The exploitation of municipal solid waste (MSW) and related waste paper streams in the production of bioalcohol

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Abstract

An organic fraction from municipal solid waste (MSW) comprised 38.9% (w/w) glucose (cellulose and starch) indicating its potential as a substrate for bioalcohol production. Microscopy indicated that the fraction was rich in waste paper fibres. Much paper waste comes from shredded office paper (50.4% w/w glucose) which is unrecyclable because of poor fibre length. This, and microbiological hazards associated with the use of MSW led to its choice as model substrate for study.

Saccharification of shredded paper waste was optimised by selection of Accellerase$^{®}$ and additional beta-glucosidase enabling digestion of 99.27% of cellulose. Sequential batch-addition of substrate permitted substrate "concentrations" equivalent to 25-30% (w/v). Saccharification was enhanced by detergent, but reduced by the presence of alcohols at over 3-4% (v/v).

Steam explosion of paper slightly enhanced saccharification. However, the approach was rejected due to high energy cost, production of fermentation inhibitors at high severities, and lack of clear benefit regarding ethanol yield. Interestingly, levels of inhibitors were low compared to other pre-treated substrates and addition of paper to other substrates greatly reduced their own production of inhibitors during pre-treatment (wheat straw 60%, filter paper 95%).

Larger pilot-scale (1.5-5 L) operations involved developing the batch-addition regime with a high-shear stirring capacity vessel. Additions equating to final substrate concentrations of ~65% (w/v) were achieved (from an initial 5% w/v) and facilitated high ethanol concentrations (11.6% v/v) with minimal enzyme input (3.7 FPU/g substrate).

Thermal tolerance of a range of yeast strains was investigated by developing a rapid screening approach with liquid-handling robotics. This identified strains able to endure temperatures up to 40°C. Evolutionary engineering may improve tolerances to temperatures nearer to enzyme optimums (50°C). Some previously unused strains exhibited superior growth to referenced industrial strains.

The above findings were integrated into a process design along with recommendations for further enhancement.

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1 Introduction

Many modern cars are now able to run on a range of fuel sources, including petrol, ethanol, butanol and diesel but this is not as new an idea as it first appears. As early as 1826, Samuel Morey patented an engine that would run on ethanol or turpentine. Henry Ford's illustrious Model T, which went into production in 1908, was the forefather of the flex fuel vehicle (FFV) being able to run on petrol, kerosene or ethanol. Ford and his colleague, George Washington Carver, also believed that it would be possible to produce enough motor fuel in the form of bioethanol from agricultural feedstocks for all road transportation needs (Jenkins, 1934). However with the discovery of massive oil fields and the presence of prohibition in the USA, it became more cost-effective to use oil derived fuels due to their abundance.

Recently, environmental, economic and social pressures have led to a resurgence and associated rise in production of bioalcohols, with ethanol production more than quadrupling between 2000 and 2010 (Earth Policy Institute, 2012). Legislatively the Kyoto protocol (United Nations, 1998) legally committed the 37 industrialized countries and the European community that participate in the United Nations Framework Convention on Climate Change (UNFCCC) to reducing greenhouse gas emissions to 1990 levels during the period 2008-2012. With these commitments coming to an end in 2012 additional proposals were agreed upon by the European Commission and EU governments. These proposals entail reducing the EU's greenhouse gas emissions by 20% (compared to 1990 levels) while at the same time increasing energy efficiency 20% and the proportion of renewable energy used to 20% all by 2020 (European Commission, 2010). Also the publication of the Stern Review (Stern, 2006), in which the economic impacts of global warming were laid out in detail put further pressure on the UK government to set targets for energy security. In addition to these, the UK has a self-imposed Renewable Transport Fuel Obligation Order which states that all fuel sold must contain at least 5% biofuel from a "renewable source"(Department for Transport, 2007).

Bioalcohols to date have predominantly been produced from sugar-rich bioresources (sugarcane bagasse, corn cobs, wheat straw, sugar beet etc), and whilst these can be considered by some to be a useful resource, their exploitation in this way has several drawbacks. Firstly, crops that would have otherwise entered the food chain are used to produce bioalcohols creating more competition in the market for these crops, thereby inflating prices by introducing abnormal market competition (Brown, 1980). Furthermore, the seasonality of crop production means that the local availability of biomass is not continuous, and would require either storage or transport from other climates, which is not appropriate for low-energy density biomass. This "First Generation" technology is primarily being exploited by Brazil and the USA who currently lead the production, together making around 86% of the world's bioethanol (20 billion gallons/year or 76 x 10^9 L/year)(Licht, 2011).

