and organic acids known to be generated under the subcritical conditions (Bicker *et al* 2005) (Fig. 5.5.). For monosaccharide analysis, the glucose peak was identified at 175 °C and reached its peak at 225°C and thereafter it decreased from 250 °C. The loss of

the sugar peaks at 250 °C and 275 °C would appear to be related to all the nonspecific reactions generating secondary products (such as HMF, levulinic acid which is indicated in the Fig. 5.5.c). Higher reaction temperature (275 °C) was used in Fig 5.5.c as the peak for levulinic acid was more pronounced at this temperature. The chromatogram which was recorded at 175 °C shows a higher reaction time (20 min) as the conversion was lower that can be shown at shorter times.

Monosaccharide analysis of the extracts clearly showed that release of glucose and mannose starts at temperatures as low as 175 °C and the change in their concentration was showed in Fig.5.6. Both compounds reached their maximum yield after 4 minutes at 225 °C and they are further dehydrated to organic acids, as the temperatures increases. Monosaccharide yield was based on the released monosaccharide (soluble in liquid phase) compared to the total amount of monosaccharide available in the substrate (untreated yeast cells) measured by carbohydrate assay described in 5.2.3.

Ethanol residues are still available in the samples even after alcohol removal step in the cider manufacturing process. The concentration of ethanol in the crude sample was measured with HPLC-RI (using the same method described in 5.2.4 and 4.2.1) and was estimated to be 25 mmole/lit (Fig 5.6)

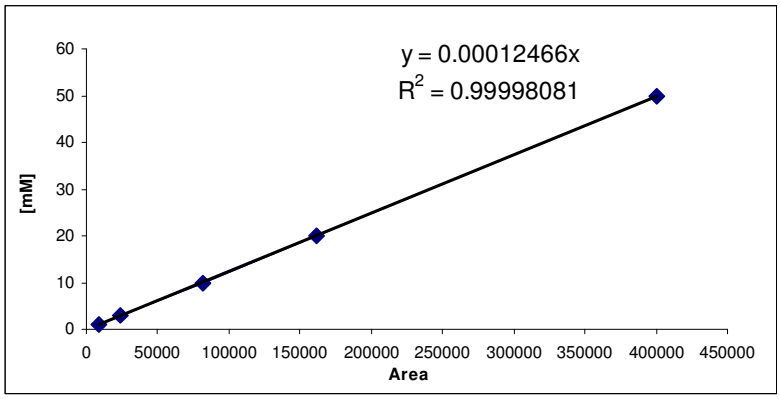


Figure 5. 5. Calibration curve for ethanol measurement using HPLC-RI

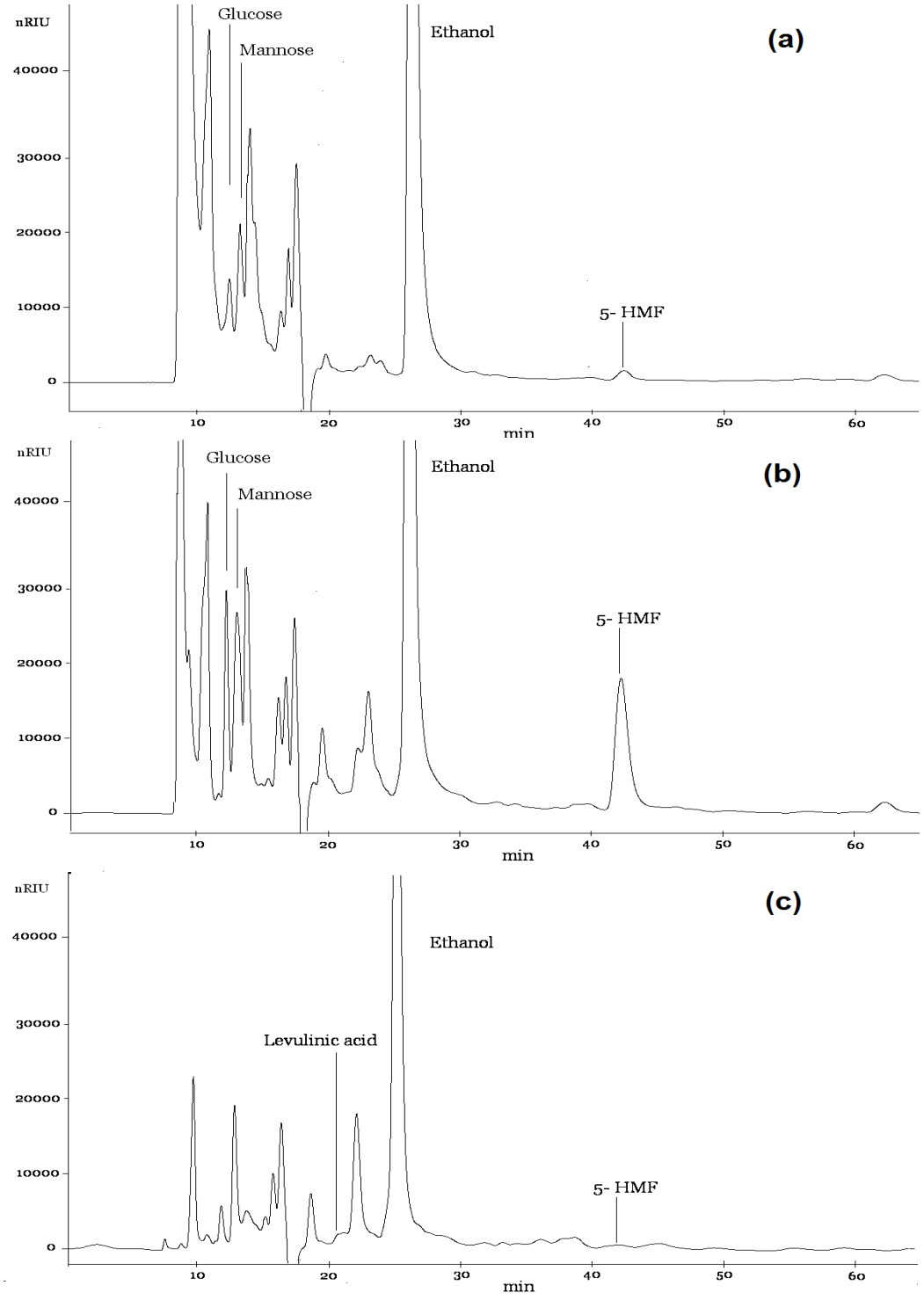


Figure 5. 6 HPLC chromatograms for the slurry after batch hydrothermal treatment (a) 175 °C for 20 minutes (b) 225 °C for 6 minutes (c) 275 °C for 6 minutes

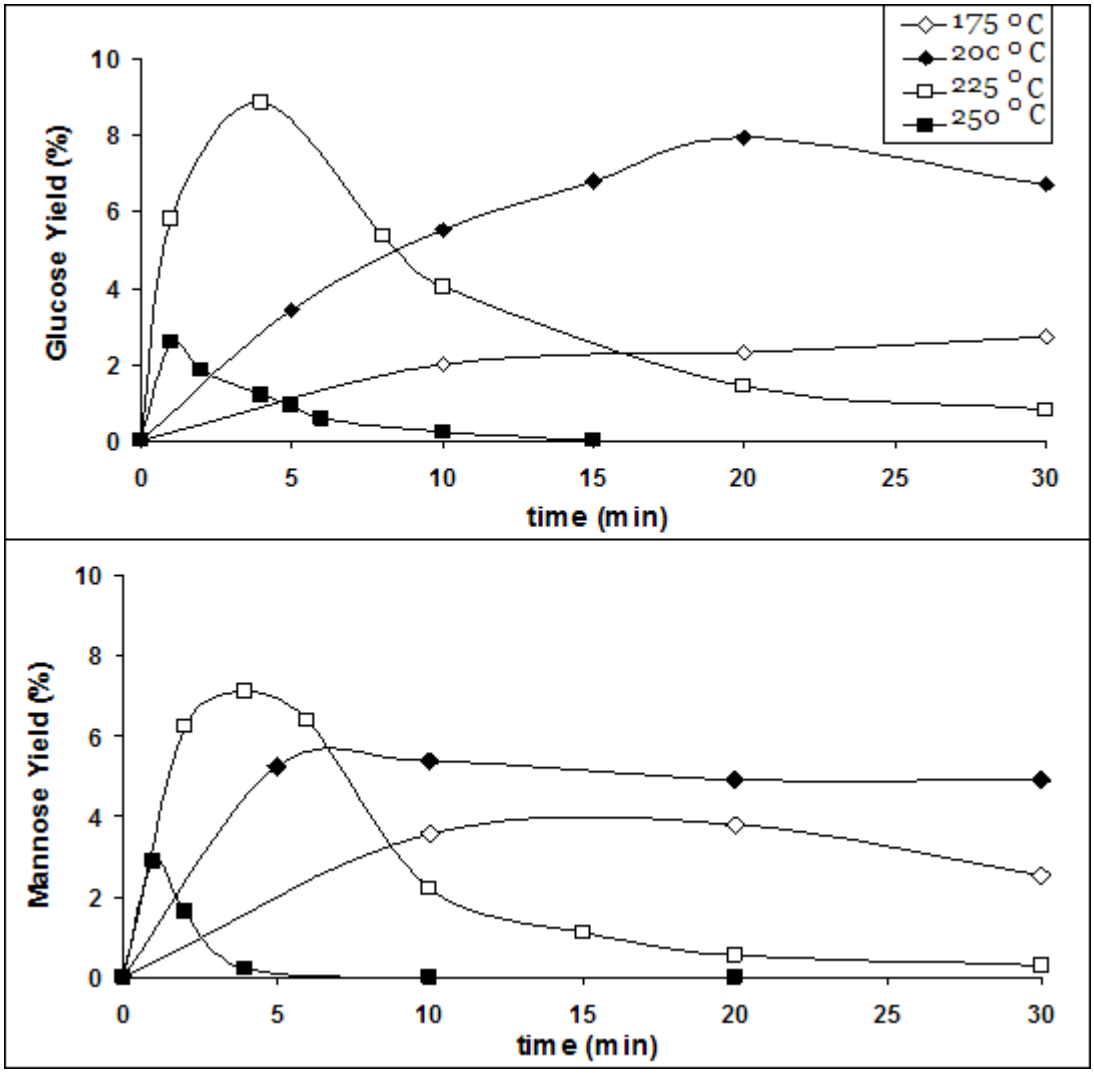


Figure 5. 7 Yield of monosaccharide release after the hydrolysis of the yeast cell wall at temperatures between 175°C and 250 °C. Yield was calculated based on the mole of monosaccharides in the liquid phase to the amount in the starting solids in feed

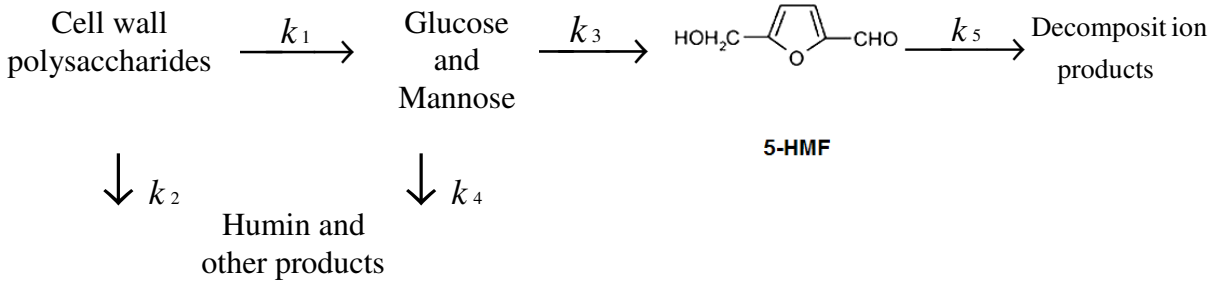
The conversion of glucose to HMF was also measured using the same HPLC method. The maximum concentration of HMF was observed after 10 minutes at 225 °C and was 21 mmole/litre. At higher temperatures (>225 °C), HMF appears to be decomposed to other compounds as previously reported by Girisuta et al. (Claude Moreau et al. 1996).

5.3.3 Reaction kinetics

In order to establish a clear understanding of the conversion rates of yeast cell wall polysaccharides to HMF and also to draw a comparison between yeast biomass and cellulose, reaction kinetics were studied and calculated.

Simple consecutive reactions have been widely employed to determine the rate constants for cellulose decomposition and product formation (Rogalinski, Ingram, et al. 2008; Rogalinski, K. Liu, et al. 2008; Jing & X. Lu 2008). Because of the similar molecular structure of the raw sample (β-glucan in yeast and cellulose), similar kinetics were also used to study the biomass decomposition in this work. 1-3-β-glucan and mannoproteins were considered to be the main substrates in the cell wall and their decomposition to glucose and mannose and further to HMF were the focus of this work.

All the reactions were assumed to be of first order and irreversible as the only reactant is biomass (and high concentration of water with constant concentration) (Rogalinski et al. 2008). The polysaccharides (β-glucan and mannoprotein) and monosaccharides (glucose and mannose) can also be converted to other by-products (humic, organic acids such as acetic acid, insoluble compounds) during the reaction. The reactions was assumed to follow the scheme 5.1.



Scheme 5.1. Yeast beta-glucan decomposition to the glucose and subsequent conversion

To solve the kinetic models and calculate the rate constants, a model was first proposed and unknown parameters were estimated based on the conversion data for each compound (glucose and HMF) as carried out by Jing *et al*. (Jing & X. Lu 2008). If $k_{\mathrm{d,CW}}$ (k_1+k_2) is the rate constant for the decomposition of the cell wall polysaccharides and $k_{\mathrm{d,MS}}$ (k_3+k_4) is for the decomposition of monosaccharides (glucose and mannose), equation (1), (2) and (3) denotes the kinetic equations for the decomposition of cell wall polysaccharides, monosaccharides and HMF respectively:

$$\frac{dC_{\mathrm{CW}}}{dt} = k_{d,\,CW} C_{\mathrm{CW}} \tag{1}$$

$$\frac{dC_{\mathrm{MS}}}{dt} = k_1 C_{d,\,CW} - k_{d,\,MS} C_{\mathrm{MS}} \tag{2}$$

$$\frac{dC_{HMF}}{dt} = k_5 C_{HMF} \tag{3}$$

Solutions of pure HMF and glucose were prepared separately and processed under the same conditions for hydrolysis experiments to measure the amount of conversion with time

for each compound. Using the conversion-time data and also calculating the analytical solution for equations (1) to (3), reaction rates were estimated and compared with the experimental data.

The rate constants were estimated using the data derived from a -ln (1-X) vs. time plot by calculating the slopes of straight lines (Fig. 5.7) and the values were summarized in Table 5.1. The fitted lines had all linear correlation of ≥ 0.93, except for data points at 250 °C where resulted in R-squared value of % 81. As temperature rises, the slope increases which is typical in the temperature related reactions. However, the slight disagreement between the lines and the experimental points at higher temperatures can be contributed to the technical limitation in temperature measurement and initial heating-up stage.

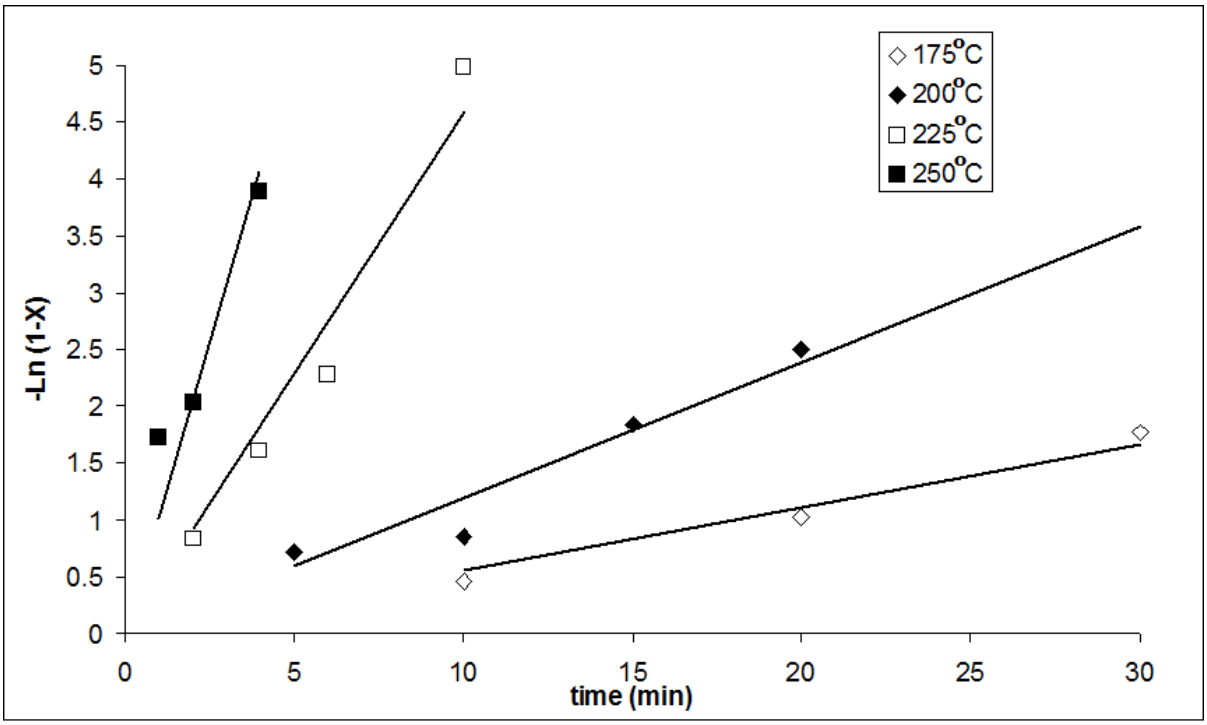


Figure 5. 8 Determination of the reaction rate constants for the hydrolytic degradation of yeast cell wall based on the data obtained from carbohydrate analysis

The measured values listed in table 5.1 were also used in the Fig 5.7 to obtain the solid line representing the kinetic model which is in good agreement with experimental values. Data obtained for cellulose and starch decomposition by Rogalinski and co-workers (Rogalinski, K. Liu, et al. 2008) in similar temperature range are provided to give a comparison of the rate constants for the hydrolysis of β-glucan, cellulose and starch.

Table 5. 1 Rate constants for the decomposition of the cell wall polysaccharides (minutes-1)

T (°C)	k_1	k_2	$k_{d,\ cell\ wall}$	$k_{d,\ cellulose}$*	$k_{d,\ Starch}$*
175	0.0047	0.0342	0.0389	n.d.	n.d.
200	0.0133	0.077	0.0903	n.d.	0.036
225	0.0629	0.2261	0.289	n.d.	0.114
250	0.0326	0.6652	0.698	0.15	0.606
275	n.d.	n.d.	n.d.	1.284	1.902

n.d.: not determined
*: The nearest temperature has been used if the data were not available for the indicated temperature (rates at 230 °C instead of 250 °C, 280°C instead of 275 °C)(Rogalinski, K. Liu, et al. 2008)

The values obtained for the cell wall reaction rate constants show higher values when compared to pure cellulose and starch, which is composed of the same glucose subunits. A plausible explanation is that the glycosidic bonds of the β-glucan, β-1-3 and β-1-6 linkages, which are abundant in the yeast cell wall and are easier to hydrolyze when compared to cellulose with β-1-4 linkages or starch with β-1-6-linkages. Milder hydrolysis condition for

β-1-3 glucan feedstocks was also observed in a study by Nagamori *et al*. where starch, with similar polysaccharide structure to the yeast cell wall, was hydrolysed in pure water (Nagamori & Funazukuri 2004). However, starch based feedstocks (mainly crops) are related to food chain supplies which limits their application for the production of bio-based chemicals.

In addition, it has been shown that the lower pH of the medium also promote the hydrolysis of the glucan polymers (Sasaki et al. 1998). Therefore, the presence of organic acids in the raw material and/or the organic acids produced during the reactions (autocatalysis) may also contribute to the higher rates of hydrolysis.