The industry is now targeting second generation processes where lignocellulosic materials, generally waste corn stalks and wood chips (Kuhad and Singh, 2007), but also lignocellulosic crops such as *Miscanthus giganteus*, are seen as potentially useful sources feedstocks from which to produce bioalcohols. However, the economic viability of lignocellulosic bioethanol is hampered by the complexity and inefficiencies of the process, much of which stems from the variability, availability and heterogeneity of the feedstocks. In particular, aggressive pre-treatments are required to enhance the digestibility of the cellulosic components, hydrolysis is not quantitative at high substrate loadings, and fermentation with yeasts can be hampered by inhibitors created during some pre-treatments (Waldron, 2010).

As lignocellulosic materials are found in such a large range of plant materials the process can be much more efficient, therefore leading to re-establishing greater biodiversity and consequently decreasing the strain on the food chain.

1.1 Municipal Solid Waste

Conversely to the problem of finding renewable sources of bioalcohol, it is becoming increasingly difficult to find environmentally viable ways of disposing of Municipal Solid Waste (MSW). Agro-industrial by-products and indeed household wastes are therefore beginning to be seriously considered as potentially fermentable resources. A large proportion of food, paper and green waste have the potential to be used to some degree in the production of second generation bioalcohols. With millions of tonnes of this resource still being sent to landfill each year in the UK alone, which cost industry and the government financially as well as resulting in significant environmental issues, this could therefore become one of the most important feed stocks for bioalcohol production in coming years.

The amount of MSW collected by local authorities in England over the last 5 years has decreased marginally by an average of 1.6% and is currently standing at 23.2 million tonnes a year (23.2 x 10^9 kg/year). Conversely however recycling now makes up for a larger proportion of waste management, rising from 12% in 2000/01 to 42% in 2011/12 (Defra, 2012). This is due in no small part to the increasing cost of landfill, both in the form of landfill tax and gate fees. Landfill tax has been increasing by £8 a year since 2011 and currently stands at £64 /tonne (1000 kg) and is set to continue to increase until at least 2014 and not to fall below £80 /tonne subsequently (Webb, 1992). Gate fees vary depending on location but range from £12-£55 /tonne for landfill (Waste & Resources Action Programme (WRAP), 2011).

MSW compositions can be seen in Figure 1, revealing organic content of approximately 60% (food waste, garden waste, other organic waste, paper and card, wood and a proportion of textiles).
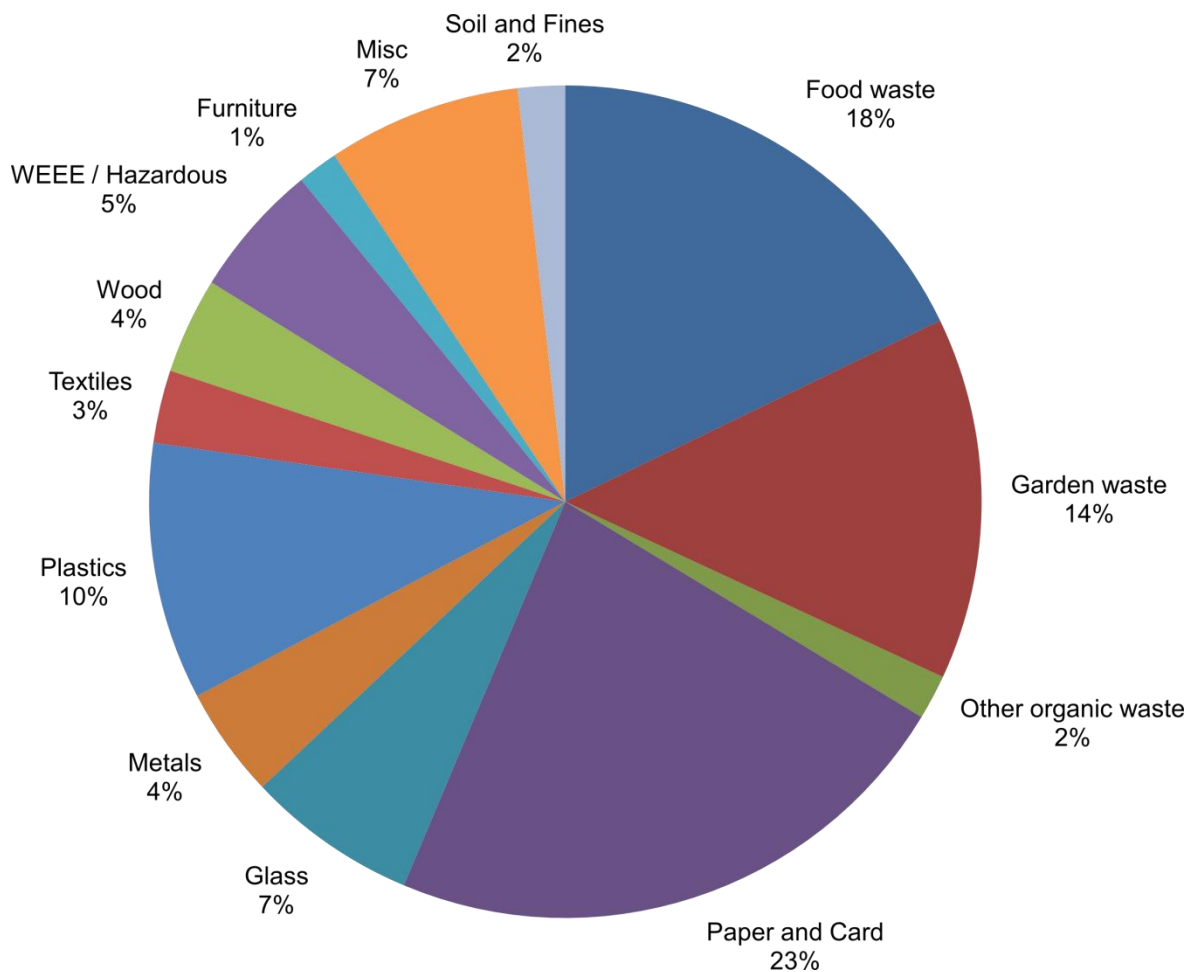
Figure 1. UK MSW Composition, by mass (Defra, 2008)

This project aims to utilise this substrate, which potentially alleviates many of the normal dis-benefits generally associated with bioalcohol feed stocks. MSW is not required as part of the food chain, it is not seasonal, in so much as plant derived lignocellulosic material from crops are, and there is also the added benefit that any substrate used saves monetary and environmental costs of landfill disposal which will therefore act as an additional driver of producing biofuels from this source. MSW therefore becomes a very important source of lignocellulosic substrate as this constitutes a large proportion of the MSW that is un-recyclable and would normally be sent to landfill or another waste management process such as composting or anaerobic digestion. Using this as a source for the bioethanol process would make an important paradigm shift towards using undesirable waste streams as useful substrate.

1.2 Paper and Card

1.2.1 Waste

Paper and cardboard make up a large proportion of lignocellulosic materials expended as waste (23% – Figure 1), Although paper and cardboard can be recycled to a degree, this process is finite since fibre length and strength eventually become too poor to be effectively re-used after four to six cycles (Confederation of Paper Industries, 2011). Equally, shredded paper is no longer suitable for recycling since the fibre length has been irrevocably reduced. There are approximately 60 grades of recovered paper and board including mixed newspapers and printed office papers set out in European Standard EN643 (CEN, 2001).

Compared to MSW, more information of the processing history is available for paper and card, leading to less unknown substances present in the mixture. Hence, uncontaminated paper wastes, such as shredded paper, make a useful and viable initial substrate for experimentation into bioalcohol production.

1.2.2 Production

Generally paper and card pulps are made using processes that are very similar to the pre-treatment steps described in §1.6, higher grade paper pulp is made using the Kraft process (Biermann, 1993), which is not unlike the alkali pre-treatment process. The Kraft (or Sulfate) process is the most widely used lignin removal process in paper manufacture and involves the use of sodium hydroxide and sodium sulphate (white liquor). The sodium hydroxide depolymerises the lignin allowing it to be solubilised out into black liquor (Roberts, 1996).