5.3.4 HMF synthesis from released monosaccharides

The conversion rates and the kinetics of HMF formation was also investigated which occur after the cell wall breakdown. In addition to the HMF pathway, there are a number of other reactions that originate from glucose and lead to other products ranging from different isomers of glucose and mannose, organic acids such as acetic acid, formic acid and etc) which have been discussed previously (Kabyemela et al. 1999; Knežević et al. 2009). When hexose molecule loses 2 molecules of H_2O, HMF would be formed, which can be converted further to other derivatives such as levulinic acid or formic acid at higher temperatures (Fig 5.5.c). In this study, however, the focus was on the synthesis and accumulation of HMF itself but other by products were detected at higher temperatures.

Fig. 5.8.a shows the yield of HMF at different reaction temperatures based on the moles of HMF produced and the glucose and mannose in the dried yeast at the start of the experiment. HMF started to accumulate at 175 °C and reached a maximum yield of 2% after 30 minutes. Yield increased at higher temperatures and reached more than 12 % at 250 °C.

At higher temperatures, the HMF yield decreases due to the decomposition to other organic acids. Due to the batch configuration it was not technically possible to measure the yield accurately at higher reaction rates at higher temperatures, because under these conditions the hydrolysis, dehydration and subsequent decomposition occurred during the initial heating-up stage.

Reaction rates for glucose conversion to HMF (HMF formation, k_3) and other soluble and insoluble products (k_4) and HMF decomposition (k_5) were calculated by using the same method (section 5.3.3) and compared with in Table 5.2. Comparing $k_{d,MS}$ and k_5 (rate constants for monosaccharides and HMF degradation respectively), the dehydration of sugars starts at low levels at 175 °C where HMF decomposition was negligible. At higher temperatures, the rate constant for HMF formation (k_3) increased while at the same time, it degrades at higher rates (k_5). However, the optimal temperature appears to be at 225 °C at which the HMF formation rate constant is higher than its decomposition reaction by 130%, resulting in the accumulation of HMF in the medium under these conditions. At higher temperatures, $k_{d,MS}$ and k_5 increased resulting in lower concentrations of HMF which were observed during very short reaction times (< 5 minutes). Based on the obtained data, the estimated rates for the synthesis of HMF are comparable to those experiments where cellulose was the substrate. However, it should be noted that the hydrolysis of cellulose requires higher temperatures (>250 °C) and this could lead to higher decomposition rates for HMF at such conditions (Jing & X. Lu 2008).

As described in previous section, the yeast cell wall was hydrolysed at higher rates when compared to cellulose-based biomass at the same temperature. For example, 90% of the yeast cell wall polysaccharides were solubilised after 15 minutes at 225°C. The same value was obtained after only 5 minutes at 250 °C which is comparable to lower rates for

cellulose (e.g. only 20% liquefaction of cellulose after 3 minutes at 240 °C (Rogalinski, K. Liu, et al. 2008)).

Initial heating up stage (induction time) is common to all batch reaction studies and its impact can be minimized by improving the heat transfer rate in the reactor. In batch configurations, one option could be the use of more novel heating techniques such as induction heating (Tsai et al. 2006). Due to the technical limitation in this research, minimising the volume of the reactor to 4 ml tube reactor was the only option to reduce the heating up time.

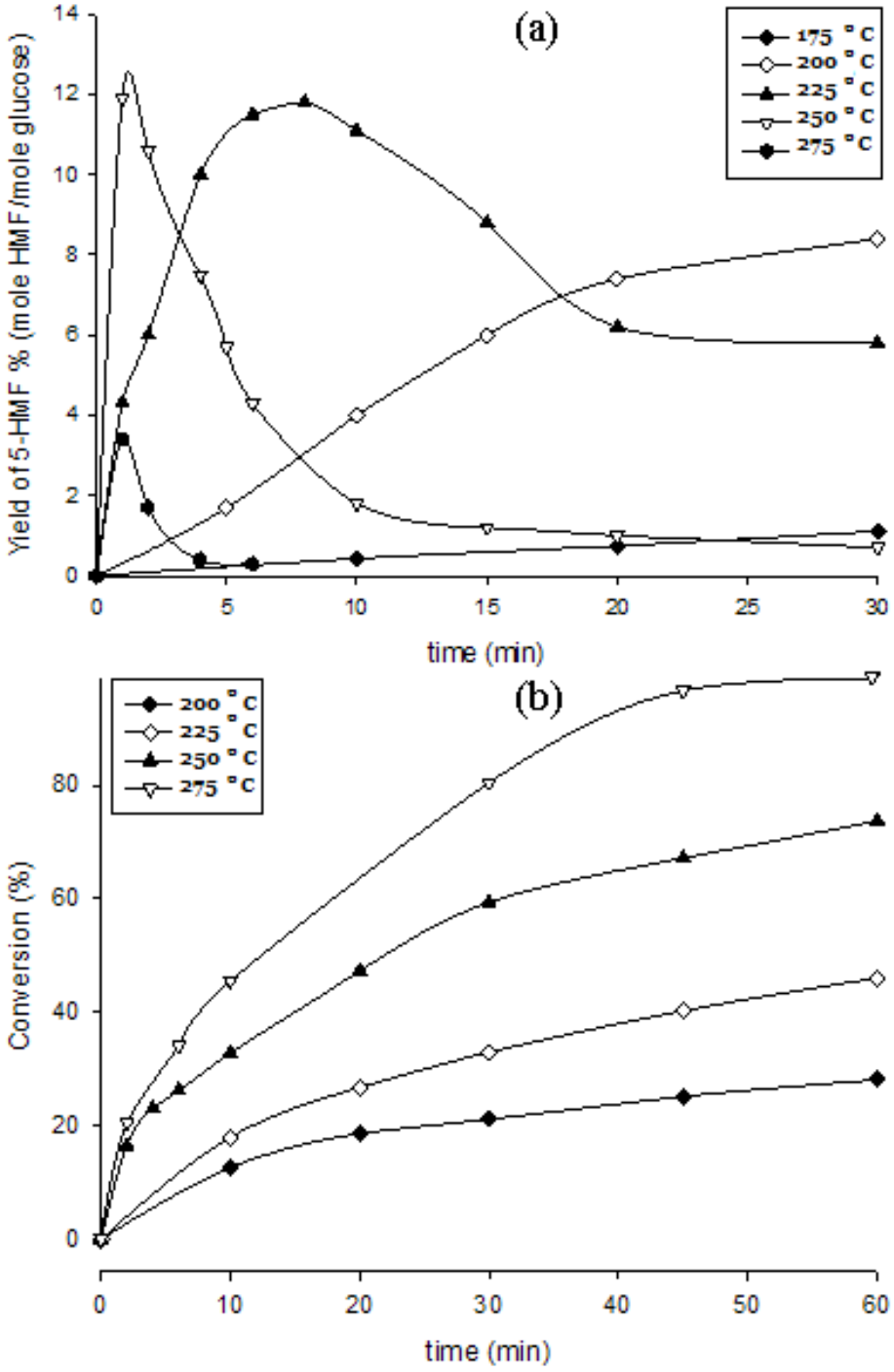


Figure 5. 9 (a) Yield of monosaccharides due to the yeast cell wall hydrolysis at temperatures between 175°C and 250 °C. Yield was calculated based on the mole of monosaccharides in the liquid phase to the amount in the starting biomass (b) β-glucan conversion at different temperatures and times (solid line are connecting lines)

In summary, it is possible to explain the observed difference between yeast cell wall and cellulose due to the more flexible structure of β-glucan and mannoprotein found in the yeast, the lack of lignin and the possible contribution of the unrefined medium on the hydrolysis/catalytic reaction.

Table 5. 2 Rate constants for glucose (and mannose) and HMF decomposition (minutes-1)

T (°C)	k_3	k_4	$k_{d,glucose}$	$k_{5(HMF,\ d)}$
175	0.0002	0.0076	0.0078	n.d.
200	0.0028	0.0391	0.0419	0.0037
225	0.0196	0.0994	0.119	0.0083
250	0.0033	0.2927	0.296	0.0202
275	n.d.	n.d.	n.d.	0.05

n.d.: not determined

5.4. Conclusion

The aim of this chapter was to demonstrate the potentials of using alternative sources of biomass to generate sugars and sugar hydrolysis products, such as HMF. Yeast biomass was composed of two polymers which were converted to monosaccharides when were subjected to hydrolysis process. It was also shown that sub-critical water can be used to hydrolyse the glucan and mannoproteins structure of the yeast cell wall into its monomers under comparatively milder operating conditions when compared to conditions used to hydrolyse lignocellulosic biomass. 90% conversion of cell wall biomass was achieved only after 15 minutes at 225 °C and less than 5 minutes at 250 °C. This is a result of more flexible structure of β-glucan in yeast, presence of no lignin and the possible contribution of the

unrefined medium to the hydrolysis reaction. The lower temperature for the production of sugars from biomass offer better conditions to target sugars as well as HMF before they are being degraded to secondary or tertiary by-products at higher temperatures. Higher reaction temperatures leaded to the increased levels of HMF and yields of more than 20% were achieved at 225 °C. This is comparable to the higher temperatures in cellulosic biomass pretreatment which is required to access to the monomers of cellulosic biomass; thus suggesting a lower temperature process that can be employed in biomass conversion to renewable chemicals. The higher reaction temperatures for lignocellulosic biomass would lead to the increased formation of secondary and tertiary reactions and lower yields of intermediate products such as HMF.

While the yields of HMF obtained with cider yeast (12%) are lower compared to the 40-60% obtained with pure substrate and catalysts (Qi *et al* 2008), an important aim of this study was to include any positive catalytic effects that may exist in this crude extract, alleviating the use of additional catalysts. However, it is believed that slight decrease of the medium pH by adding some acid can improve the yields for the HMF synthesis. Eliminating the use of any auxiliary organic solvent during the reaction was also a target that was tried to address, which may have resulted in lower yields in our single phase (aqueous) medium.

Considering other potential products that may be present in such feedstocks (yeast components such as amino acids or vitamins, or compounds that originate from apple), this processes can establish a platform for both extraction and synthesize of valuable compounds by integrating sequential batches operating at optimum conditions that is designed for each product/fraction. Therefore, in summary our results indicate the feasibility of using benign solvents to add value to organic waste by generating multiple revenues for the cider, brewing and ethanol industries that produce large volumes of spent yeast waste annually.

In terms of waste treatment and disposal issues, the studied process can be a step forward for the brewery industries to introduce an alternative approach for the large volumes of waste they produce annually Biomass transformation can be a solution to these concerns as it has been suggested for similar feeds previously (Lamoolphak et al. 2007; Ferreira et al. 2010). Authors were not able to identify any similar work which had suggested an alternative approach for cider lees waste. This can be due to its different composition such as the acidic medium, different species of yeast (in this case, *S. bayanous* in cider fermentation instead *of S. ceravisiae* in beer fermentation) or the existence of other microorganisms or undesired compounds.

Furthermore, the obtained yields should be viewed within a potential integrated biorefinery context where an organic waste material is cascaded into multiple products of which HMF is but one. In particular, similar waste is generated in bioethanol fermentation waste (the energy section of most biorefineries) which this study can propose a solution for their application. This approach can lead to further improvements in bioeconomy merits of current biorefinery technology by fully capturing the chemical and energy of potentials of biomass and secondary yeast wastes. However, more concentrated feed (in terms of yeast concentration) is necessary in order to find out how this process operates in order to perform the economic analysis. In a techno-economic study by Kazi et al (Kazi et al. 2011), HMF production from pure fructose was studied in a catalytic reaction and it was showed that feedstock price accounts for 48% of the final cost of the HMF ($1.33/lit). In comparison, the suggested process in this study have access to a very low cost feedstock which can improve the feasibility of the process.

Chapter 6

Cider Spent Yeast and the Phenolics: The Release of Phenolics Using Subcritical Water

6.1 Introduction

Apple cider is known to contain significant amounts of phenolics which are originated from apple fruits and these phenolics largely contribute to its final nutritional values. The major part of these soluble materials, except sugar which has been consumed during the fermentation, are still present in the cider lees. Preliminary results, which were presented in chapter 3 showed that the crude sample contains some phenolics which its concentration increases significantly after running the subcritical water hydrolysis. These compounds are believed to be the main cause of antioxidant activity in the extracts highlighting their role in the final properties of extracts. The phenolic concentration profile against temperature showed an increasing trend to temperatures up to 225 °C while it decreased at higher temperatures. This pattern proposes that these compounds are either had been produced or most probably had been released from yeast cells as will be discussed in this chapter.

As discussed earlier in chapter 2, yeast cells have been shown to interact and adsorb the available phenolics in wine process during the fermentation step by adsorbing them on their outer surface. This interaction has been studied specially in the wine making process as it can change the final properties of wine by removing certain phenolics from the product (e.g. anthocyanidins) and change the properties of the final product (such as colour). While the same interaction should occur in the cider process, this have not been addressed before which could be due to the its negligible effect on the final properties of cider.

This chapter discusses more detailed experiments which carried out to investigate the nature of phenolic fraction in the hydrothermal treated extracts and their adsorption and comparing their release from the yeast cells using both organic solvent extractions and subcritical water treatment. In these experiments, the same batch stirred reactor (which was described in Chapter 3) was used at temperatures between 100°C and 250 °C and 10 MPa pressure. In order to measure only the phenolics which have been released during the desorption process, yeast cells were separated from the original slurry after centrifugation, washed and re-suspended in distilled water. HPLC analysis of the extracts was also carried out and the chlorogenic acid level was shown to increase following the same trend which confirms its prior adsorption on the yeast cell wall and its release due to being subjected to subcritical water. UV-spectrum of the HPLC column eluent (which recorded by Diode Array Detector) also indicated a significant change in the composition of the extracts at higher temperatures.

6.2 Materials and Methods

6.2.1 Raw Material

Spent cider lees was collected from cider manufacturer site (Bulmer, Herefordshire, UK), stored in -20 °C freezer and used as described before in section 3.2.1. Total solid concentration in the slurry was 12% (w/v) and the total concentration of the yeast cells were determined by filter paper (Whatman, grade No. 1) and was 10% (w/v). To prepare washed yeast cells, crude sample was first centrifuged at 5000 g for 10 minutes to separate the yeast cells from the liquid fraction. Yeast cells were washed three times by repetitive re-suspension in distilled water and centrifugation using the same speed. In the final step, yeast residues were suspended in distilled water at a concentration of 10 % (w/v) and used for the experiments. Folin-Ciocalteau reagent (2 N solution), sodium bicarbonate (≥ 98.0%) and gallic acid (97.5-102.5%) which were used for total phenolic measurements were all purchased from Sigma-Aldrich, United Kingdom. Standard phenolics were including (with purity grade): chlorogenic acid (≥ 95%), epicatechin (≥ 98%), *p*-coumaric acid (≥ 98.0%), phloridzin dehydrate (≥ 99%), quercetin glucoside (≥ 90%) and caffeic acid (≥98.0%) were also purchased from Sigma-Aldrich, United Kingdom.

In order to eliminate the interference of the suspended phenolics in the substrate, 3 times washed cells were used in subcritical water reactions instead of original substrate. The washed cells were prepared by consecutive centrifugation and re-suspension of the cells in distilled water until the supernatant became clear. In the final step, cells were suspended in distilled water in the same weight ratio as original sample.

6.2.2 Reactions in Subcritical water

For subcritical water reactions, the same setup and process which described earlier in Chapter 3 was used. Briefly, 200 ml of washed cider spent yeast was used for each experiment in the 300 ml high pressure stirred reactor where it was heated using an electrical heater at temperatures between 100 and 250 °C. Stirrer speed was set to 500 rpm and the reactor was pressurized with Nitrogen gas at 10 MPa to maintain the contents in the liquid phase. Cooling water was circulated through a pre-installed coil to lower the reactor temperature at the end of each run and before opening the reactor. To remove the effect of heating-up stage (which described in chapter 5), a sample was taken when the reactor reached the set temperature (t= 0 minutes). Samples were collected from the reactor using a cooled sampling valve at different times and centrifuged at 3500 g (Beckman,US) for 10 minutes after quenched in a mixture of ice and water. The supernatants were stored at-20°C freezer and defrosted before being analysed. Supernatants were directly subjected to total phenolics assays (Folin-Ciocalteu assay) and HPLC analysis without any pretreatment.

6.2.3. Phenolics extraction with organic solution

The cells which were washed three times with distilled water were submitted to repetitive extraction using a solution of Acetone:Water:HCl (70:29:1) (Fig 6.1). This solution was selected as it had been used previously to remove the phenolics from wine lees (Mazauric & J.-M. Salmon 2006). Using this method, the phenolic compounds that had been adsorbed on the cider yeast during the fermentation were expected to be extracted. Measuring the TPC in these extracts shows the total content of phenolics that can be released and facilitates the comparison between organic solvent extraction and subcritical water treatment.

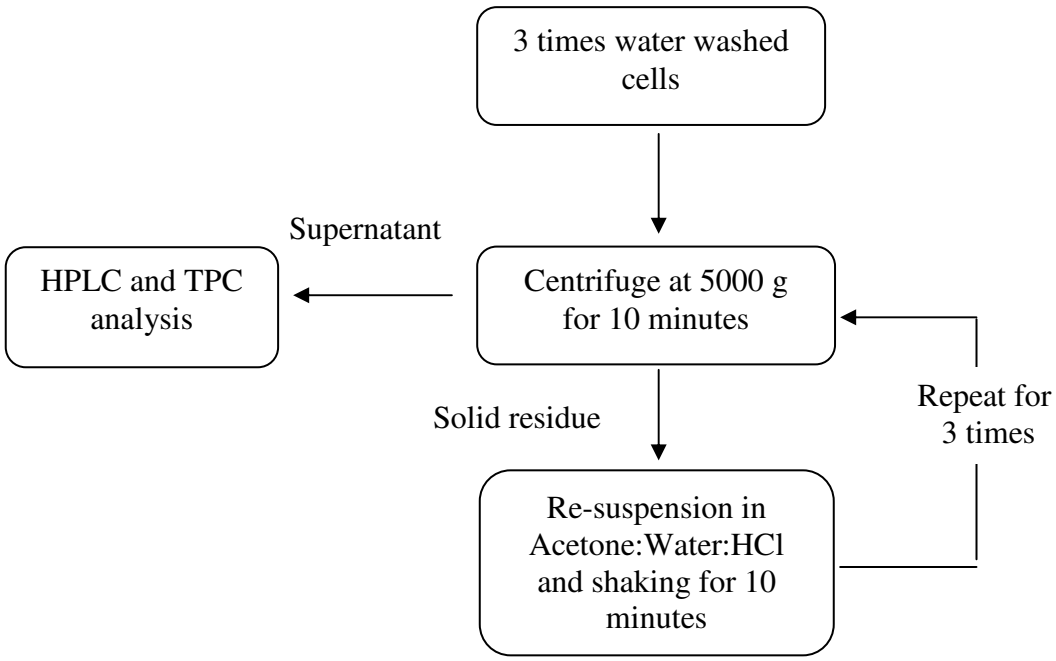


Figure 6. 1 Extraction scheme using a solution of Acetone:Water:HCl (70:29:1) to release the desorbed phenolic compounds from yeast cells

To achieve this, washed cells were first centrifuged at 5000g for 10 minutes to harvest the cells from the suspension and the precipitated cells were re-suspended in the solvent mixture. After 10 minutes of mixing in a shaker at room temperature, the slurries were centrifuged and the cells were re-suspended in fresh solvent again. This was repeated for three times until the supernatant became clear. Extractions were carried out in four solid ratios (10%, 7.5 %, 5% and 2.5 % w/v) to measure the extraction power at different solid concentrations.