Virgin pulp
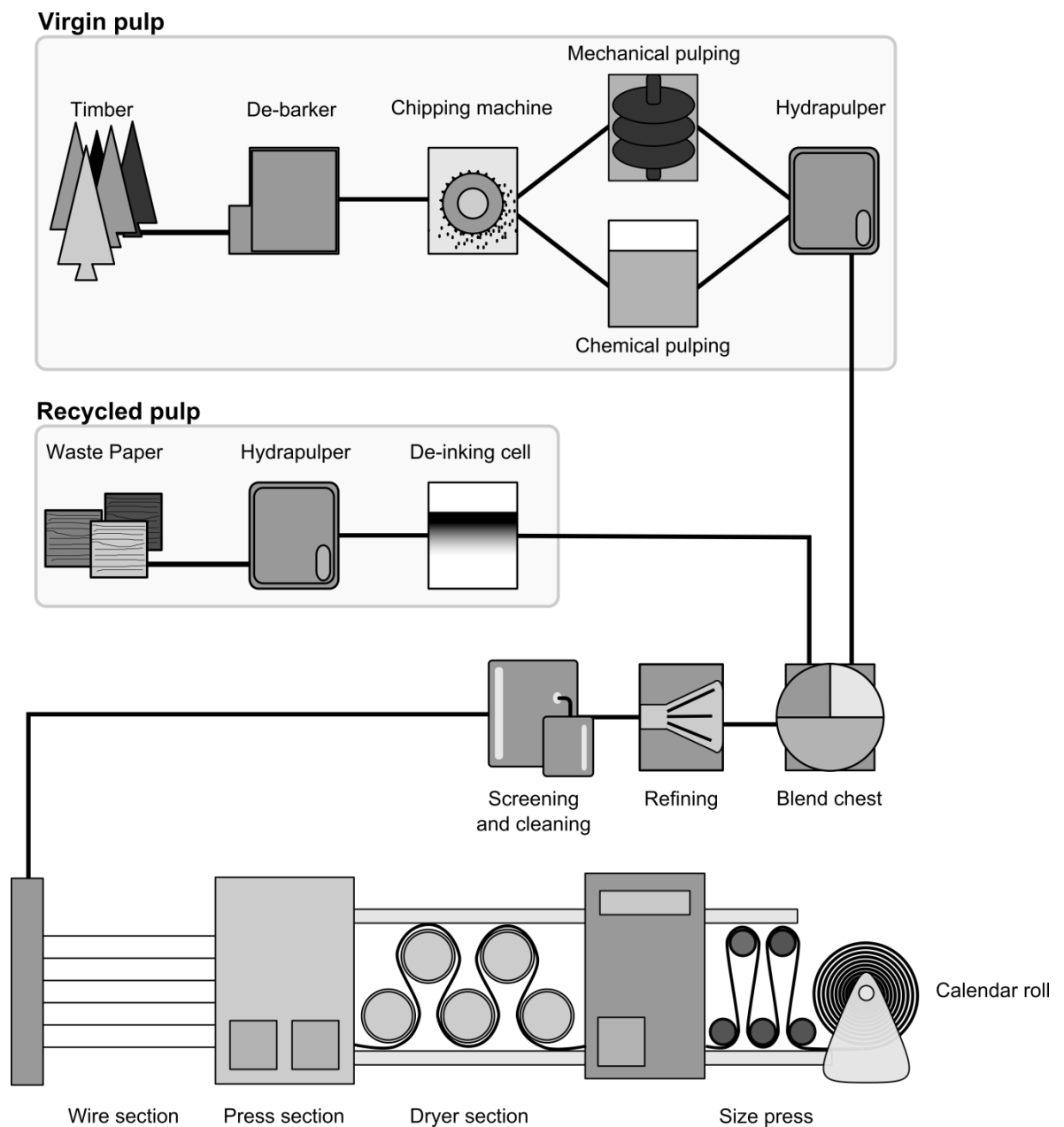
Recycled pulp

Figure 2. Paper mill – process flow diagram

Figure 2 shows the process flow of a typical paper mill. Raw materials come from either managed forests in the case of virgin pulp or waste paper in the case of recycled pulp. A large proportion of paper is made from softwood pulp as it has longer and stronger fibres, but entangle forming poor visual quality so it is mixed with hardwood pulp to make a product of the desired quality (Roberts, 1996). The trees are first debarked as this cannot be used for paper manufacture; the remaining wood is then chipped and pulped. Pulping is completed either mechanically, simply ground with water, or chemically as per the Kraft process.

Mechanically treated pulp tends to be used in high commodity printing such as newspaper and magazines but is likely to contain higher quantities of lignin. Pulp is then broken down in water and unwanted materials are removed in the hydrapulper. This is the first procedure used when processing waste paper, which then additionally has to be de-inked in a flotation process. Depending on the final paper quality required the two steams of pulp (virgin and recycled) are mixed, in given ratios, into the final paper making stock. The pulp is refined, screened and cleaned to the desired level required for the output paper. The paper pulp is then sprayed onto wires to form a wet fibre mat which is then; pressed to remove water, dried, sized and finished with additions such as starch and clay then placed on calendar rolls ready for delivery.

1.3 Substrate advancement

First generation biofuel plants are currently beset with many problems involving competition from food chain manufacturers, seasonality of feedstock, high transportation and agricultural costs. This has led to the search for a commercially viable second generation process, using lignocellulosic materials as a feedstock, alleviating first generation problems but introducing ones of its own, such as enzyme cost and poor efficiency (Gray et al., 2006, Black and Veatch Limited, 2008). Pre-treatment technologies and advances in enzymology and fermentation herald the possibility that fully operational second generation plants are very nearly a reality. However using MSW and paper waste streams as a substrate may effectively step over second generation plants by alleviating operational costs by taking a substrate that is a cost to the producer, due to rising landfill costs, and making it a benefit or even an income source to the bioalcohol industry.

1.4 Plant Cell Walls

Bioalcohol production exploits the abundance of cellulose in plant cell walls. Plant cell walls are comprised of two phases; microfibrillar and matrix (Brett and Waldron, 1996). The microfibrillar phase is constituted from microfibrils, long structures formed from parallel cellulose (§1.4.1) chains, and has a high degree of crystallinity when compared to the matrix phase. The matrix phase is made up

from a number of, but not always all of, the following: pectin, hemicellulose (§1.4.2), protein and lignin (§1.4.3).