6.2.4 Total phenolic content (TPC) assay

6.2.4.1 An Introduction to Folin-Ciocalteau (FC) Assay

This method is a well-known assay which introduced in chapter 3 in detail. It has been widely reported as the method of choice in analysis of numerous biological and non-biological samples. However, the main drawback of this assay is the potential interference of non-phenolic materials such as proteins, reducing sugars, ascorbic acid, SO_2 and sorbic acid which are largely distributed in wine (Wrolstad et al. 2001). The main reason is shown to be the high reactivity of Folin-Ciocalteu reagent that oxidizes other compounds. This may lead to overestimation of phenolics in the studied extracts and hence, suitable removal procedures must be applied in order to consider any error. This problem can be partially avoided by adding acetaldehyde (to bind the bisulphate) or apply the correction factors (Waterhouse, 2005). As an example, Escarpa and Gonzalez addressed the interference problem in green bean samples and suggested the use of HPLC as a tool to measure the total phenolics in addition to Folin-Ciocalteau assay (Escarpa & González 2001).

6.2.4.2 Folin-Ciocalteau Assay-The method

To measure the total phenolic content of the crude sample and the obtained extracts, the Folin-Ciocalteau assay was used following a modified version of the original method (Singleton & Rossi 1965) and results were reported as grams of gallic acid equivalent (GAE). The actual method used for the assay was earlier discussed in section 3.2.8 based on the micro method which was developed by University of Davis, California, US (Waterhouse, 2005) and is described below:

Gallic Acid Stock Solution: In a 100-mL volumetric flask, 0.500 g of dry gallic acid was dissolved in 10 ml of ethanol and was diluted to volume with distilled water. The flask was kept closed in a refrigerator and fresh solution was prepared every two weeks.

Sodium Carbonate Solution: 20 g of anhydrous sodium carbonate was dissolved in 80 ml of distilled water and heated until boiling. After cooling, a few crystals of sodium carbonate was added and left at room temperature for 24 hr. The solution was filtered with filter paper (Grade 1, Whatman, UK) and diluted to the volume of 100 ml with distilled water.

Calibration curve: To prepare a calibration curve, 0, 1, 2, 3, 5, and 10 ml of the prepared phenol stock solution was added into a 100 ml volumetric flasks, and then was diluted to volume with distilled water. These solutions had the phenol concentrations of 0, 50, 100, 150, 250, and 500 mg/L gallic acid, the effective range of the assay and used to prepare the calibration curve (Fig 6.2).

Measurement: From each calibration solution, sample, or blank, 20 µl was added into separate cuvettes and diluted with 1.58 ml water. 100 µl of the Folin-Ciocalteu reagent was also added and the solution was mixed well. After 2 minutes, 300 µl of the sodium carbonate

solution was added and mixed. Solutions were left at room temperature for 2 hr and the absorbance of each solution was determined at 765 nm against the blank (the "0 ml" solution). The absorbance vs. concentration plot was prepared (calibration curve) and used to calculate the unknown amount of phenolics in the extracts for each new set of measurement.

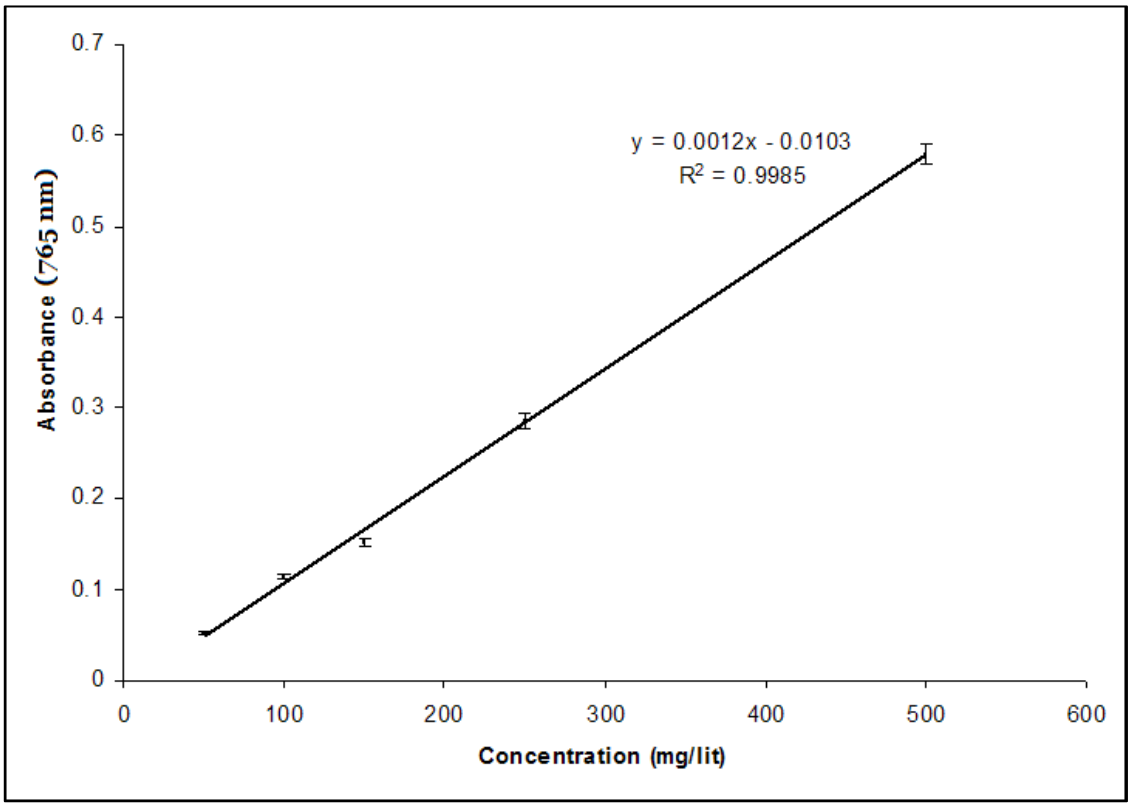


Figure 6. 2. The calibration curve in the Folin-Ciocalteu assay (absorbance measured at 765 nm and 3 replicates were prepared for each point)

Samples were collected from the reactor and the solids were separated by centrifugation at 3500 g for 10 minutes and the supernatants were used for the analysis.

6.2.4.3 Protein interference in Folin-Ciocalteau assay

As described in result section 3.3.6, the hydrolysed samples were showed to contain soluble proteins which may interfere in the FC assay (Escarpa & González 2001). This may apply to samples which were prepared at temperatures between 150 and 200 °C as the protein quantity was higher compared to other temperatures. To study the possible interference of proteins, a protein precipitation technique was used to remove the proteins from the samples and the resulting supernatant was checked with FC-assay again to find out if there is any variation in the reported values of phenolics in samples containing proteins and the ones which proteins were removed.

6.2.4.4 TCA protein precipitation protocol

Protein precipitation using mineral acids is based on changing the medium pH to get closer to the pI (isoelectric point) of the proteins which is the pH at which the net protein charge becomes zero. Protein aggregation occurs in these conditions which facilitates their removal using centrifuge. In TCA protocol, 1 volume of Trichloroacetic acid solution (100% w/v) is added to 4 volumes of the sample and the mixture is incubated at 4°C for 10 minutes followed by centrifugation at 14,000 rpm for 5 minutes (Sanchez 2001). The protein pellet was formed as the precipitate leaving behind the non-protein components in the solution. After removing the proteins from all the hydrolysed extracts that prepared at different temperatures, no change in phenolic concentration was observed before and after the protein removal step suggesting that there is no protein interference in the FC assay.

Figure 6.3 shows the comparison between samples' total phenolic concentration before and after protein removal at selected conditions which showed maximum protein concentration.

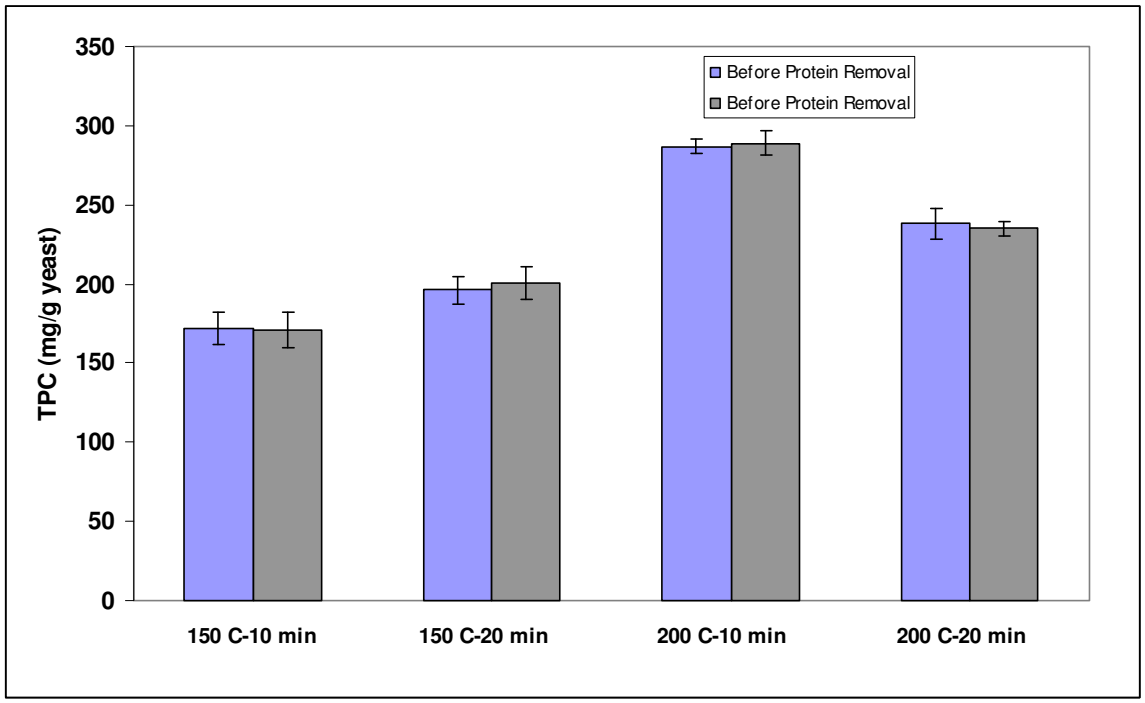


Figure 6. 3. Total phenolic concentration before and after protein removal (n=3)

6.2.5 HPLC with Diode Array Detection (DAD)

To separate and determine individual phenolics in the extracts, samples from hydrothermal treatment process and organic solvent extraction were analyzed with the discussed RP-HPLC technique. Pure phenolics were used to investigate the efficiency of the developed method. Phenolics (Chlorogenic acid, *p*-coumaric acid, caffeic acid, phloridzin dihydrate, epicatechin, quercetin glucoside and phloretin) were purchased from Sigma-Aldrich, UK and 1 mg/ml solution of each standard was prepared in methanol (HPLC grade, Sigma-Aldrich, UK). The HPLC system was comprised of an Agilent 1100 HPLC system (Agilent, USA) and was comprised of an auto-sampler, high pressure quaternary pump,

online degasser, column oven and diode-array detector (Agilent, USA). The chromatograms and spectrum data was collected with ChemStation® software (Version B.01.02) which was provided from Agilent to control and carry on the measurements on their LC system.

The HPLC column was a 4 micron Synergi-Fusion (250 × 4.6 mm) column (Phenomenex®, USA) which was equipped with a column guard with the same stationary phase (4 × 2.0 mm). To provide a better separation (resolution) of the studied phenolics, a new method was developed in our laboratory based on using varying ratios of aqueous and organic solvents in mobile phase throughout each analysis (more discussion in section 6.3.1). Two mobile phase were prepared for the method: First, the aqueous phase (solvent A) which was HPLC-grade water (Sigma Aldrich, UK) with 0.1% formic acid (pH=3.6) and second, the organic phase (solvent B) which was HPLC- grade methanol (Sigma Aldrich, UK) with 0.1% formic acid (HPLC grade-Sigma Aldrich) (pH=4.2) and were used as follows (flow rate was constant at 1 ml/minutes): Pure A for the first 5 minutes, 0-20% B linear from 5 to 15 minutes, 20-45% B from 15 to 35 minutes, 45-60% from 35 to 45 minutes and back to 0% B from 50 to 52 minutes. The method was validated by running a mixture of 6 phenolics which were expected to be present in our substrate and checking the separation of peaks. Diode-array detector provided the spectrum of the column eluent at wavelengths of 200-600 nm between 0-54 minutes of elution from the HPLC column. Based on the previous studies about phenolics in cider apples (B. Suárez et al. 2005), phenolic standards were purchased and used to prepare standard solution of each compound. By injecting the standards and comparing their retention time with the peaks at same retention time in the crude sample and extracts, available phenolics were identified.

6.3 Results

6.3.1 RP-HPLC Method Development and Determination of Phenolics

In order to identify and quantify the individual phenolic acids, a new HPLC technique was developed based on the elution profile of different compounds in the reversed phase HPLC. A mixture of phenolic compounds where prepared as standard sample to evaluate the elution of different phenolic compounds. The major phenolic compounds which are expected to be present in apple were identified and were purchased (Sigma-Aldrich, UK).

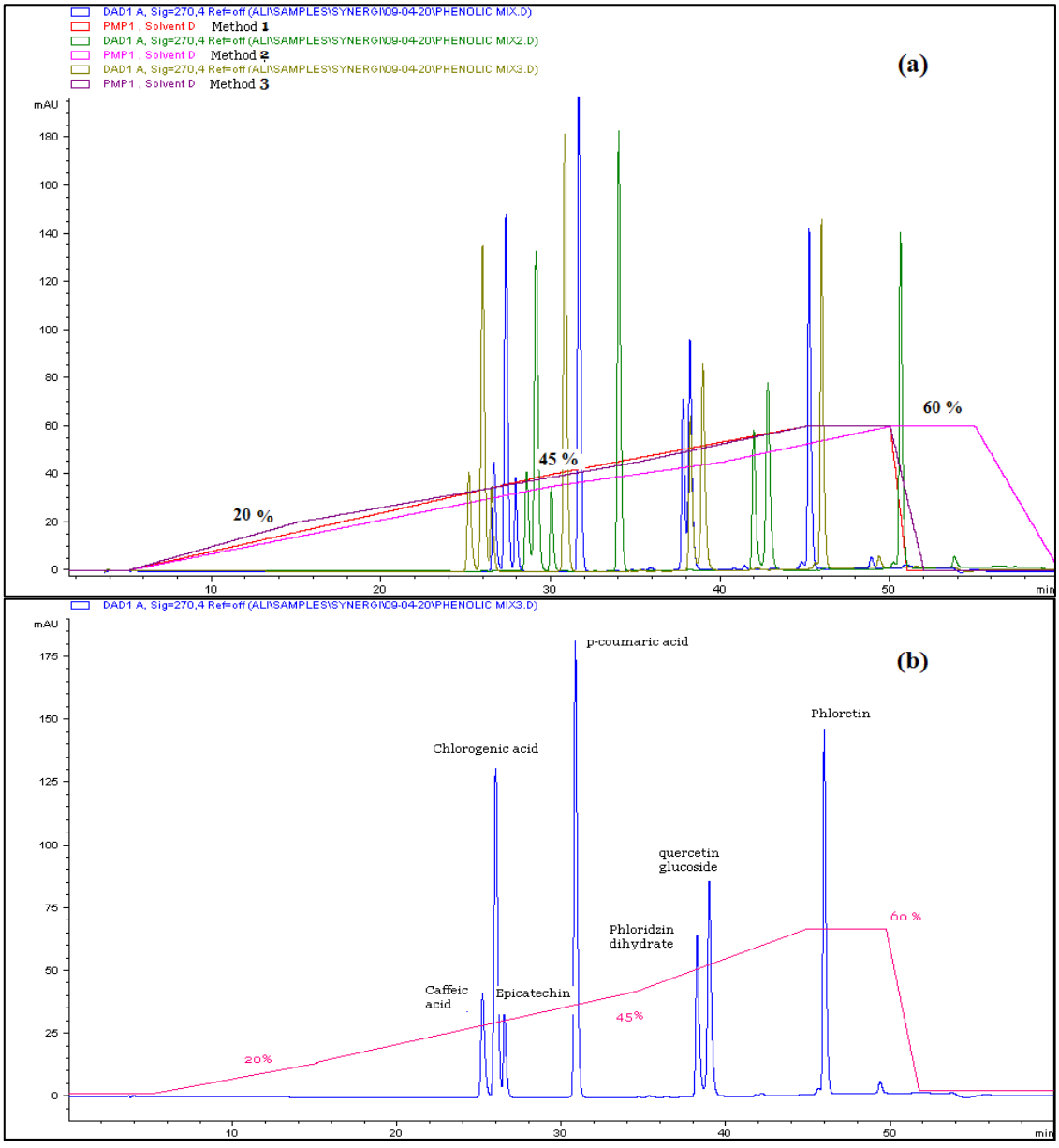


Figure 6. 4. Method development and the chromatogram of phenolic mixtures at different organic phase ratio (a) and the optimized final method which used for the study (b)

The final standard mixture was a solution of 1 mg/ml of following phenolics in HPLC water (Chlorogenic acid, *p*-coumaric acid, caffeic acid, phloridzin dihydrate, epicatechin and quercetin glucoside). For the mobile phase, two main solvents were used including acidified HPLC water (0.1 % formic acid) and acidified HPLC methanol (0.1% formic acid) and were used in different ratios to obtain the best separation of resulting peaks. Typically, analysis started with highest ratios of aqueous phase. In this work, the HPLC method was started with 100% water as a Synergi-Fusion column was used which is a modified reversed phase column with enhanced separation for very polar compounds that elute very fast in a reversed phase column. After 5 minutes of running the column with pure water after injection, a gradient of organic solvent introduced which its ratios changed during the run. Three different ratios of 20 %, 40% and 60% was used. For the detection, a DAD detector was used at 270 nm and flow rate was set to 1 ml/min. Table 6.1 listed the condition used in 3 separate methods developed during this step and Table 6.2 shows the retention time of each phenolic standard in each method. Method 3 was selected as the running method as it provided better separation for all the phenolic in shorter time.