1.4.1 Cellulose

Cellulose is a linear chain, β-(1$\rightarrow$4) linked polysaccharide of D-glucopyranose, in repeating blocks of cellobiose, as shown in Figure 3. Cellulose is able to form highly polymerised chains, with wood typically having a degree of polymerisation (DP) in the region of 10,000. Cellulose crystallises in a number of subtlety different polymorphs which are classified as types and numbered I-IV by their differing diffraction patterns. Type I, being the principal form found in nature, is subdivided into Type Iα which is found in bacteria and algae, and Type Iβ which constitutes the higher plants (Brown and Saxena, 2007). Type I cellulose can be converted into Type II, the most structurally stable polymorph, by the mercerization process which involves the use of cold sodium hydroxide (NaOH). This process is typically used on cotton based cellulose for use in the clothes industry. Type III cellulose is made by treatment with amines, giving rise to either Type III$_I$ or III$_{II}$ depending on whether Type I or II was the starting polymorph. Finally Type IV is created at high temperatures (approx. 240°C), again in either Type IV$_I$ or IV$_{II}$ depending on the starting material.
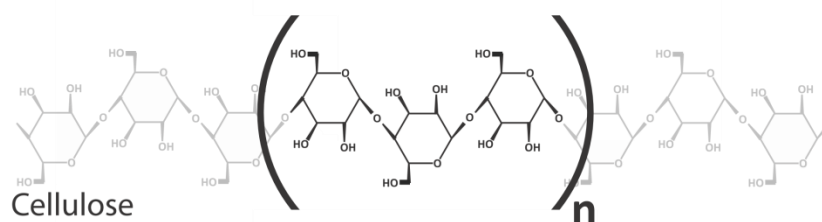
Figure 3. Cellulose chemical structure

Cellulose forms regions of differing crystallinity, with the centres of the microfibrils having a higher crystallinity than the outside and also being interrupted occasionally by amorphous regions (Figure 4). More highly crystalline sections of the microfibril are less susceptible to enzyme attack.

Side view

End view
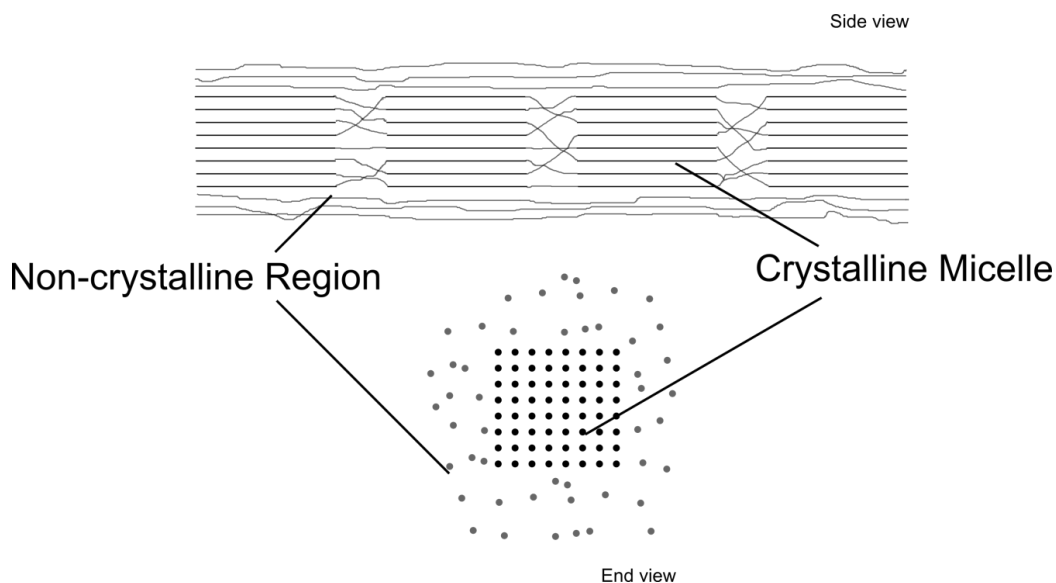
Figure 4. Cellulose crystallinity regions (Brett and Waldron, 1996)

As cellulose can be hydrolysed to D-glucose monomers this makes it desirable as a substrate for second generation bioalcohol production. Furthermore it is the most abundant polysaccharide on earth.

1.4.2   Hemicellulose

Hemicelluloses are the second most abundant polysaccharide in plant cell walls and the term originally included cell wall components that were removed by alkali treatment (Heldt, 1997). Typical examples of common hemicelluloses are shown in Table 1.

Hemicellulose	Backbone
Xylan	β-(1→4) Xylose
Mannan	β-(1→4) Mannose
Glucomannan	β-(1→4) Glucose - Mannose
Galactomannan	β-(1→4) Galactose - Mannose
Xyloglucan	β-(1→4) Xylose - Glucose
Callose	β-(1→3) Glucose

Table 1. Common hemicelluloses (Scheller and Ulvskov, 2010)

1.4.3 Lignin

Lignin is a phenolic polymer which surrounds other cell wall components in a hydrophobic network which binds cellulose fibres together, adds strength and protects the cell wall from attack (Brett and Waldron, 1996). Lignin does also however have the effect of reducing the effectiveness of enzyme digestion on the plant cell wall (Yu et al., 2012).