Table 6. 1 Description of gradient elutions used in method development

Method 1		**Method 2**		**Method 3**	
Time (min)	**Organic phase (B) ratio (%)**	**Time (min)**	**Organic phase (B) ratio (%)**	**Time (min)**	**Organic phase (B) ratio (%)**
0-5	0	0-5	0	0-5	0
5-30	45	5-30	40	5-15	20
30-45	60	30-40	45	15-35	45
45-50	60	40-50	60	35-45	60
50-51	0	55-60	0	50-52	0

Table 6. 2 Retention time(Rt) of the phenolic peaks in different methods

Phenolic compound	Rt(min) Method 1	Rt(min) Method 2	Rt(min) Method 3
Caffeic acid	26.5	28.6	25.1
Chlorogenic acid	27.2	29.3	26
Epicatechin	28	30	26.5
p-Coumaric acid	31.9	34	31
Phloridzin dihydrate	38.9	42	38.1
Quercetin glucoside	39.1	42.8	39.3
Phloretin	45.6	50.7	46

Figure 6.4(a) shows an overlay of phenolic chromatograms in different gradient systems (straight lines with the indicated ratios of methanol). As the chromatogram suggests, the ratios of organic phase and its duration has a significant effect on the separation of phenolic compounds. The optimized method (method 3) was selected and used throughout the phenolic study to determine and measure the individual phenolics (Figure 6.4(b)). To provide some comparison, figure 6.4 shows the chromatograms of the crude substrate (a) and the extract at 225 °C, 15 min and 100 bar (b). Only 5 of the 7 standard phenolic were detected in the studied lees samples (section 6.3.3). As can be seen from fig 6.5 , the major phenolic also break down at 200 °C

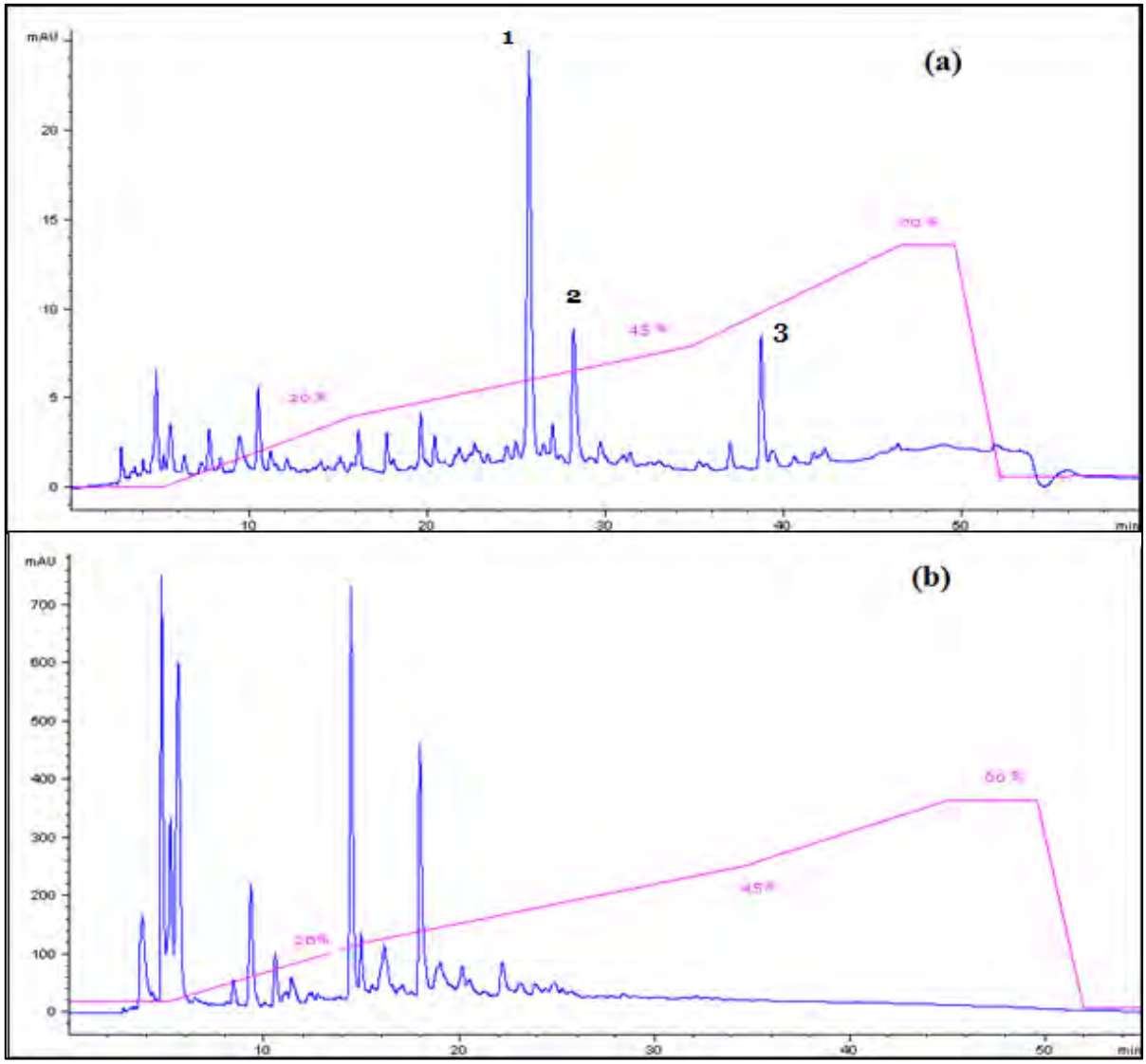


Figure 6. 5. Chromatogram of the crude substrate (a) and the subcritical water extract at 225 °C and 15 minutes and breakdown of major detected phenolic compounds (phenolic peaks were 1-Chlorogenic acid, 2- *p*-Coumaric acid and 3-Phloridzin dihydrate)

6.3.1 Phenolic desorption using subcritical water

Figure 6.6 shows the concentration of total phenolics (in terms of mg of equivalent gallic acid per g of yeast) in the washed cells after being subjected to subcritical water. Concentrations were divided by the dry weight of yeast cells in the starting substrate to better represent the relation between phenolic content and the yeast cells concentration. In addition, this makes the comparison between subcritical water and organic solvent extraction more convenient. The suspended (or measurable) total phenolic content of the washed cells was also measured and was zero which confirms that no phenolic is present in the liquid phase before the reaction. Time zero is for the samples which were taken from the reactor after it reached to the set temperature.

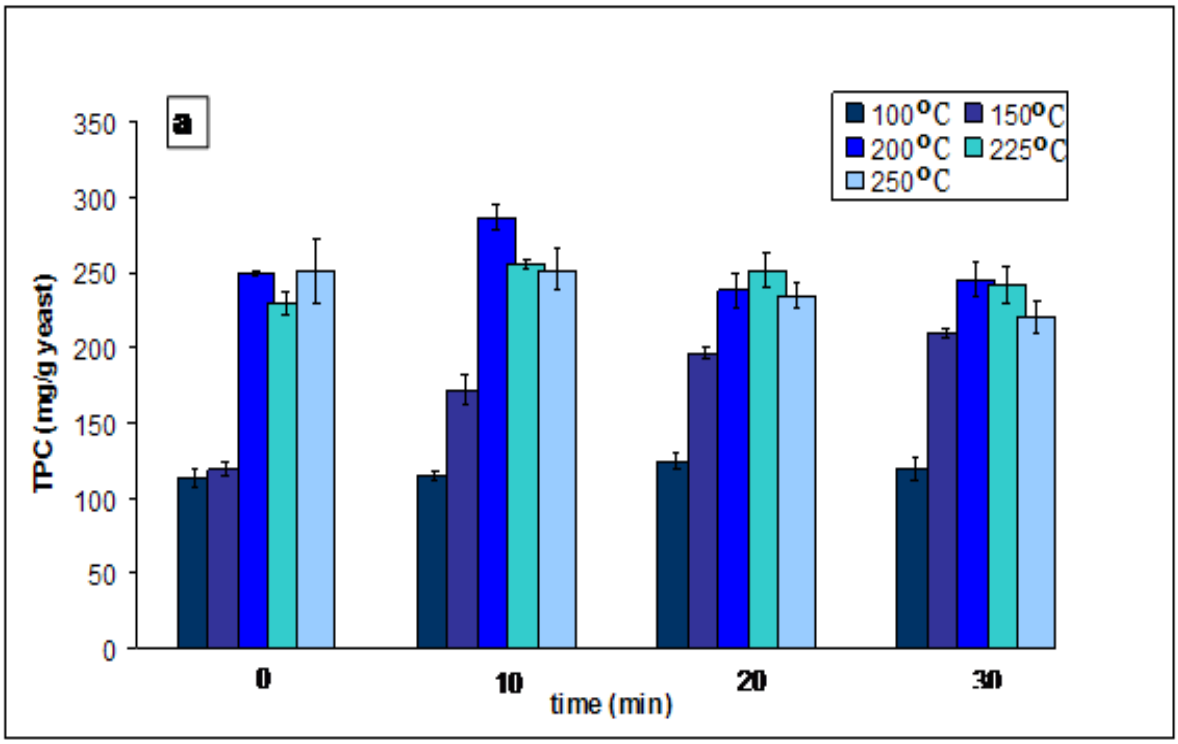


Figure. 6. 6. Total phenolic content (TPC) of cider yeast released through hydrothermal treatment process. Error bars represent standard deviation (n=3).

Comparing the TPC content of the extract to the reaction temperature suggest that increasing the temperature has improved the yield of extraction. Residence time, however, is only important at temperatures below 200 °C as TPC increased at longer residence times. At higher temperatures (225 and 250 °C), it seems that phenolic desorption had long been begun during the heating up stage suggesting the high rate of desorption and consecutive degradation at these temperatures while the effect of time becomes minimum at these conditions.

The value of TPC in the starting sample (the original substrate before any treatment) was 58 mg/g of dry yeast. Higher concentrations of phenolics in the extracts suggest that most of the phenolics were previously adsorbed (more than 3 times) comparing to original substrate (cider lees), and shows the strong adsorption of phenolic compounds with the yeast cells.

The results from these experiments clearly show the accumulation of phenolic compounds in the liquid phase. The desorption could be due to the cleavage of the bonds which had been formed between the phenolic compounds and the polysaccharide or proteins in the cell wall of yeast.

Results also suggest that the total phenolic concentration increases significantly with increasing temperature. TPC was negatively affected at temperatures higher than 200 °C due to higher rates of phenolic decomposition. The total phenolic concentration was reached to a maximum of 280 mg/g of dry yeast (3441 mg of gallic acid/ml) after 10 minutes at 200 °C.

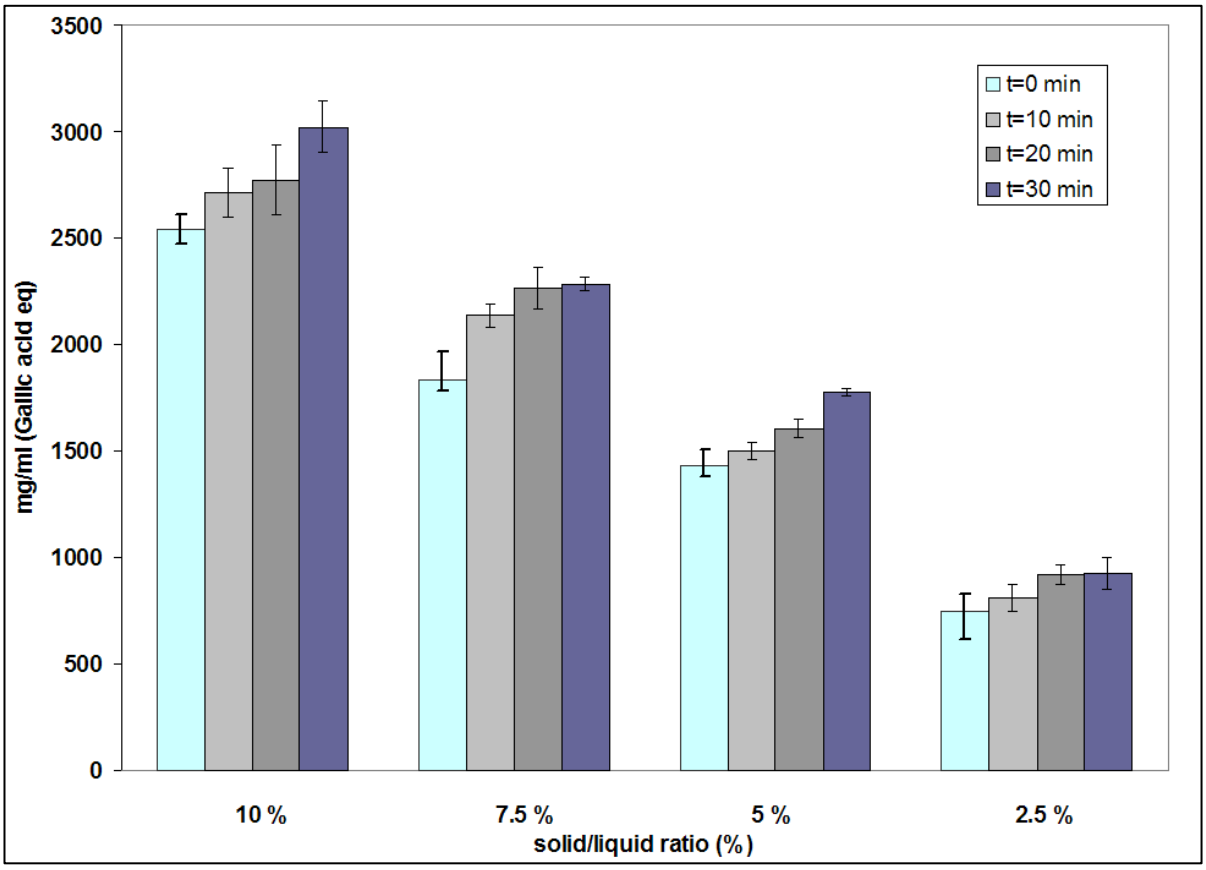


Figure 6. 7 Total phenolic contents of yeast extracts at different times after being treated at 200 °C in different solid concentration (P=100 bar)

The hydrolysis experiments were also carried out using different concentrations of washed cells in the medium, in order to identify any possible correlation between the solid and solvent ratios and the amount of released phenolics from the cells (Fig 6.7). These experiments were only performed in 200 °C. As indicated in Fig. 6.7, the concentration of released phenolics changed in almost the same order as the solid concentration in the liquid phase. In other terms, the desorption rate was not effected in the maximum studied solid concentration.

6.3.2 Solvent extraction

Subjecting the washed cells to the organic solution showed a similar effect on releasing the phenolics from biomass. Extracts became yellow-brown which is believed to be due to the presence of chromatic phenolic compounds. Extracts also became clearer after repeating the extraction. Extraction step was repeated for 3 times and as Figure 6.8 suggests, more than 30% of the phenolics were extracted in the 2nd and 3rd step together. The effect of solid ratio and organic solvent was studied using one-way ANOVA (p>0.05) (Microsoft Excel 2003, statistical analysis functions) which suggested that it was negligible (Table 6.3)

Table 6. 3. Details of one-way ANOVA to investigate correlation of solid ratio and solvent extraction

Anova: Single Factor -Solvent Extraction

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
2.5	3	722.3466	240.7821973	623.8366
5	3	597.9275	199.3091573	5.431791
7.5	3	664.8718	221.6239467	327.3151
10	3	706.9535	235.65116	121.0859

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3096.625	3	1032.208214	3.831261	0.04714	4.066181
Within Groups	2155.339	8	269.4173489			
Total	5251.963	11				

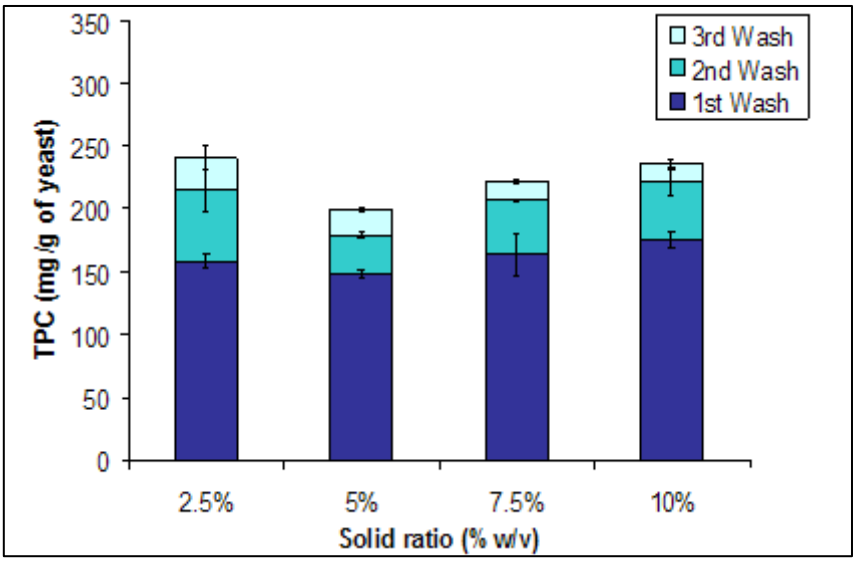


Figure 6. 8 Total phenolic content (per g of yeast) of washed cells being extracted with organic solvent in a 3-step solvent wash process. Error bars represent standard deviation (n=3).

Using organic solution, the total amount of released phenolics was between 200-240 mg/g of dry yeast and was lower to what was observed in hydrothermal treatment experiments at higher temperatures (270 mg/g of yeast at 200 °C). The higher phenolic concentration in the hydrolysed samples could be caused by the parallel or secondary reactions that have resulted in further amounts of phenolic compounds. As discussed in Chapter 3, the hydrolysed samples contain proteins which have been released to the medium after yeast cell breakage.

6.3.3 HPLC analysis- Single wavelength (270 nm)

Figure 6.8 compares the chromatograms of the yeast samples before and after being treated with subcritical water at 100 °C, 150°C and 200°C for 30 minutes. Five phenolic compounds were identified in the original substrate as chlorogenic acid, *p*-coumaric acid, phloridzin dihydrate, caffeic acid and epicatechin (in order of the peak area respectively). These phenolics were detected by injecting the solution of standard phenolics and comparing the obtained retention times of the standard peaks with the peaks which were detected in

extracts at the same retention time. In addition, DAD analysis provided a complimentary UV-spectrum of peaks, helping to confirm their specific structure.

The detected phenolic compounds were eluted at longer elution time and start to appear after introducing the organic mobile phase solvent (Methanol) to the system (Fig 6.9). UV-detector recording at 270 nm of the raw sample indicates the main phenolic compounds (chlorogenic acid, caffeic acid, epicatechin, *p*-coumaric acid and phloridzin dihydrate) which were identified. Chlorogenic acid was the most concentrated compound (Rt=24 min) with the concentration of 146 µg/ml in the substrate. It was selected as the individual phenolic acid to study its release from yeast cells after hydrothermal treatment and solvent extraction (discussed in next section).

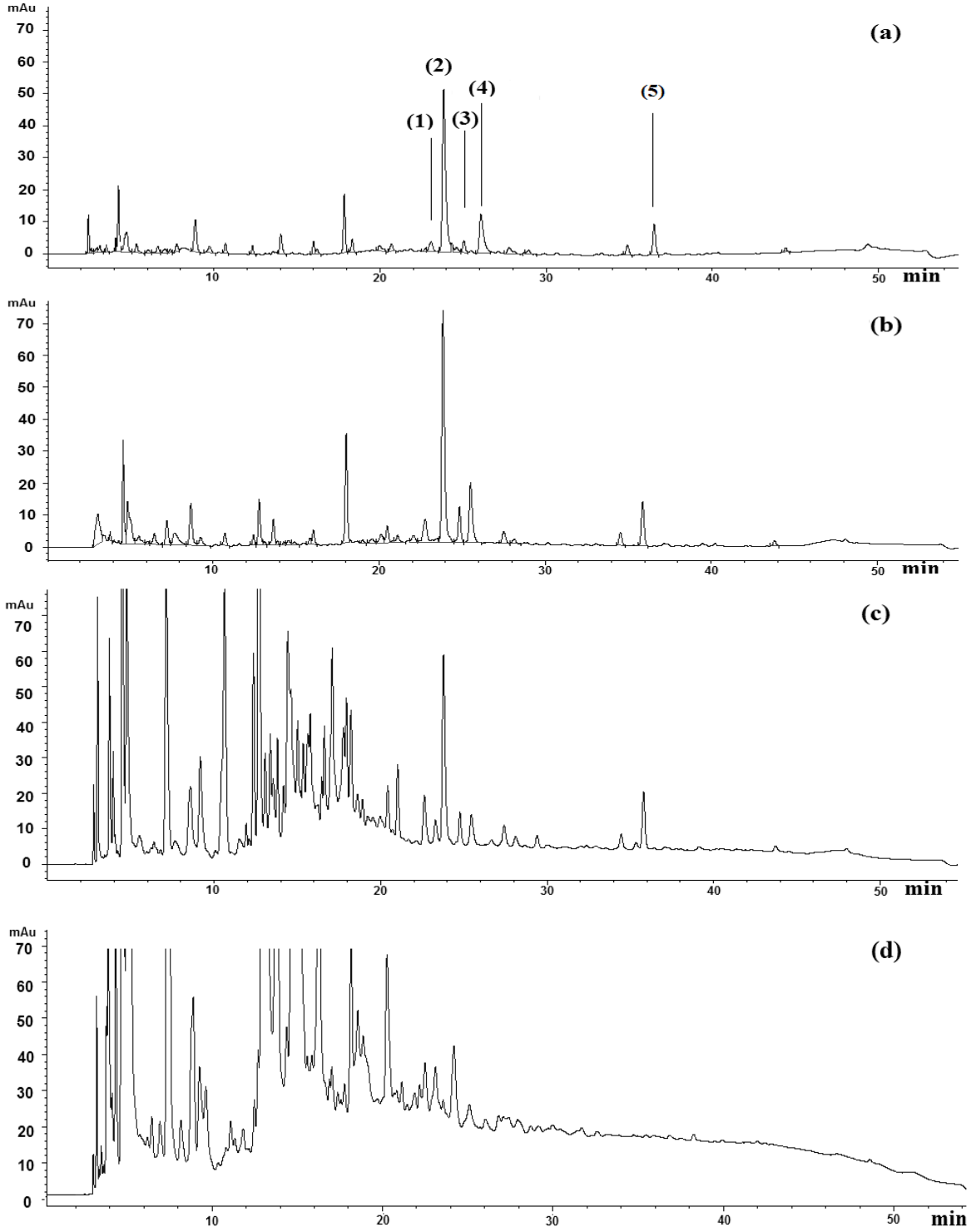


Figure 6. 9 HPLC chromatogram of (a) crude sample and treated ones at different temperatures ((b) 100°C after 30min, (c) 150 °C after 30 minutes and (d) 200°C after 30 minutes). Compounds were identified by comparing the retention time of standards with existing peaks in the samples. Identified phenolics were (1) Caffeic acid (2) Chlorogenic acid, (3) Epicatechin, (4) *p*-coumaric acid and (5) phloridzin dihydrate. (wavelength= 270nm)

The high concentration of chlorogenic acid enabled its convenient detection and accurate quantification in the crude biomass and hydrolysed extracts. Hence it was selected as a marker for phenolic acids to further study their release and concentration in the experiments.