1.4.4 Starch

Whilst starch is not a cell wall component it is an important plant derived carbohydrate. Starch is a mixture of amylose, α-(1→4) polymer of D-glucose, and amylopectin, α-(1→4) based polymer of D-glucose with α-(1→6) branch points. Starch is likely to be found as a component of the organic fraction of MSW due to its role as an energy storage molecule in plants, and also as it is added to paper as a binding and strengthening agent (Roberts, 1996).

1.5 Production of bioalcohols

Second generation bioalcohol processes convert cellulose-containing plant biomass into bioalcohol in four main steps (Figure 5):

I. **Pre-treatment:**
    To improve accessibility of hydrolytic enzymes to the cellulosic substrate by removing lignin and hemicellulose.

II. **Hydrolysis:**
    The conversion of polysaccharides to soluble carbohydrates, typically by enzymatic or acid hydrolysis.

III. **Fermentation:**
    Glucose and other usable sugars are fermented to produce alcohols; yeast strains are generally used to create ethanol but other micro-organisms such as *Clostridium acetobutylicum* can be used to produce higher-chain alcohols, in this case butanol (Fouad et al., 1976).

IV.   **Distillation/purification:**

Finally the alcohol is distilled or otherwise purified to concentrations that are commercially usable.

These process steps can all be achieved in a myriad of different ways, this leads to the complex array of methodologies moving towards the same goal, that of producing bioalcohol in an efficient manner. An integrated approach to tackling the problems associated with this process is therefore needed if this goal is ever to become reality. Therefore this project tries to look at the whole process and the effects one section has on the others.

Biomass

↓

Pre-treatment

↓ **Cellulose**

Hydrolysis

↓ **Sugar**

Fermentation

Co-products ↓ **Alcohol**

Distillation

↓
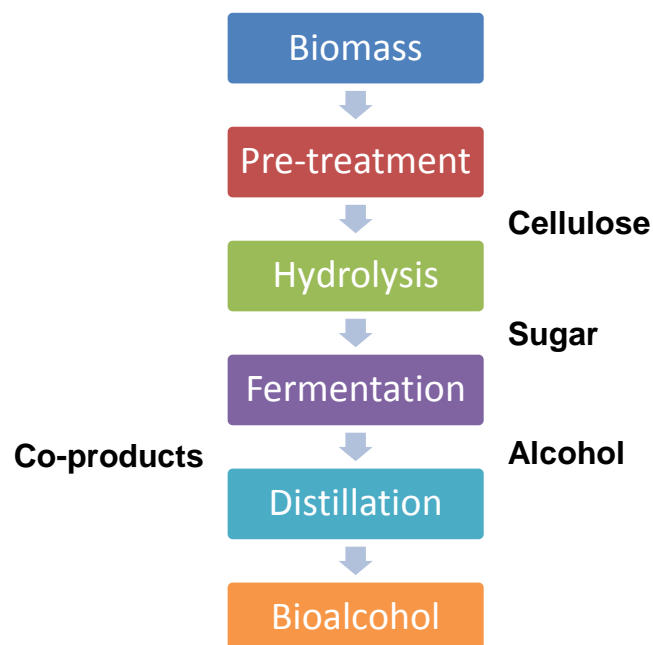
Bioalcohol

Figure 5. Production of bioalcohol from ligno-cellulose – process flow

1.6   Pre-treatment

A wide variety of delignification and cellulose accessibility steps have been postulated, for the use in both paper pulp manufacture and also as pre-treatment steps for bioethanol production. Lignin poses the difficult problem of inhibiting the hydrolysis action of cellulase on lignocellulosic biomass; this can be attributed as one of the main drawbacks of second generation bioethanol plants along with inaccessibility of crystalline cellulose fibres. However there are also a number of different pre-treatment steps that can be used to remove the lignin from the plant

material, and also increase the accessibility to the substrate of the enzymes used in hydrolysis stages by breaking down the crystallinity of the cellulose. The costs and benefits of a number of these processes have been looked at in Taherzadeh and Karimi (2008) and Hendriks and Zeeman (2009). Currently the most widely used pre-treatments include, but are not limited to; steam explosion, alkaline hydrolysis, ammonia fiber expansion (AFEX), liquid hot-water pre-treatment, organosolv, ozonolysis, and dilute- and concentrated-acid hydrolyses.

1.6.1 Organosolv

Organosolv involves using organic solvents, mainly alcohols, such as ethanol, methanol or butanol at high temperatures (100-250°C) and pressures to remove the lignin in a given substrate (Johansson et al., 1987). One of the advantages of using this method is that the dissolved lignin can be simply recovered from the organic solvent by distillation. Organic solvents are also sometimes mixed with acids or alkalis to increase the efficiency of the process; however this can then lead to more complicated methods to recover the solvents used.