6.3.4 Chlorogenic acid desorption

Chlorogenic acid was selected also as a marker to provide further understanding on the release of phenolic acids in the extracts. The high concentration of chlorogenic acid is due to the significant concentration of this compound in apple and cider (Schieber et al. 2001). The concentration of chlorogenic acid was 146 µg/ml in the starting sample (crude slurry) and increased to 196 µg/ml after being heated to 100 °C for 20 minutes (Fig. 6.10). The 30 % increase in the concentration is believed to be due to the release from the yeast cell wall. To confirm this, chlorogenic acid concentration was also measured in the organic solution extracts which were prepared previously. Results suggest that the amount of released chlorogenic acid due to subcritical water is almost the same as the organic solution extracts suggesting that this compound had been adsorbed on the yeast cell wall released during these experiments

6.3.5 HPLC analysis and results from DAD detector

Using diode array detector, it was possible to determine the UV absorbance of eluent at different times and at different wavelengths (Fig 6.11). This provided a better picture of the compositional change in the samples using its UV spectrum for different peaks helping to identify and determine the structures. UV absorbance data were normalized to the maximum value of absorbance to facilitate analysing the effect of temperature on the sample. Comparing the spectrum of these samples suggest that at later elution times (which

corresponds to more nonpolar components based on the used reversed-phase mode in the chromatography), UV absorption increased at higher wavelengths (phenolic structures). The increased phenolic content was due to either their formation synthetically, being desorbed from the yeast cell wall or have been released due to the cleavage of bonds in highly polymerized polyphenolics.

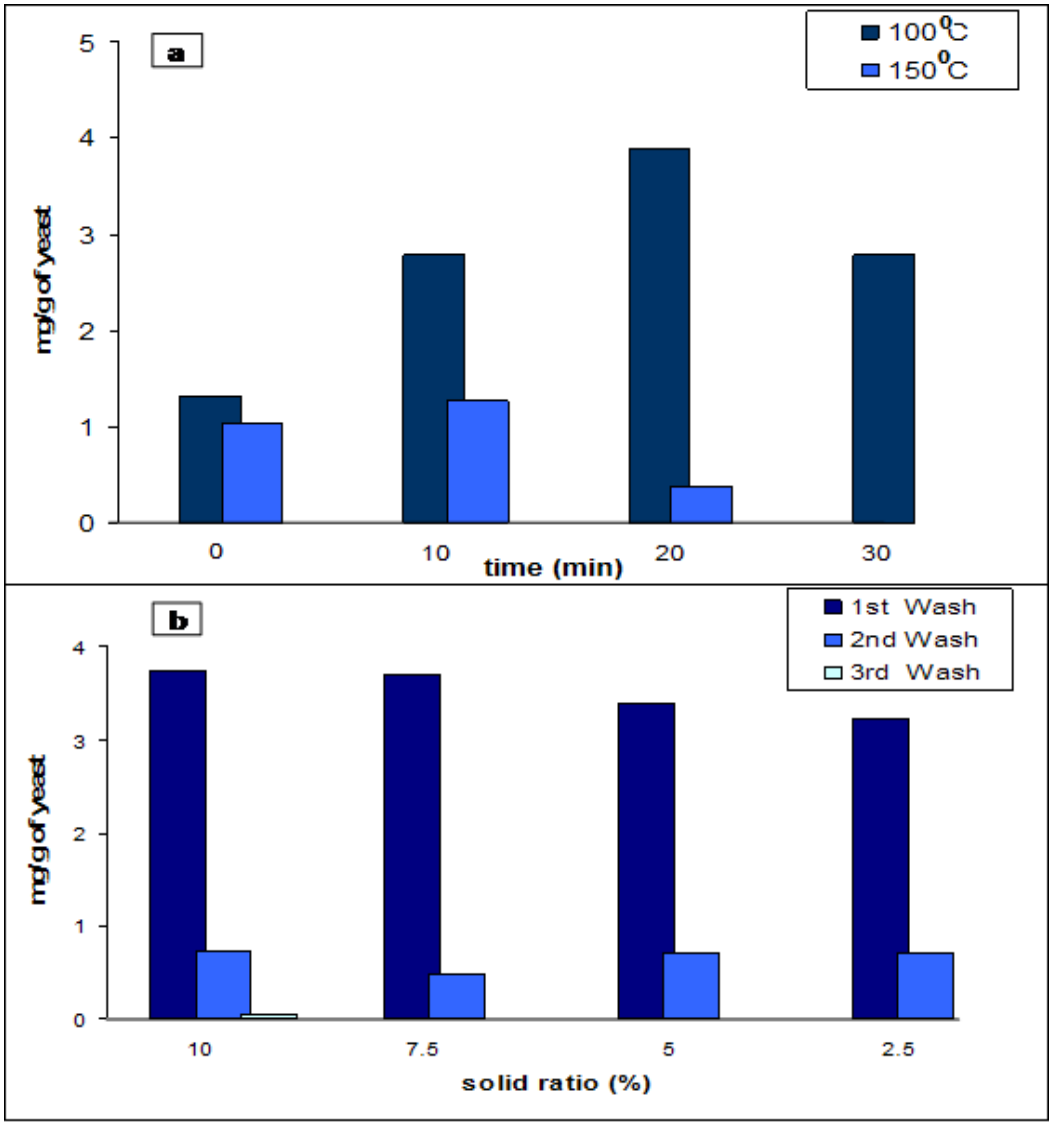


Figure 6. 10 .Chlorogenic acid desorption from yeast cells after hydrothermal treatment (a) and organic solvent extraction (b).

As Fig 6.11.a shows, the individual phenolic peaks which were discussed in section 6.3.3 can be observed at the same retention time mostly by their higher wavelength UV absorbance (> 280 nm) compared to the other components in the spectrum. In addition, there are other unidentified peaks, with absorbance above 300 nm, which represent other soluble phenolic compounds. Most of the phenolic compounds start to elute and emerge after the introduction of the organic mobile phase, as they tend to adsorb to the reversed phase stationary phase in a highly aqueous mobile phase. Comparing the Fig 6.11.b with the crude sample, two new compounds can be observed in the 150 °C sample with retention times of 29 and 31 minutes. The retention times of these new compounds and their significance absorbance at wavelengths more than 300 nm suggest that they are phenolic compounds formed or released under such conditions.

To confirm their phenolic structure, their UV spectrum were obtained using the DAD detector and are shown in Fig 6.12. Considering these spectrums, there are two peaks at 280 nm and 360 nm which are indications of their flavonol-like structure that corroborates their phenolic origin. As discussed in chapter 2, apple has been reported as a good source for flavonols as well as other discussed phenolic compounds. These two peaks were not detected in the samples at other temperatures. As these two compounds were not detected in extracts prepared at lower temperatures, there is the possibility that they may have been released from the yeast or being synthesized or derived from other condensed polyphenolics in reactive subcritical water. They could have also been released by the breakdown of other phenolic acids to their monomers. In the absence of a HPLC-MS system, no more information could be obtained about their characteristics or what compounds they could be. Comparing the release profile of these compounds with the one for chlorogenic acid, the new compounds follow a different release profile to chlorogenic acid as their maximum

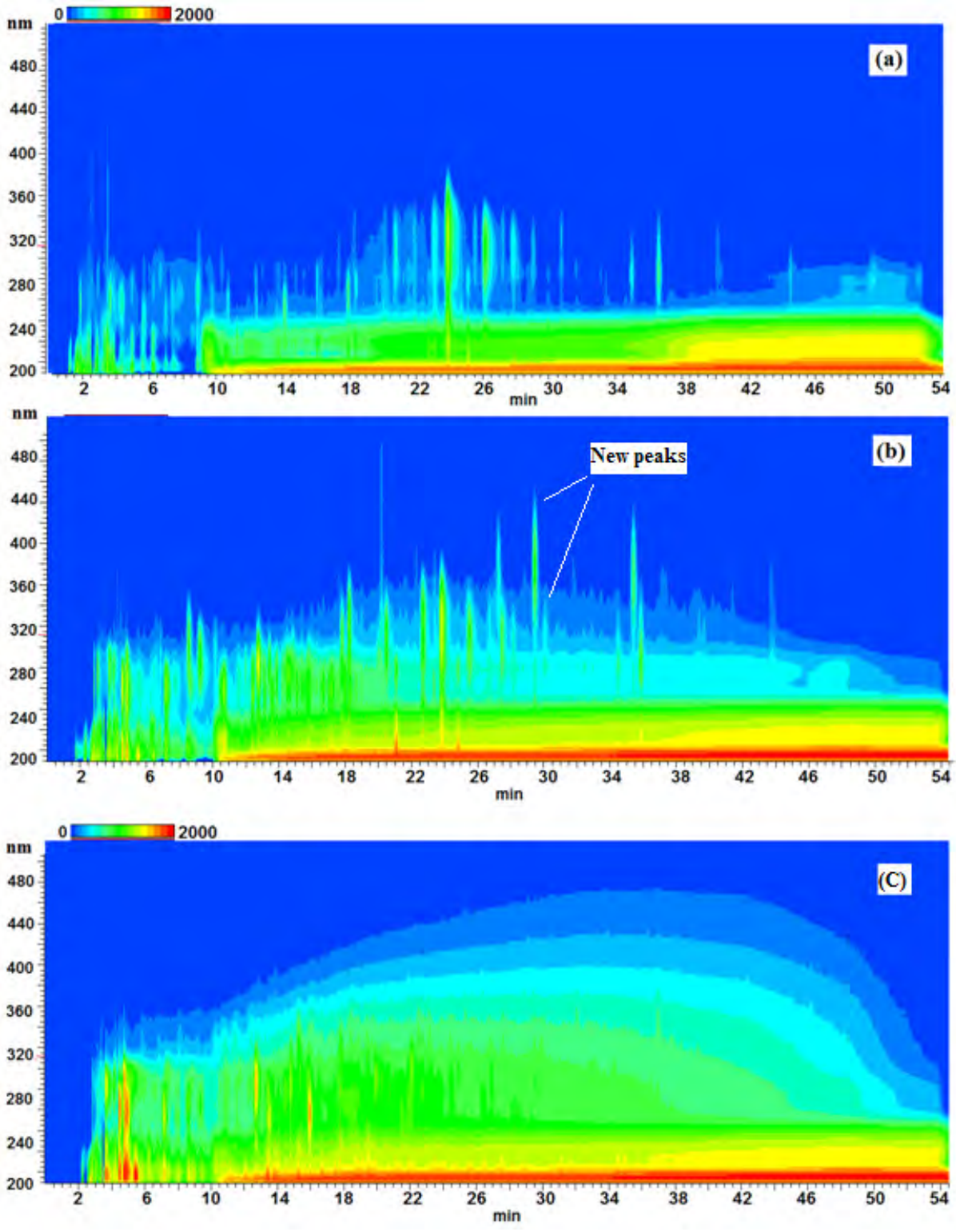


Figure 6. 11 DAD-UV spectrum of the crude and treated samples eluting from the HPLC column. (a) crude sample, (b) 150 °C after 30 mins and (c) 225 °C after 30 mins. Chromatograms are normalized to the in the logarithmic scale

concentration was at 150 °C in contrast to 100 °C for chlorogenic acid which supports the occurrence of secondary reactions (other than desorption) that results in their accumulation in the hydrolysed samples. The formation of these compounds also highlights the potentials of subcritical water as a solvent-free reaction medium to obtain new derivatives from phenolic fraction in waste.

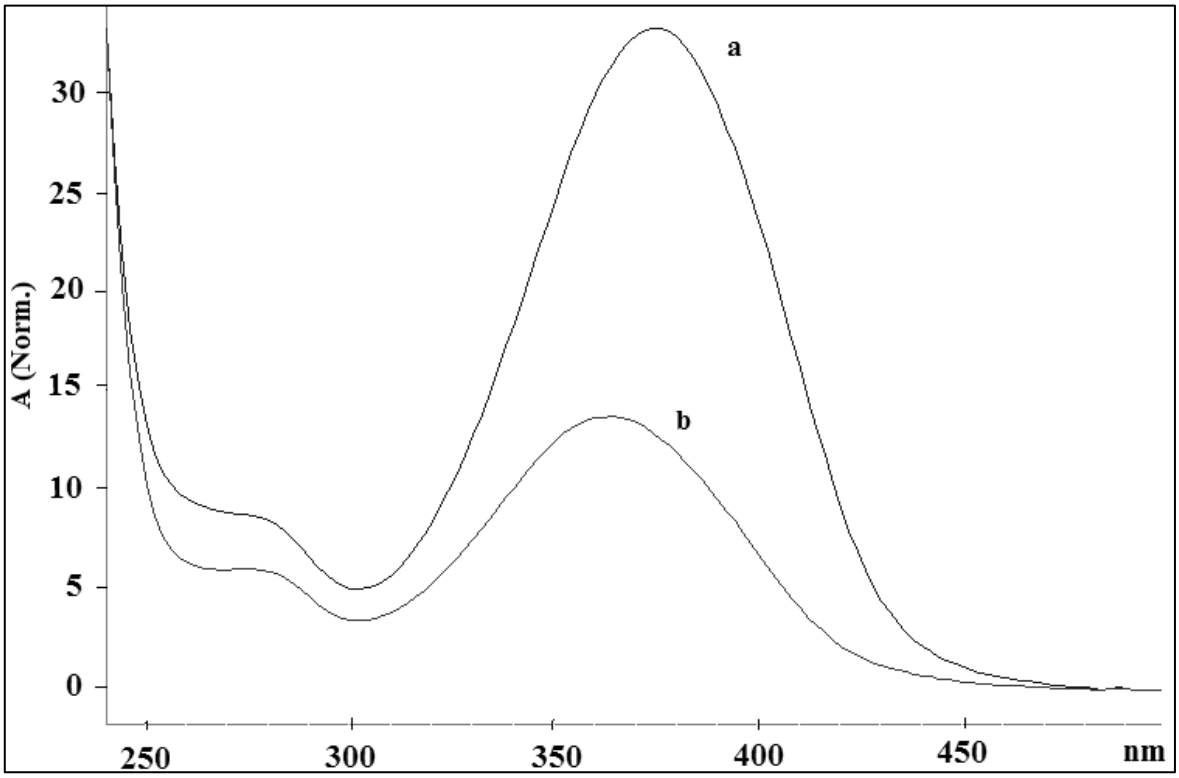


Figure 6. 12 UV-spectra of the two identified compounds in the extract of subcritical water treated samples

6.4 Conclusion

A combination of HPLC analysis coupled with DAD detector (for individual phenolics) and the Folin-Ciocalteau assay (for total phenolics) provided extensive data on the chemical composition of the samples in terms of phenolic content and a profile of individual phenolics. Using these assays, it was showed that the main cause for the increase of phenolic concentration was the desorption from the yeast cells.

HPLC analysis was employed to identify the major phenolic compounds in the cider spent yeast and how their concentration was changed during the hydrothermal reactions. Chlorogenic acid was detected as the most concentrated phenolic in the crude biomass and results suggested that 50% of its available content was already adsorbed on the yeast cell wall. The release profile of individual phenolics suggest that at 100 °C, the maximum concentration of chlorogenic acid can be obtained from the yeast. The same profile may be present in higher temperatures but the reactor configuration and the heating up stage prohibited accurate measurements of its release at earlier times. More structural information was obtained using DAD detector based on its superiority or the identification and characterisation of phenolic compounds which facilitate the determination of two new compounds in the extracts.

The release of chlorogenic acid and other phenolics was studied at different ratios of solids, different temperatures and residence time showing that 1) subcritical water is able to release the phenolics from the yeast cells in a process that is not fully elucidated yet and 2) their release is mainly the function of temperature while the release started during initial heating up stage at higher temperatures or maximized at certain temperatures (225 °C).

Data from total phenolic assay showed that the concentration of released phenolic compounds increased with temperature from 58 (mg/g of dry yeast) in crude extract to 280

(mg/g of dry yeast) after 30 minutes in 200°C. Almost similar concentration was observed when the cells were subjected to repetitive organic solvent extraction (250 mg/g of yeast). This proved that subcritical water treatment could effectively be used for the release of phenolics from yeast cells using a solvent free reaction. However, during the hydrothermal treatment step, there might be secondary reactions which lead to degradation of highly polymeric tannins to phenolic monomers which is expected to occur more significantly at higher temperatures. These secondary reactions may lead to partly overestimated results in the total phenolic content of the extracts. Furthermore, it can modify the structure of both the suspended phenolics in the crude biomass and the ones which had been desorbed later. Therefore, to study and determine the original composition of released phenolics (which can take place in smaller scales experiments), organic solvent extraction leads to less modification in their structure. In addition, subcritical water may also promote the formation of complex polymerized molecules between different compounds (polysaccharides, proteins and phenolics) leading to more condensed structures as it was suggested in section 3.3.3. Yet, the role of phenolic compounds in these reactions and other chemical routes has not yet been determined which offers an opportunity for further studies.

The release of phenolic compounds in the subcritical water treated resulted a mixture of phenolics which show significant antioxidant properties (Section 3.3.9) as well as unique UV spectrum were make them useful in certain products. More biological and toxicological studies is needed to better understand the possibility of using these extracts in consumer products (e.g. supplements and cosmetic products).

The results from this chapter, which introduces a solvent-free and simple process to prepare an extract enriched with apple phenolic, must be seen as a supplementary product stream to the other discussed compounds which reported in previous chapters. By identifying

the main products of interest, it is possible to adjust process parameters (i.e. temperature and residence time) in order to obtain fractions with a noticeable concentration of each class of compounds which already have been discussed. As an example, temperatures up to 150 °C are optimum for release of monomeric phenolics from yeast, while higher temperatures (200 °C) can result in higher content of phenolics in the samples. Further increase in the temperature will result in the formation of sugar monomers and their dehydrated derivatives.

Chapter 7

CONCLUSION AND RECOMMENDATION FOR FURTHER RESEARCH

7.1 Overall conclusions

i) A multidisciplinary approach was used to study the utility of subcritical water to convert the cider spent yeast (lees) into a mixture of valuable compounds and address how the biomass changes under such conditions. Two experimental setups, 5 ml (for kinetic study) and 300 ml (for all other measurements) were prepared to carry out batch hydrolysis reactions of cider spent yeast (lees) under subcritical water conditions. At the same time, various analytical assays were developed and used to evaluate the chemical and physical properties of the extracts. The results showed that various reactions took place in subcritical water and cider lees samples including hydrolysis and bond cleavage (i.e. cell wall solubilisation), dehydration (i.e. conversion of sugars to HMF), caramelisation (based on the colour and odour of the extracts) and as a result of dry weight measurement, it was reported that the condensation of heavier molecules have also occurred.

ii) Yeast cells were the major carbohydrate fraction in the studied feedstocks and no significant change was observed in their overall structure at temperatures up to

150 °C. The concentration profile for proteins, however, suggested that they have been released from the cells under such conditions which is believed to have been accompanied with other soluble intracellular compounds. Phenolic compounds, especially the identified individual phenolics, showed their highest concentration at 100 °C while the total phenolic content increased with temperature. At higher temperatures (up to 225 °C), cells started to lose their oval shape as an indication of cell wall solubilisation which was further confirmed by the accumulation of monosaccharides in the extracts. Protein concentration also dropped at these conditions, which could be due to their decomposition to smaller peptides with sizes below the detection limit of the assay. In addition to proteins, other heat stable intracellular compounds will be released at these conditions including nucleotides, amino acids and other minerals and nutrients. Despite all the attempts to identify vitamin B3 in subcritical water treated extracts, this compound was not detected which later was shown that it has not been available in its free form in the substrate and enzymatic reaction may be needed for its transformation. At elevated temperatures (250 °C and 300 °C) the solid residues had turned into char and the formation of gaseous products was more noticeable. The extracts had more characteristics of crude oil in terms of colour and oily structure.

iii) Subcritical water was shown to completely hydrolyse the yeast cells after 30 minutes at 200 °C. As a result, β-glucan and mannoprotein in the cell wall were converted to glucose and mannose (monosaccharides) respectively and their concentration was measured in both liquid and solid phase at different times and temperatures. Based on these data, monosaccharides' mass balance was calculated which provided reliable and accurate data about the kinetics of yeast cell wall solubilisation. A simple first order reaction was used to model the glucan and mannoprotein hydrolysis and other consecutive steps, which were fitted perfectly with experimental data. Since milder conditions are needed for glucan

conversion to sugars, this feedstock (or any other yeast biomass) can be used for the production of intermediate bio-based chemicals (e.g. HMF) using subcritical water. Subjecting the reaction medium to higher temperatures can lead the reaction to final derivatives such as organic acids. Comparing the kinetics of glucan conversion in subcritical water with cellulose conversion showed faster conversion in a less energy-intensive reaction and the feasibility of the production of bio-based chemicals. It worth noting that the reported yields were from a non-optimized reaction as no added catalyst was used. However, the acidic pH of the substrate may catalyse the hydrolysis reaction to some extents. Comparing the reaction rates constant for different compounds in the polysaccharide and mannoprotein conversion can provide useful details about how to perform the reaction in order to increase the selectivity of a specific fraction.

iv) Apple phenolics were identified in the original substrate and in the extracts suggesting that they have been carried over by the yeast cells after being adsorbed during the fermentation. Subcritical water was able to release these phenolics from yeast cells in levels comparable to organic solvent extraction. At the same time newly formed phenolic compounds were identified which may have been formed or released from more condensed polymeric phenolics. The release of phenolic compounds resulted in the significant change of the antioxidant activity of the extracts at 225 °C which showed their potentials as a source of highly active natural phenolics. HPLC-DAD method was proved to be an effective way to study the soluble phenolics and identifying the unknown structures using the UV spectrum.

v) In most studies, biomass conversion have been modelled with pure polysaccharides (such as cellulose or starch) making the study of kinetics more convenient but introduces some degrees of inaccuracy in the results. Our carbohydrate feedstock in particular (yeast cells) consists of a mixture of different polysaccharides that may behave differently in their natural

form compared to their purified individual structure. In addition, there is the possibility of synergistic effect of different compounds in the substrate (such as organic acids) which may act as catalysts reducing the need of using additives. Using crude and un-purified biomass in our study can also be useful towards filling the existing gap between model studies and larger industrial exploitation of real biomass. However, studying crude mixtures usually necessitates the use of sample pretreatment steps or more tedious procedures for method development as there are numerous impurities in the extracts that may interfere in the selected assays.

vi) Since the major goal of biomass processing with subcritical water is to employ a sustainable route for the production of bio-based chemicals, extracts were analysed to identify potential valuable fractions. Multiple product streams were identified in the extracts due to the different reactions that occur in subcritical water. Comparing the yields of different compounds at different temperatures, it may be possible to design a sequential hydrolysis process where different products are produced and separated from the reaction medium using selective separation techniques while the remaining reactants can proceed to the next steps for other derivatives. Offering different products can provide higher revenues for a single process and make the industrial applications of biomass conversion more appealing. In particular, this process could be modified in order to be carried out in parallel to the biofuel production (ethanol fermentation) and use the large amount of yeast waste produced after the fermentation similar to the proposed research. This parallel approach can further improve the economical viability of biorefinery platforms by reusing their main waste.

7.2 RECOMMENDATION FOR FURTHER RESEARCH

The outcomes of this research which were discussed in the thesis are currently under further investigation in few projects in the School of Chemical Engineering, University of Birmingham. They include the process-ability and scale-up of the reaction, application of the generated extracts for health and cosmetic products, furthering the HMF process to other derivatives applications and the possibility of using supercritical carbon dioxide to extract phenolics from the aqueous medium.

The idea of further reforming the generated chemical intermediate to produce other chemical feedstocks was considered a promising area for further research. However, higher yields of products are required for such applications as there should be enough concentrations of material for the analysis of secondary derivatives. A solution could be the use of more concentrated yeast slurries as the reaction substrate. Using higher concentration of yeast or fabricating a setup for continuous hydrolysis of cider lees will enable to better evaluate the economical feasibility of the process where higher amount of product will be formed per volume of the reactor.

While the interaction of wine phenolics with the yeast cells and its effect on the final product is previously discussed, not much data is available for cider. Detailed compositional studies of the adsorbed phenolics, which are released in our extracts, may lead to better understanding of the reaction and also the potentials of yeast cells for extracting specific phenolics (such as procyanidins) during the fermentation of cider or other alcoholic beverages. This improves the economic feasibility of this reaction and proves the advantages of subcritical water hydrolysis of such wastes.

While much food chemistry has focused on the Maillard reaction and interaction of sugars and proteins to give melanoidins, with proved antioxidant activity in food products,

not much research is available on the role of phenolic compounds in Maillard reaction. Further investigation of biopolymers in the extracts using size-exclusion chromatography and studying the separated fractions in terms of their possible beneficial effects can be suggested as a new topic for further research. Parenthetically, these reactions can be of special interest of cosmetic companies as phenolics offer strong UV adsorbing properties while self-tanning creams promote the Maillard reaction in the skin of consumer. Melanoidins have already been addressed for their role in flavour and colour of heated food products.

Major breakthroughs were achieved in the project after the identification of individual compounds via rigorous analysis using HPLC and Mass Spectroscopy. It is believed that using a dedicated LC-MS machine can facilitate the product characterisation, studying the involved reactions and carrying out the process optimisation steps. More novel analytical techniques could be specially valuable for studies involving the phenolics, especially to study the phenolics which have interacted with the yeast cells or other intracellular compounds during the subcritical water hydrolysis.

References

Adschiri, T. et al., 1993. Noncatalytic Conversion of Cellulose in Supercritical and Subcritical Water. *JOURNAL OF CHEMICAL ENGINEERING OF JAPAN*, 26(6), pp.676-680.

Afonso, C.A.M. & Crespo, J.G., 2005. *Green separation processes: fundamentals and applications*, Wiley-VCH.

Aida, T.M. et al., 2007. Dehydration of d-glucose in high temperature water at pressures up to 80 MPa. *The Journal of Supercritical Fluids*, 40(3), pp.381-388.

Akiya, N. & Savage, P.E., 2002. Roles of Water for Chemical Reactions in High-Temperature Water. *Chemical Reviews*, 102(8), pp.2725-2750.

Alonso-Salces, R. et al., 2001. Determination of Polyphenolic Profiles of Basque Cider Apple Varieties Using Accelerated Solvent Extraction. *J.Agric.Food Chem*, 49(8), pp.3761-3767.

Alonso-Salces, Rosa M. et al., 2004. Technological Classification of Basque Cider Apple Cultivars According to Their Polyphenolic Profiles by Pattern Recognition Analysis. *Journal of Agricultural and Food Chemistry*, 52(26), pp.8006-8016.

Antal, M.J., Mok, W.S. & Richards, G.N., 1990. Mechanism of formation of 5-(hydroxymethyl)-2-furaldehyde from D-fructose an sucrose. *Carbohydrate Research*, 199(1), pp.91-109.

Antal, Michael Jerry et al., 1991. Mechanism of formation of 2-furaldehyde from -xylose. *Carbohydrate Research*, 217, pp.71-85.

Appell, H. et al., 1971. *Converting organic wastes to oil:A Replenishable Energy Source*, Bureau of Mines Report of Investigation.

Armaroli, T. et al., 2000. Acid sites characterization of niobium phosphate catalysts and their activity in fructose dehydration to 5-hydroxymethyl-2-furaldehyde. *Journal of Molecular Catalysis A: Chemical*, 151(1-2), pp.233-243.

Arup, U. et al., 1993. High-performance thin-layer chromatography (HPTLC), an improved technique for screening lichen substances. *Lichenologist*, 25, pp.61-71.

Asghari, F.S. & Yoshida, H., 2006. Dehydration of fructose to 5-hydroxymethylfurfural in sub-critical water over heterogeneous zirconium phosphate catalysts. *Carbohydrate Research*, 341(14), pp.2379-2387.

Baek, J.Y., Lee, J.M. & Lee, S.C., 2008. Extraction of nutraceutical compounds from licorice roots with subcritical water. *Separation and Purification Technology*, 63(3), pp.661-664.

Baker, E. et al., 1989. Catalytic Destruction of Hazardous Organics in Aqueous Wastes: Continuous Reactor System Experiments. *Hazardous Waste and Hazardous Materials*, 6(1), pp.87-94.

Basile, A., Jimenez-Carmona, M.M. & Clifford, A.A., 1998. Extraction of Rosemary by Superheated Water. *Journal of Agricultural and Food Chemistry*, 46(12), pp.5205-5209.

Bezuidenhout, L. & Brett, M., 2008. Ultrathin layer chromatography on nanostructured thin films. *Journal of Chromatography A*, 1183, pp.179-185.

Bhattacharjee P & Singhal R. S, Extraction of squalene from yeast by supercritical carbon dioxide. Available at: http://www.ingentaconnect.com/content/klu/wibi/2003/00000019/00000006/05127077 [Accessed November 4, 2010].

Bicker, M., Hirth, J. & Vogel, H., 2003. Dehydration of fructose to 5-hydroxymethylfurfural in sub- and supercritical acetone. *Green Chemistry*, 5, pp.280-284.

Bicker, M. et al., 2005. Dehydration of d-fructose to hydroxymethylfurfural in sub- and supercritical fluids. *The Journal of Supercritical Fluids*, 36(2), pp.118-126.

Binder, J.B. et al., 2010. Mechanistic insights on the conversion of sugars into 5-hydroxymethylfurfural. *Energy & Environmental Science*, 3(6), pp.765-771.

Bitsch, R. et al., 2000. Bioavailability of antioxidative compounds from Brettacher apple juice in humans. *Innovative Food Science & Emerging Technologies*, 1(4), pp.245-249.

Bobleter, O., 1994. Hydrothermal degradation of polymers derived from plants. *Progress in Polymer Science*, 19(5), pp.797-841.

Bonilla, F. et al., 1999. Extraction of phenolic compounds from red grape marc for use as food lipid antioxidants. *Food Chemistry*, 66(2), pp.209-215.

Le Bourvellec, C., Guyot, S. & Renard, C.M.G.C., 2004. Non-covalent interaction between procyanidins and apple cell wall material. Part I: Effect of some environmental parameters. *Biochimica et Biophysica Acta (BBA) - General Subjects*, 1672(3), pp.192-202.

Boyer, J. & Liu, R., 2004. Apple phytochemicals and their health benefits. *Nutrition Journal*, 3(1), p.5.

Bozell, J.J. & Petersen, G.R., 2010. Technology development for the production of biobased products from biorefinery carbohydrates-the US Department of Energy's "Top 10" revisited. *Green Chemistry*, 12(4), pp.539-554.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72, pp.248-254.

Brunner, G., 2009. Near critical and supercritical water. Part I. Hydrolytic and hydrothermal processes. *The Journal of Supercritical Fluids*, 47(3), pp.373-381.

Cacace, J., 2003. Mass transfer process during extraction of phenolic compounds from milled berries. *Journal of Food Engineering*, 59(4), pp.379-389.

Carlini, C. et al., 2004. Heterogeneous catalysts based on vanadyl phosphate for fructose dehydration to 5-hydroxymethyl-2-furaldehyde. *Applied Catalysis A: General*, 275(1-2), pp.111-118.

Chae, H.J., Joo, H. & In, M., 2001. Utilization of brewer's yeast cells for the production of food-grade yeast extract. Part 1: effects of different enzymatic treatments on solid and protein recovery and flavor characteristics. *Bioresource Technology*, 76(3), pp.253-258.

Chen, S., 2008. Industrial Biosystems Engineering and Biorefinery Systems. *Chinese Journal of Biotechnology*, 24(6), pp.940-945.

Cherubini, F., 2010. The biorefinery concept: Using biomass instead of oil for producing energy and chemicals. *Energy Conversion and Management*, 51(7), pp.1412-1421.

Chheda, J.N., Roman-Leshkov, Y. & Dumesic, J.A., 2007. Production of 5-hydroxymethylfurfural and furfural by dehydration of biomass-derived mono- and poly-saccharides. *Green Chemistry*, 9(4), pp.342-350.

Cimpoiu, C. & Hosu, A., 2007. Thin Layer Chromatography for the Analysis of Vitamins and Their Derivatives. *Journal of Liquid Chromatography & Related Technologies*, 30(5), p.701.

Clark, J.H. & Deswarte, F.E.I., 2008. *Introduction to chemicals from biomass*, Wiley.

Clifford, T., 1998. *Fundamentals of supercritical fluids*, Oxford University Press, New York.

Coitino, E.L., Tomasi, J. & Ventura, O.N., 1994. Importance of water in aldol condensation reactions of acetaldehyde. *Journal of the Chemical Society, Faraday Transactions*, 90(12), p.1745.

Conde, E. et al., 2009. Antioxidant activity of the phenolic compounds released by hydrothermal treatments of olive tree pruning. *Food Chemistry*, 114(3), pp.806-812.

Corma, A., Iborra, S. & Velty, A., 2007. Chemical Routes for the Transformation of Biomass into Chemicals. *Chemical Reviews*, 107(6), pp.2411-2502.

Cruz, J.M., Dominguez, H. & Parajo, J.C., 2004. Assessment of the Production of Antioxidants from Winemaking Waste Solids. *Journal of Agricultural and Food Chemistry*, 52(18), pp.5612-5620.

Cui, L., Wu, G. & Jeong, T., 2010. Adsorption performance of nickel and cadmium ions onto brewer's yeast. *The Canadian Journal of Chemical Engineering*, 88(1), pp.109-115.

Dallies, N., François, J. & Paquet, V., 1998. A new method for quantitative determination of polysaccharides in the yeast cell wall. Application to the cell wall defective mutants of Saccharomyces cerevisiae. *Yeast*, 14(14), pp.1297-1306.

Demirbas, A., 2000. Mechanisms of liquefaction and pyrolysis reactions of biomass. *Energy Conversion and Management*, 41(6), pp.633-646.

Diñeiro García, Y., Valles, B.S. & Picinelli Lobo, A., 2009. Phenolic and antioxidant composition of by-products from the cider industry: Apple pomace. *Food Chemistry*, 117(4), pp.731-738.

Dunning, J.W. & Lathrop, E.C., 1945. Saccharification of Agricultural Residues. *Industrial & Engineering Chemistry*, 37(1), pp.24-29.

Ederer, H.J. et al., 1999. Modelling of the pyrolysis of tert-butylbenzene in supercritical water. *The Journal of Supercritical Fluids*, 15(3), pp.191-204.

Eggersdorfer, M. et al., 2005. *Vitamins in Ullmann's Encyclopeida of Industrial Chemistry (online)*, Weinham: Wiley-VCH Verlag GmbH & Co. KGaA.

Elliott, D.C., Hart, T.R. & Neuenschwander, G.G., 2006. Chemical Processing in High-Pressure Aqueous Environments. 8. Improved Catalysts for Hydrothermal Gasification. *Industrial & Engineering Chemistry Research*, 45(11), pp.3776-3781.

Escarpa, A. & González, M., 2001. Approach to the content of total extractable phenolic compounds from different food samples by comparison of chromatographic and spectrophotometric methods. *Analytica Chimica Acta*, 427(1), pp.119-127.

Escarpa, A. & González, M., 1999. Fast separation of (poly)phenolic compounds from apples and pears by high-performance liquid chromatography with diode-array detection. *Journal of Chromatography A*, 830(2), pp.301-309.

Feng, W., van der Kooi, H.J. & de Swaan Arons, J., 2004. Biomass conversions in subcritical and supercritical water: driving force, phase equilibria, and thermodynamic analysis. *Chemical Engineering and Processing*, 43(12), pp.1459-1467.

Ferreira, I. et al., 2010. Brewer's Saccharomyces yeast biomass: characteristics and potential applications. *Trends in Food Science & Technology*, 21(2), pp.77-84.

Franden, M.A., Pienkos, P.T. & Zhang, M., 2009. Development of a high-throughput method to evaluate the impact of inhibitory compounds from lignocellulosic hydrolysates on the growth of Zymomonas mobilis. *Journal of Biotechnology*, 144(4), pp.259-267.

G.L. Huang, 2010. Extraction of proteins from yeast cell wall. *African Journal of Biotechnology*, 9(21), pp.3207-3209.

Guha, B., 1931. Investigations on the preparation and behaviour of vitamin B1 concentrates from yeast. *Biochem J,*, 25(3), pp.931-944.

Gullon, B. et al., 2007. Evaluation of apple pomace alternative applications as a raw material for in food industries. *Food Technology and Biotechnology*, 45(4), pp.426-433.

Halsz, A. & Lsztity, A., 1991. *Use of Yeast Biomass in Food Production*, CRC Press.

Harbertson, J.F., Kennedy, J.A. & Adams, D.O., 2002. Tannin in Skins and Seeds of Cabernet Sauvignon, Syrah, and Pinot noir Berries during Ripening. *Am. J. Enol. Vitic.*, 53(1), pp.54-59.

Hashimoto, S., 2004. Remediation of soil contaminated with dioxins by subcritical water extraction. *Chemosphere*, 54(1), pp.89-96.

He, W. et al., 2008. Application of hydrothermal reaction in resource recovery of organic wastes. *Resources, Conservation and Recycling*, 52(5), pp.691-699.

Hippler, H., 1997. Elementary reactions in supercritical fluids. In *Proceedings of the Fourth Italian Conference on Supercritical Fluids and their Applications*. p. 279.

Hong, N. et al., 2001. Microwave-assisted extraction of phenolic compounds from grape seed. *Natural Product Letters*, 15(3), pp.197-204.

Ibañez, E. et al., 2003. Subcritical Water Extraction of Antioxidant Compounds from Rosemary Plants. *Journal of Agricultural and Food Chemistry*, 51(2), pp.375-382.

Illanes, A. & Gorgollón, Y., 1986. Kinetics of extraction of invertase from autolysed bakers' yeast cells. *Enzyme and Microbial Technology*, 8(2), pp.81-84.

In, M., Kim, D. & Chae, H., 2005. Downstream process for the production of yeast extract using brewer's yeast cells. *Biotechnology and Bioprocess Engineering*, 10(1), pp.85-90.

Ishida, H. & Seri, K., 1996. Catalytic activity of lanthanoide(III) ions for dehydration of -glucose to 5-(hydroxymethyl) furfural. *Journal of Molecular Catalysis A: Chemical*, 112(2), pp.L163-L165.

Japón-Luján, R. & Luque de Castro, M., 2006. Superheated liquid extraction of oleuropein and related biophenols from olive leaves. *Journal of Chromatography A*, 1136(2), pp.185-191.