1.6.2 Hot water treatment

Hot water treatment, also termed hydrothermolysis, aqueous or steam/aqueous fractionation, uncatalyzed solvolysis and aquasolv (Mosier et al., 2005) has been around at least since the 70's with Bobleter producing a lot of literature on the subject (Bobleter, 1976, Bobleter, 1979, Bobleter, 1981, Bobleter, 1994). Hot water treatment is much like organosolv pre-treatment but using super-heated water, (around 200°C) under high pressure rather than an organic solvent. This treatment is able to remove up to 60% lignin and 90% hemicellulose, there are three different types of reactor used for this process, these are co-current, where a slurry of the substrate is pumped with heated water, counter current, where the water is pumped in the opposite direction as a slurry of substrate, through a reactor, and flow through where hot water is pumped over a bed of substrate and removes the lignin and hemicelluloses as it passes (Mosier et al., 2005).

1.6.3 Ozonolysis

Ozonolysis uses the oxidizing effect of ozone; ozone can be produced by either using ultraviolet light sources or by using a plasma producing electric arc. Lignin is broken down using ozonolysis when the ozone reacts with the C=C bond reducing the lignin content in the substrate by up to 95% (w/w). Low levels of ozone are used to disrupt the inter-monomer bonds and produce aromatic compounds that can be removed easily from the substrate (Quesada et al., 1998), the oxidizing process also increases the accessibility of the substrate to the enzymes.

1.6.4 Acid and alkali

Acid (Sun and Cheng, 2005) and alkali pre-treatment (Hu and Wen, 2008) steps are again much as organosolv and hot water treatment processes, using either acid or alkali in dilute solutions to disrupt the recalcitrant structure of the lignocellulose under a raised temperature and pressure. Although only a dilute acid or base is needed to have the desired effect on the substrate there is the added problem with this method of having to neutralize the acid/alkali before moving on to the enzyme hydrolysis step so as to not disrupt the saccharification. Also there is the increased difficultly over organosolv to recycle the acid/alkali, adding to processing costs.

AFEX (Holtzapple et al., 1991) utilises aqueous ammonia (5-15% v/v) flowing through a biomass packed column at temperatures of 160-180°C (Mosier et al., 2005). This process removes lignin and hemicellulose from the biomass and alters the cellulose from type$_I$ to type$_{III}$ increasing it accessibility.

1.6.5 Steam Explosion

Steam explosion was originally used as a pulping technique in the paper industry but has since been used as a pre-treatment process in bioethanol production (Kokta et al., 1992). Essentially the process involves heating the substrate with high temperature steam under increased pressure for a short period then releasing the pressure of the reactor suddenly, thus making the substrate actively explode under controlled conditions. The high temperatures used in this method cause the

cellulose to actively thermally degrade leading to depolymerisation of the cellulose chain and also the production of fermentation inhibitors such as 5-Hydroxymethylfurfural (5-HMF) and 2-Furaldehyde (2-FA) which are themselves breakdown products of carbohydrate monomers (glucose and xylose, Figure 6) (Jacquet et al., 2011). These products can have the effect of lowering the pH of the reaction thereby increasing the degradation of the cell wall materials. This all has the effect of reducing the lignin content much as in hot water or acid treatments and also severely breaking apart the crystal structure of the cellulose due to the explosion. More details of steam explosion methodology can be found in §2.5.
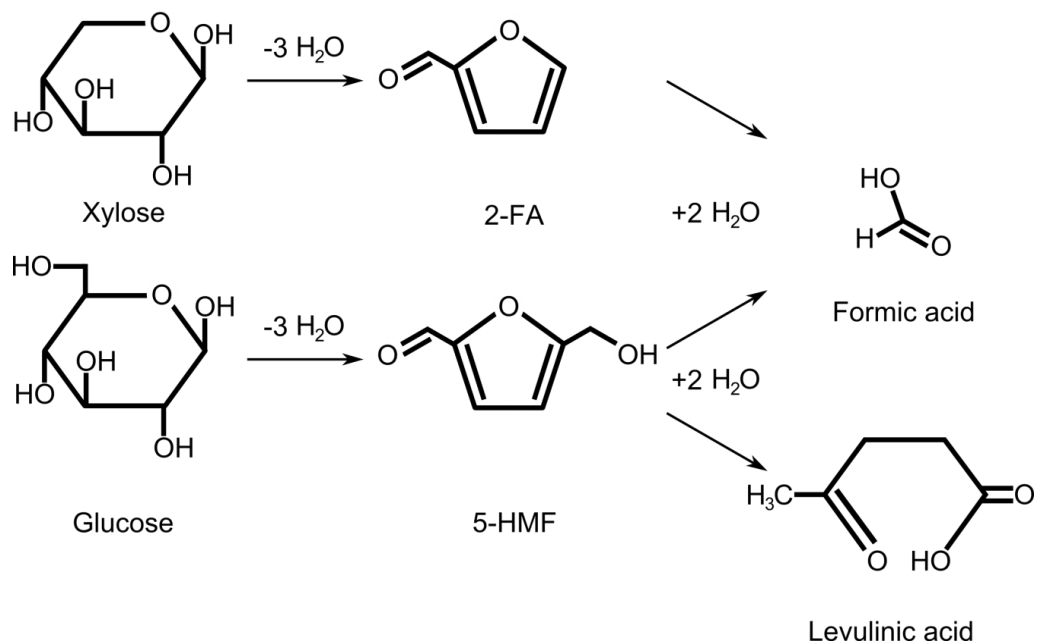
Figure 6. Steam explosion inhibitor formation (Meyer and Pedersen, 2010)