Jiang, M. et al., 2009. Succinic Acid Production by Actinobacillus succinogenes Using Spent Brewer's Yeast Hydrolysate as a Nitrogen Source. *Applied Biochemistry and Biotechnology*, 160(1), pp.244-254.

Jing, Q. & Lu, X., 2008. Kinetics of Non-catalyzed Decomposition of Glucose in High-temperature Liquid Water. *Chinese Journal of Chemical Engineering*, 16(6), pp.890-894.

Kabyemela, B. et al., 1999. Glucose and Fructose Decomposition in Subcritical and Supercritical Water: Detailed Reaction Pathway, Mechanisms, and Kinetics. *Industrial & Engineering Chemistry Research*, 38(8), pp.2888-2895.

Kamm, B., Gruber, P.R. & Kamm, M., 2006. *Biorefineries - Industrial Processes and Products: Status Quo and Future Directions (2 Volume Set)*, Wiley-VCH.

Kazi, F.K. et al., 2011. Techno-economic analysis of dimethylfuran (DMF) and hydroxymethylfurfural (HMF) production from pure fructose in catalytic processes. *Chemical Engineering Journal*, 169(1-3), pp.329-338.

Kim, K.S. & Yun, H.S., 2006. Production of soluble [beta]-glucan from the cell wall of Saccharomyces cerevisiae. *Enzyme and Microbial Technology*, 39(3), pp.496-500.

Kim, K.S. & Yun, H.S., 2006. Production of soluble [beta]-glucan from the cell wall of Saccharomyces cerevisiae. *Enzyme and Microbial Technology*, 39(3), pp.496-500.

King, J.W., 2008. Critical fluid technology for the processing of lipid-related natural products. *Comptes Rendus Chimie*, 7(6-7), pp.647-659.

Kirk-Othmer ed., 2000. *Kirk-Othmer Encyclopedia of Chemical Technology*, Hoboken, NJ, USA: John Wiley & Sons, Inc. Available at: http://onlinelibrary.wiley.com/book/10.1002/0471238961.

Kivilompolo, M. & Hyotylainen, T., 2009. On-line coupled dynamic sonication-assisted extraction–liquid chromatography for the determination of phenolic acids in Lamiaceae herbs. *Journal of Chromatography A*, 1216(6), pp.892-896.

Klis, F. M., 1994. Review: cell wall assembly in yeast. *Yeast (Chichester, England)*, 10(7), pp.851-869.

Knekt, P. et al., 2002. Flavonoid intake and risk of chronic diseases. *The American Journal of Clinical Nutrition*, 76(3), pp.560-568.

Knežević, D., 2009. *Hydrothermal Conversion of Biomass*. PhD thesis. University of Twente.

Knežević, D., van Swaaij, W.P.M. & Kersten, S.R.A., 2009. Hydrothermal Conversion of Biomass: I, Glucose Conversion in Hot Compressed Water. *Industrial & Engineering Chemistry Research*, 48(10), pp.4731-4743.

Kollár, R. et al., 1997. Architecture of the Yeast Cell Wall. *Journal of Biological Chemistry*, 272(28), pp.17762-17775.

Kruse, A. & Dinjus, E., 2007a. Hot compressed water as reaction medium and reactant: 2. Degradation reactions. *The Journal of Supercritical Fluids*, 41(3), pp.361-379.

Kruse, A. & Dinjus, E., 2007b. Hot compressed water as reaction medium and reactant: Properties and synthesis reactions. *The Journal of Supercritical Fluids*, 39(3), pp.362-380.

Kulkarni, A., Suzuki, S. & Etoh, H., 2007. Antioxidant compounds from Eucalyptus grandis biomass by subcritical liquid water extraction. *Journal of Wood Science*, 54(2), pp.153-157.

Lamoolphak, W., Goto, M. & Sasaki, M., 2007. Hydrothermal decomposition of yeast cells for production of proteins and amino acids. *Journal of Hazardous Materials*, B137, pp.1643-1648.

Lazarus, S.A. et al., 1999. High-Performance Liquid Chromatography/Mass Spectrometry Analysis of Proanthocyanidins in Foods and Beverages. *Journal of Agricultural and Food Chemistry*, 47(9), pp.3693-3701.

Lee, K.W. et al., 2003. Major Phenolics in Apple and Their Contribution to the Total Antioxidant Capacity. *Journal of Agricultural and Food Chemistry*, 51(22), pp.6516-6520.

Lee, S.K. et al., 1998. Evaluation of the antioxidant potential of natural products. *Combinatorial Chemistry & High Throughput Screening*, 1(1), pp.35-46.

Lewkowski, J., 2003. Synthesis, Chemistry and Applications of 5-Hydroxymethyl-furfural and Its Derivatives. *ChemInform*, 34(2).

Li, C., Zhang, Z. & Zhao, Z.K., 2009. Direct conversion of glucose and cellulose to 5-hydroxymethylfurfural in ionic liquid under microwave irradiation. *Tetrahedron Letters*, 50(38), pp.5403-5405.

Liu, Q., Cai, W. & Shao, X., 2008. Determination of seven polyphenols in water by high performance liquid chromatography combined with preconcentration. *Talanta*, 77(2), pp.679-683.

Liu, X.Y., Wang, Q., et al., 2008. A new isolation method of [beta]-d-glucans from spent yeast Saccharomyces cerevisiae. *Food Hydrocolloids*, 22(2), pp.239-247.

Loev, B. & Goodman, M.M., 1967. Dry-Column Chromatogaphy- A preparative chromatographic technique with resolution of thin-layer chromatography. *Chemistry and Industry*, (48), p.2026.

Lu, Q., 2004. Adsorption of phenolics on activated carbon—impact of pore size and molecular oxygen. *Chemosphere*, 55(5), pp.671-679.

Luijkx, G.C.A., van Rantwijk, F. & van Bekkum, H., 1993. Hydrothermal formation of 1,2,4-benzenetriol from 5-hydroxymethyl-2-furaldehyde and -fructose. *Carbohydrate Research*, 242, pp.131-139.

Lyko, H., Deerberg, G. & Weidner, E., 2009. Coupled production in biorefineries--combined use of biomass as a source of energy, fuels and materials. *Journal of Biotechnology*, 142(1), pp.78-86.

Malin, A.S. et al., 2003. Intake of fruits, vegetables and selected micronutrients in relation to the risk of breast cancer. *International Journal of Cancer. Journal International Du Cancer*, 105(3), pp.413-418.

Manach, C. et al., 2005. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *American Journal of Clinical Nutrition*, 81(1), p.230S-242.

Mane, C. et al., 2007. Assessment of the Molecular Weight Distribution of Tannin Fractions through MALDI-TOF MS Analysis of Protein-Tannin Complexes. *Analytical Chemistry*, 79(6), pp.2239-2248.

Marshall, A.-L. & Alaimo, P.J., 2010. Useful Products from Complex Starting Materials: Common Chemicals from Biomass Feedstocks. *Chemistry - A European Journal*, 16(17), pp.4970-4980.

Matsumura, Y., Yanachi, S. & Yoshida, T., 2006. Glucose Decomposition Kinetics in Water at 25 MPa in the Temperature Range of 448−673 K. *Industrial & Engineering Chemistry Research*, 45(6), pp.1875-1879.

Mattila, P. & Kumpulainen, J., 2002. Determination of Free and Total Phenolic Acids in Plant-Derived Foods by HPLC with Diode-Array Detection. *Journal of Agricultural and Food Chemistry*, 50(13), pp.3660-3667.

Mazauric, J.-P. & Salmon, J.-M., 2006. Interactions between yeast lees and wine polyphenols during simulation of wine aging. II. Analysis of desorbed polyphenol compounds from yeast lees. *Journal of Agricultural and Food Chemistry*, 54(11), pp.3876-3881.

McNeff, C.V. et al., 2010. Continuous production of 5-hydroxymethylfurfural from simple and complex carbohydrates. *Applied Catalysis A: General*, 384(1-2), pp.65-69.

Medina, K. et al., 2005. Yeast Interactions with Anthocyanins during Red Wine Fermentation. *Am. J. Enol. Vitic.*, 56(2), pp.104-109.

Jason S. Midgett, 2008. Assessing a Hydrothermal Liquefaction Process Using Biomass Feedstocks. Available at: http://etd.lsu.edu/docs/available/etd-01162008-150910/ [Accessed October 23, 2010].

Miniati, E., 2007. Assessment of phenolic compounds in biological samples. *Ann Ist Super Sanita*, 43(4), pp.362-368.

Minowa, T. & Ogi, T., 1998. Hydrogen production from cellulose using a reduced nickel catalyst. *Catalysis Today*, 45(1-4), pp.411-416.

Mochidzuki, K., Sakoda, A. & Suzuki, M., 2000. Measurement of the hydrothermal reaction rate of cellulose using novel liquid-phase thermogravimetry. *Thermochimica Acta*, 348(1-2), pp.69-76.

Mok & Antal, 1992. Uncatalyzed solvolysis of whole biomass hemicellulose by hot compressed liquid water. *Industrial & Engineering Chemistry Research*, 31(4), pp.1157-1161.

Molton, P.M. et al., 1978. Mechanism of conversion of cellulosic wastes to liquid fuels in alkaline solution. In Symposium on energy from biomass and wastes, Washington, DC, USA.

Montedoro, G. et al., 1992. Simple and hydrolyzable phenolic compounds in virgin olive oil. 2. Initial characterization of the hydrolyzable fraction. *Journal of Agricultural and Food Chemistry*, 40(9), pp.1577-1580.

Morata, A. et al., 2003. Adsorption of Anthocyanins by Yeast Cell Walls during the Fermentation of Red Wines. *Journal of Agricultural and Food Chemistry*, 51(14), pp.4084-4088.

Morata, A. et al., 2005. Cell wall anthocyanin adsorption by different Saccharomyces strains during the fermentation of Vitis vinifera L. cv Graciano grapes. *European Food Research and Technology*, 220(3), pp.341-346.

Moreau, C, Finiels, A. & Vanoye, L., 2006. Dehydration of fructose and sucrose into 5-hydroxymethylfurfural in the presence of 1-H-3-methyl imidazolium chloride acting both as solvent and catalyst. *Journal of Molecular Catalysis A: Chemical*, 253(1-2), pp.165-169.

Moreau, Claude et al., 1994. Preparation of 5-hydroxymethylfurfural from fructose and precursors over H-form zeolites. *Industrial Crops and Products*, 3(1-2), pp.85-90.

Moreau, Claude et al., 1996. Dehydration of fructose to 5-hydroxymethylfurfural over H-mordenites. *Applied Catalysis A: General*, 145(1-2), pp.211-224.

Moreno-Arribas, M.V. & Polo, M.C. eds., 2009. *Wine Chemistry and Biochemistry*, New York, NY: Springer New York. Available at: http://www.springerlink.com/content/w704520506336128/export-citation/ [Accessed November 14, 2010].

Mosier, N., 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology*, 96(6), pp.673-686.

Musau, R.M. & Munavu, R.M., 1987. The preparation of 5-hydroxymethyl-2-furaldehyde (HMF) from d-fructose in the presence of DMSO. *Biomass*, 13(1), pp.67-74.

Nagamori, M. & Funazukuri, T., 2004. Glucose production by hydrolysis of starch under hydrothermal conditions. *Journal of Chemical Technology & Biotechnology*, 79(3), pp.229-233.

Neilands, J.B. et al., 1950. Concentration of bound pantothenic acid. *The journal of Biological Chemistry*, 185, pp.335-346.

Nogueira, A. et al., 2008. Effect of alcoholic fermentation in the content of phenolic compounds in cider processing. *Brazilian Archives of Biology and Technology*, 51(5).

Pedersen, M., Viksø-Nielsen, A. & Meyer, A.S., 2010. Monosaccharide yields and lignin removal from wheat straw in response to catalyst type and pH during mild thermal pretreatment. *Process Biochemistry*, 45(7), pp.1181-1186.

Peterson, A., 2009. *Biomass Reforming Processes in Hydrothermal Media*. PhD thesis. Massachusetts Institute of Technology.

Ponder, E.L., Fried, B. & Sherma, J., 2004. Thin-layer chromatographic analysis of hydrophilic vitamins in standards and from Helisoma trivolvis snails. *ACTA CHROMATOGRAPHICA*, 14, pp.70-81.

Pourali, O., Asghari, F.S. & Yoshida, H., 2010. Production of phenolic compounds from rice bran biomass under subcritical water conditions. *Chemical Engineering Journal*, 160(1), pp.259-266.

Proestos, C., Kapsokefalou, M. & Komaitis, M., 2008. Analysis of Naturally occuring phenolics compounds in aromatic plants by RP-HPLC and GC-MS after silylation. *Journal of Food Quality*, 31(3), pp.402-414.

Pueyo, E. et al., 2000. Release of Lipids during Yeast Autolysis in a Model Wine System. *Journal of Agricultural and Food Chemistry*, 48(1), pp.116-122.

Quail, M.A. & Kelly, S.L., 1996. The extraction and analysis of sterols from yeast. *Methods in Molecular Biology (Clifton, N.J.)*, 53, pp.123-131.

Rajamanickam, S. & Agarwal, R., 2008. Natural products and colon cancer: current status and future prospects. *Drug Development Research*, 69(7), pp.460-471.

Raman, G. & Gaikar, V.G., 2002. Microwave-Assisted Extraction of Piperine from Piper nigrum. *Industrial & Engineering Chemistry Research*, 41(10), pp.2521-2528.

Rangsriwong, P. et al., 2009. Subcritical water extraction of polyphenolic compounds from Terminalia chebula Retz. fruits. *Separation and Purification Technology*, 66(1), pp.51-56.

Renard, Catherine M. G. C. et al., 2001. Interactions between apple cell walls and native apple polyphenols: quantification and some consequences. *International Journal of Biological Macromolecules*, 29(2), pp.115-125.

Rezk, B.M. et al., 2002. The antioxidant activity of phloretin: the disclosure of a new antioxidant pharmacophore in flavonoids. *Biochemical and Biophysical Research Communications*, 295(1), pp.9-13.

Rice-Evans, C., Miller, N. & Paganga, G., 1997. Antioxidant properties of phenolic compounds. *Trends in Plant Science*, 2(4), pp.152-159.

Rizzo, M. et al., 2006. HPLC determination of phenolics adsorbed on yeasts. *Journal of Pharmaceutical and Biomedical Analysis*, 42(1), pp.46-55.

Rogalinski, T., Ingram, T. & Brunner, G, 2008. Hydrolysis of lignocellulosic biomass in water under elevated temperatures and pressures. *The Journal of Supercritical Fluids*, 47(1), pp.54-63.

Rogalinski, T., Liu, K., et al., 2008. Hydrolysis kinetics of biopolymers in subcritical water. *The Journal of Supercritical Fluids*, 46(3), pp.335-341.

Roldán-Gutiérrez, J.M., Ruiz-Jiménez, J. & Luque de Castro, M.D., 2008. Ultrasound-assisted dynamic extraction of valuable compounds from aromatic plants and flowers as compared with steam distillation and superheated liquid extraction. *Talanta*, 75(5), pp.1369-1375.

Roman-Leshkov, Y. et al., 2007. Production of dimethylfuran for liquid fuels from biomass-derived carbohydrates. *Nature*, 447(7147), pp.982-985.

Rubin, S.H., Scheiner, J. & Hirschberg, E., 1947. The availability of vitamin B6 in yeast and liver for growth of saccharomyces carlsbergenesis. *The Journal of Biological Chemistry*, 167, pp.599-611.

Saksinchai, S., Suphantharika, M. & Verduyn, C., 2001. Application of a simple yeast extract from spent brewer's yeast for growth and sporulation of Bacillus thuringiensis subsp. kurstaki: a Physiological Study. *World Journal of Microbiology and Biotechnology*, 17(3), pp.307-316.

Salak Asghari, F. & Yoshida, H., 2006. Acid-Catalyzed Production of 5-Hydroxymethyl Furfural from d-Fructose in Subcritical Water. *Industrial & Engineering Chemistry Research*, 45(7), pp.2163-2173.

Salmon, J.M., 2006. Interactions between yeast, oxygen and polyphenols during alcoholic fermentations: Practical implications. *LWT - Food Science and Technology*, 39(9), pp.959-965.

Salvador, F. & Merchán, M.D., 1996. Study of the desorption of phenol and phenolic compounds from activated carbon by liquid-phase temperature-programmed desorption. *Carbon*, 34(12), pp.1543-1551.

Sanchez, L., 2001. TCA protein precipitation protocol. Available at: www.its.caltech.edu/~bjorker/Protocols/TCA_ppt_protocol.pdf.

Sandula, J. et al., 1999. Microbial (1-->3)-[beta]--glucans, their preparation, physico-chemical characterization and immunomodulatory activity. *Carbohydrate Polymers*, 38(3), pp.247-253.

Sasaki, M. et al., 2000. Dissolution and Hydrolysis of Cellulose in Subcritical and Supercritical Water. *Industrial & Engineering Chemistry Research*, 39(8), pp.2883-2890.

Sasaki, M., Kabyemela, B. & Malaluan, R., 1998. Cellulose hydrolysis in subcritical and supercritical water. *Journal of Supercritical Fluids*, 13, pp.261-268.

Schieber, A. et al., 2003. A new process for the combined recovery of pectin and phenolic compounds from apple pomace. *Innovative Food Science & Emerging Technologies*, 4(1), pp.99-107.

Schieber, A., Keller, P. & Carle, R., 2001. Determination of phenolic acids and flavonoids of apple and pear by high-performance liquid chromatography. *Journal of Chromatography A*, 910(2), pp.265-273.

Schwald, W. & Bobleter, O., 1989. Hydrothermolysis of Cellulose Under Static and Dynamic Conditions at High Temperatures. *Journal of Carbohydrate Chemistry*, 8(4), p.565.

Sereewatthanawut, I. et al., 2008. Extraction of protein and amino acids from deoiled rice bran by subcritical water hydrolysis. *Bioresource Technology*, 99(3), pp.555-561.

Seri, K.-ichi, Inoue, Y. & Ishida, H., 2000. Highly Efficient Catalytic Activity of Lanthanide(III) Ions for Conversion of Saccharides to 5-Hydroxymethyl-2-furfural in Organic Solvents. *Chemistry Letters*, 29(1), p.22.

Sherma, J. & Fried, B., 2005. Thin Layer Chromatographic Analysis of Biological Samples. A Review. *Journal of Liquid Chromatography & Related Technologies*, 28(15), p.2297.

Shibata, K. & Taguchi, H., 2000. Nicotinic Acid and Nicotinamide. In W. Lambert, A. De Leenheer, & J. Van Bocxlaer, eds. *Modern Chromatographic Analysis Of Vitamins*. CRC Press.

Singleton, V.L. & Rossi, J.A., 1965. Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. *Am. J. Enol. Vitic.*, 16(3), pp.144-158.

Sombutyanuchit, P., Suphantharika, M. & Verduyn, C., 2001. Preparation of 5-GMP-rich yeast extract from spent brewer's yeast. *World Journal of Microbiology and Biotechnology*, 17(2), pp.163-168.

Sommer, R., 1996. Yeast Extracts: Production, Properties and Components. In 9th International Symposium on Yeasts. Sydney.

Stephen, A.M., 1995. *Food polysaccharides and their applications*, Marcel Dekker.

Subramaniam, B. & McHugh, M.A., 1986. Reactions in supercritical fluids - a review. *Industrial & Engineering Chemistry Process Design and Development*, 25(1), pp.1-12.

Sun, J. et al., 2002. Antioxidant and Antiproliferative Activities of Common Fruits. *Journal of Agricultural and Food Chemistry*, 50(25), pp.7449-7454.

Suárez, B. et al., 2005. Liquid chromatographic method for quantifying polyphenols in ciders by direct injection. *Journal of Chromatography A*, 1066(1-2), pp.105-110.

Svobodová, A., Psotová, J. & Walterová, D., 2003. Natural phenolics in the prevention of UV-induced skin damage. A review. *Biomedical Papers of the Medical Faculty of the University Palacký, Olomouc, Czechoslovakia*, 147(2), pp.137-145.

Swami Handa, S. et al., 2008. Extraction Technologies for Medicinal and Aromatic Plants- International Centre For Science and High Technology (UNIDO).

Szmant, H.H. & Chundury, D.D., 2007. The preparation of 5-hydroxymethylfurfuraldehyde from high fructose corn syrup and other carbohydrates. *Journal of Chemical Technology and Biotechnology*, 31(1), pp.135-145.

Tanguler, H. & Erten, H., 2008. Utilisation of spent brewer's yeast for yeast extract production by autolysis: The effect of temperature. *Food and Bioproducts Processing*, 86(4), pp.317-321.

Teply, L.J., Strong, F.M. & Elvehjem, C.A., 1941. The distribution of nicotinic acid in foods. *The Journal of Nutrition*, p.14.

Thanardkit, P. et al., 2002. Glucan from spent brewer's yeast: preparation, analysis and use as a potential immunostimulant in shrimp feed. *World Journal of Microbiology and Biotechnology*, 18(6), pp.527-539.

Tonnies, I., 2008. *Subcritical water hydrolysis of spent yeast for the recovery of high value compounds*. UK: Hamburg University of Technology, University of Birmingham.

Torget, R.W., Kim, J.S. & Lee, Y.Y., 2000. Fundamental Aspects of Dilute Acid Hydrolysis/Fractionation Kinetics of Hardwood Carbohydrates. 1. Cellulose Hydrolysis. *Industrial & Engineering Chemistry Research*, 39(8), pp.2817-2825.

Tsai, W.T., Lee, M.K. & Chang, Y.M., 2006. Fast pyrolysis of rice straw, sugarcane bagasse and coconut shell in an induction-heating reactor. *Journal of Analytical and Applied Pyrolysis*, 76(1-2), pp.230-237.

Tsang, S., Lee, C.H. & Rha, K., 1979. Disintegration of cell wall and extraction of protein from Candida Lipolytica. *Journal of Food Science*, 44(1), pp.97-99.

Tseng, M.M.-C. & Phillips, C.R., 1982. The kinetics of yeast growth and nicotinic acid production. *Biotechnology and Bioengineering*, 24(6), pp.1319-1325.

Tyrlik, S.K. et al., 1999. Selective dehydration of glucose to hydroxymethylfurfural and a one-pot synthesis of a 4-acetylbutyrolactone from glucose and trioxane in solutions of aluminium salts. *Carbohydrate Research*, 315(3-4), pp.268-272.

Ullmann, F., 2007. *Ullmann's Encyclopedia of Industrial Chemistry*, John Wiley & Sons, Inc.

Urquiaga, I. & Leighton, F., 2000. Plant Polyphenol Antioxidants and Oxidative Stress. *Biological Research*, 33(2).

Vatai, T., Skerget, M. & Knez, Z., 2009. Extraction of phenolic compounds from elder berry and different grape marc varieties using organic solvents and/or supercritical carbon dioxide. *Journal of Food Engineering*, 90(2), pp.246-254.

Vendruscolo, F. et al., 2008. Apple pomace: a versatile substrate for biotechnological applications. *Critical Reviews in Biotechnology*, 28(1), pp.1-12.

Watanabe, M., Inomata, H. & Arai, K., 2002. Catalytic hydrogen generation from biomass (glucose and cellulose) with ZrO2 in supercritical water. *Biomass and Bioenergy*, 22(5), pp.405-410.

Waterhouse, A., 2005. Folin-Ciocalteau Micro Method for Total Phenol in Wine. *University of California, Davis*. Available at: http://waterhouse.ucdavis.edu/phenol/folinmicro [Accessed March 1, 2010].

Wheatcroft, R., 1997. Production of beta-glucan-mannan preparations by autolysis ofcells under certain pH, temperature and time conditions. , pp.15, 20,21, 22.

Wrolstad, R.E. et al. eds., 2001. *Current Protocols in Food Analytical Chemistry*, Hoboken, NJ, USA: John Wiley & Sons, Inc. Available at: http://onlinelibrary.wiley.com/book/10.1002/0471142913/toc.

Xu, C. et al., 2010. Extraction, distribution and characterisation of phenolic compounds and oil in grapeseeds. *Food Chemistry*, 122(3), pp.688-694.

Yaman, S., 2004. Pyrolysis of biomass to produce fuels and chemical feedstocks. *Energy Conversion and Management*, 45(5), pp.651-671.

Yanagida, A et al., 2000. Fractionation of apple procyanidins according to their degree of polymerization by normal-phase high-performance liquid chromatography. *Journal of Chromatography. A*, 890(2), pp.251-259.

Yanagida, Akio, Shoji, T. & Shibusawa, Y., 2003. Separation of proanthocyanidins by degree of polymerization by means of size-exclusion chromatography and related techniques. *Journal of Biochemical and Biophysical Methods*, 56(1-3), pp.311-322.

Yu, J.-xia et al., 2009. Poly(Amic Acid)-Modified Biomass of Baker's Yeast for Enhancement Adsorption of Methylene Blue and Basic Magenta. *Applied Biochemistry and Biotechnology*, 160(5), pp.1394-1406.

Yu, Y., Lou, X. & Wu, H., 2008. Some Recent Advances in Hydrolysis of Biomass in Hot-Compressed Water and Its Comparisons with Other Hydrolysis Methods†. *Energy & Fuels*, 22(1), pp.46-60.

Zakaria, M., Gonnord, M.F. & Guiochon, G., 1983. Application of two-dimensional thin-layer chromatography. *Journal of Chromatography*, 271(2), pp.127-192.

Zakrzewska, M.E., Bogel-Łukasik, E. & Bogel-Łukasik, R., 0. Ionic Liquid-Mediated Formation of 5-Hydroxymethylfurfural—A Promising Biomass-Derived Building Block. *Chemical Reviews*, 0(0).

Zhang, B., Huang, H.-J. & Ramaswamy, S., 2007. Reaction Kinetics of the Hydrothermal Treatment of Lignin. *Applied Biochemistry and Biotechnology*, 147(1-3), pp.119-131.

Zuo, Y., Wang, C. & Zhan, J., 2002. Separation, Characterization, and Quantitation of Benzoic and Phenolic Antioxidants in American Cranberry Fruit by GC−MS. *Journal of Agricultural and Food Chemistry*, 50(13), pp.3789-3794.

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DETERMINATION OF HYDROXYMETHYLFURFURAL IN CIDER SPENT YEAST TREATED WITH SUBCRITICAL WATER

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Abstract: In this work, subcritical water treatment was used as an environmentally friendly technique to release available nutraceuticals or convert other potential components in the biomass to active and more useful products. The resulting extracts of the subcritical water batch treatment were analyzed with liquid chromatography and hydroxymethylfurfural was identified as the major component in the aqueous fraction. This may suggest that the available or decomposed monosaccharides may provide the necessary raw material required for dehydration process to manufacture HMF. The concentration profile was also investigated at different reaction temperatures to find out the effect of heating on the reaction output. The results could be more advantageous when the process include other prospective products in the medium to increase the productivity.

Keywords: Cider spent yeast, subcritical water, HMF

1. INTRODUCTION

Within the European food and drink industry over 200 million tonnes of organic waste are produced each year. The brewing and cider making industry produces for example spent grain and yeast, both are wastes. On a European scale spent yeast amounts to 700,000 tonnes per annum. Traditional markets for spent yeast are as dried whole extracts serving the nutraceutical markets and as a slurry, with 24% solid content, for animal feed. Available markets which used to employ the available nutrients in the biomass for the feed industry products are also declining. Therefore, alternative applications are being sought by the brewery industries to find sustainable routes to convert or capture the existing valuable fractions. It has been the focus of a large number of research groups to investigate the possible alternatives instead of traditionally land filling or disposal.

Green processing and new manufacturing routes have been recommended worldwide by governmental and legislation bodies to decrease the reliance on petroleum based chemicals. Subcritical water, because of its benign nature and adjustable solvent power, dielectric constant and ion product has been largely studied as a green extraction solvent (Herrero et al., 2006). With its unique chemical and physical conditions that it provides, it is possible to employ it as a reaction medium for different purposes simultaneously (Smith, 2002).

In this work, subcritical water (pressurised hot or superheated water as been addressed in some studies (Smith, 2002)) was used to hydrolyze suspended yeast cells in a slurry, and release other biomolecules which required further extraction procedures. Initially the hydrophilic fraction was investigated and the focus was on identification and characterisation of the main components which were available in this fraction. It is believed that there are many other valuable compounds which are present in the medium, but have not been included in this study (like the ones in lipophilic fraction or solid particles) and are currently being investigated in this project.

2. MATERIALS AND METHODS

2.1 Subcritical Water Hydrolysis

200 ml of spent cider yeast slurry was supplied by Bulmers® with a solid content of 13% (w/v) was subject to batch hydrolysis using a 300 ml stainless steel vessel (Parr Instrument Company ® 5500 mini bench top reactor). The vessel was heated electrically and cooled through the installation of additional cooling loop. The pressure was provided by a nitrogen gas cylinder at the indicated pressure.

2.2 Sample preparation

After subcritical water hydrolysis the samples were cooled and divided into 50 ml tubes and centrifuged at 17000 g for 10 mins (Beckman) and stored in -20 °C freezer for analysis. Samples were filtered prior to HPLC using 0.2 µm syringe driven filters (Millex- Millipore®).

2.3 High Performance Liquid Chromatography

An Agilent1100 coupled with a UV detector/analyzer (270 nm) fitted with a Phenomenex Synergi, RP-18, 250 × 4.6 mm column. The column was coupled with a guard cartridge packed with the same stationary phase (Phenomenex, UK) to capture all the compounds in the injected sample which may interact with or be retained by the column stationary phase. The column temperature was set to 30 °C with a flow rate of 1.0 ml/min. Gradient mobile phase condition was started from 99:1 water:acetonitrile and changed to steep gradient of 20% acetonitrile to remove insoluble compounds after the peak elution. All the solvents were purchased from Fisher Scientific®. The hydroxymethylfurfural standard was bought from Sigma-Aldrich to prepare the standard solution for the calibration and also validation.

2.4 EI-Mass spectroscopy

To identify the major peak in the chromatograms, samples were analyzed by taking fractions from HPLC column and analyzing them with a EI-MS (VG-ZabSpec). This was made possible by adjusting the HPLC method in terms of considering the compatibility of the chemicals in the analysis and also prohibiting the use of any buffer to prevent any negative effect on the analysis.

3. RESULTS

As discussed earlier, the applied analytical techniques were developed to include only the compounds available in the aqueous phase and categorized as hydrophilic compounds. All the samples were stored in -20 °C freezer prior to analysis.

3.1 HPLC analysis of the extract

Figure 1 shows the resulting chromatogram of the prepared sample which was recorded at 270 nm. Other wavelengths were also investigated but the most significant peak was present in this wavelength.

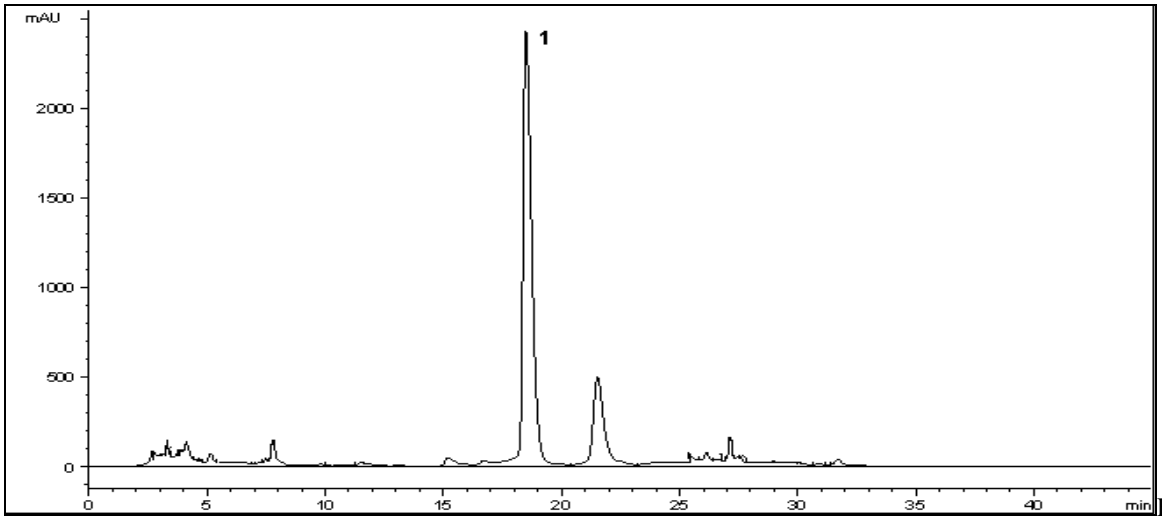

Fig. 1- Chromatogram resulted from the analysis of the heat treated cider spent yeast with modified C18 Chromatography running at high ratio of water. Peak No. 1(Rt =18.2 min) was collected for further analysis

3.2 Mass Spectroscopy

The eluent peak was collected from the column and checked separately with the mass spectroscopy and because of its unidentified structure, the fragments were prepared and compared with the existing library of spectrums (Fig. 2).

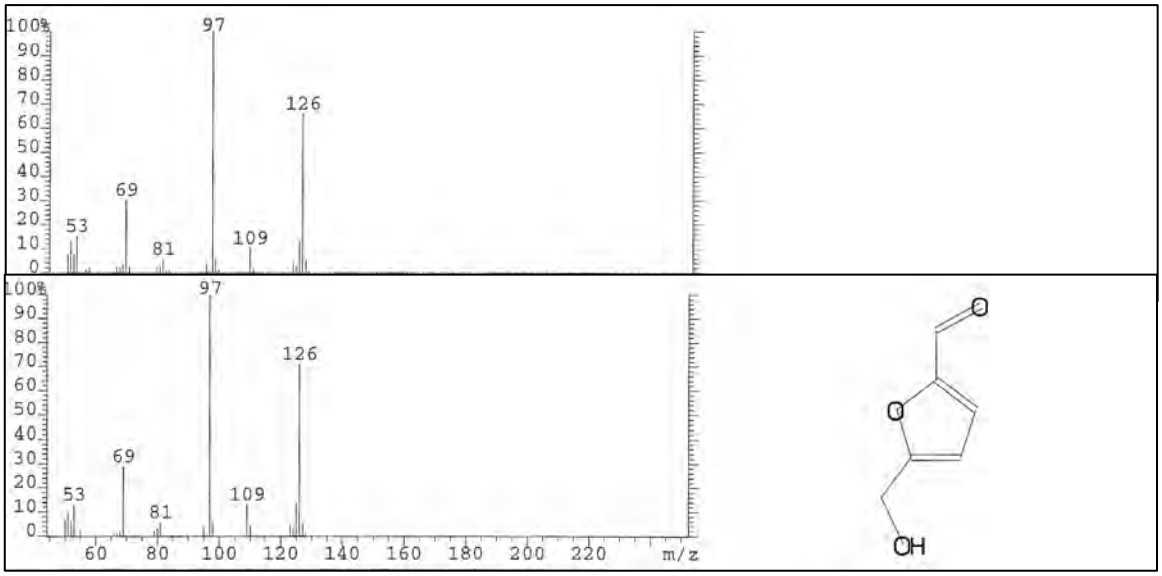

Fig. 2- The spectrum of the eluting compound (on the top) and the suggested library entry which indicates that the it corresponds to 5-Hydroxymethylfurfural

To validate this conclusion, a standard solution of HMF was prepared and checked with the same HPLC method and showed the exact retention time and absorption for the eluting peak.

3.3 Effect of temperature on the concentration

Keeping other subcritical water reaction parameters (pressure and time) constant, concentration profile of the HMF was studied at different temperatures and the results are shown in Figure 3. The pressure and residence time were set to 30 bar and 15 min respectively.

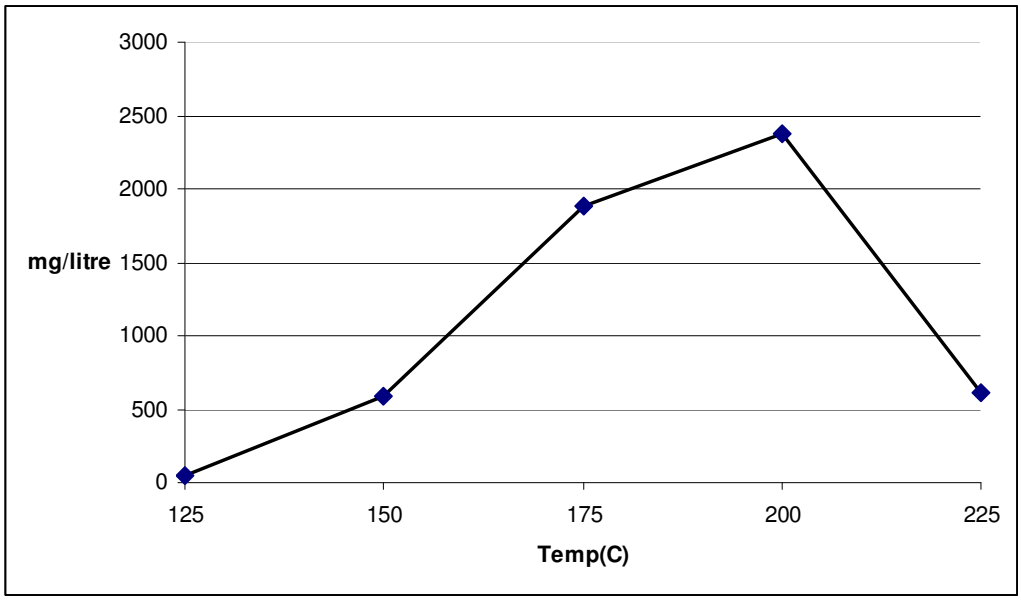

Fig. 3- Concentration of HMF vs. different reaction temperatures

As the figure suggests, the concentration continue to rise until 200 °C and falls sharply at higher temperatures. As suggested earlier, this can be due to the dehydration of the available monosaccharides in the medium or degradation of the yeast cells' wall to simple sugars then followed by dehydration in the subcritical water reaction. The HMF loss at higher temperatures could be due to its decomposition to other components, such as levulinic acid which was detected at very high temperature range as well.

4. CONCLUSION

This work is a part of a larger study whose philosophy is based on the design of a platform which uses environmentally friendly techniques to extract valuable products from biomass wastes. Considering different analytical techniques, we were able to prepare an easy and robust way to determine and quantify a major compound in the hydrophilic fraction of the extract. While studies suggest lower yields of HMF in aqueous solutions (due to degradation to other chemicals) compared to organic mediums (Bicker et al., 2003; Lewkowski, 2003), there is the need of removing this organic solvents in the following steps. A lower concentration of this compound may not seem competitive with other manufacturing routes but it may be advantageous by offering it as an alternative technique to exploit valuable compounds in waste which may have been produced due to thermal reaction or released from the yeast cells during their decomposition of the cells. Using straight forward extraction techniques based on subcritical fluid technology and chromatography separation concepts we are attempting to isolate compounds in other fractions using several fractionation stages.

References

Bicker,M., Hirth,J., and Vogel,H. (2003). Dehydration of fructose to 5-hydroxymethylfurfural in sub- and supercritical acetone. Green Chemistry *5*, 280-284.

Herrero,M., Cifuentes,A., and Ibanez,E. (2006). Sub- and supercritical fluid extraction of functional ingredients from different natural sources: Plants, food-by-product, algae and microalgae: A review. Food Chemistry *98*, 136-148.

Lewkowski,J. (2003). Synthesis, Chemistry and Applications of 5-Hydroxymethyl-furfural and Its Derivatives. ChemInform *34*.

Smith,R.M. (2002). Extractions with superheated water. Journal of Chromatography A *975*, 31-46.