There are several other pre-treatment steps that have not been described above, but the ones listed are currently the most widely used. As well as using a single pre-treatment step some research is also being carried out on the use of multiple or combined methods, such as using ethanol and acid at the same time. Again more information is given in the reviews of pre-treatment by (Hendriks and Zeeman, 2009, Taherzadeh and Karimi, 2008, Eggeman and Elander, 2005). Pre-treatment will likely also be a necessity with regard to sterilisation of the MSW feedstock due to its high microbial activity. Many of the above pre-treatment steps

also have the added consequence of sterilising the substrate, for example hot water and ozone, but it remains to be seen through research which of them will be the most commercially viable and also have the desired pre-treatment and sterilisation effects.

1.7   Enzymatic Hydrolysis

In addition to improving process methods, the demand to develop commercial enzymes preparations that are able to hydrolyse lignocellulosic biomass efficiently has increased to match the rise in ethanol production in recent years. A number of proprietary cellulase enzyme mixes are now being specifically formulated with improved yield, reduced reaction times and, importantly, reduced cost implications in mind for these processes. The increased requirement for these enzymes has meant that the cost, which was until recently the main hurdle to second generation lignocellulose bioalcohol production (Black and Veatch Limited, 2008), has fallen dramatically increasing the possibility of commercially viability.
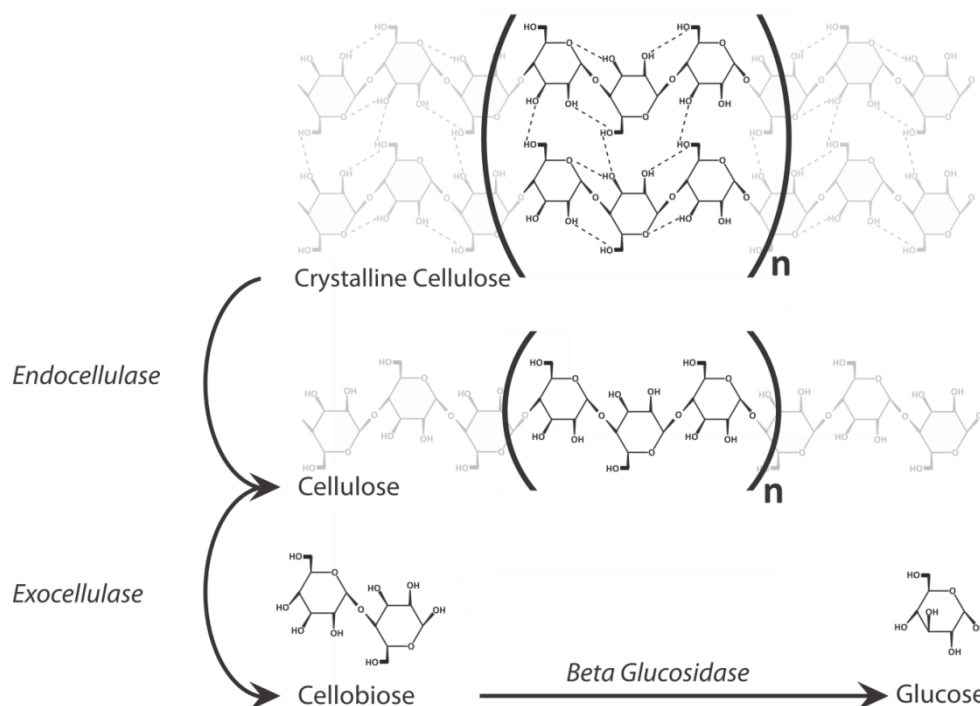
Figure 7. Cellulase action on cellulose substrate

Cellulase is a complex enzyme system and contains a number of different sub-enzymes all performing different processes. Endo-glucanases (EG) are known to breakdown the structure of cellulose from mid-chain active sites focusing on amorphous regions, exocellulases (cellobiohydrolases, CBH) cleave the cellulose chains, from the reducing and non-reducing ends (depending on type), into cellodextrins and ultimately cellobiose, finally beta-glucosidase (βG) degrades the cellobiose into glucose monomers (Figure 7). However, if there is insufficient βG in the enzyme complex then cellobiose will build up as an end product of the CBH and EG. Cellobiose is a well-known and powerful inhibitor of cellulase hydrolysis (Gruno et al., 2004) therefore as its concentration increases the reaction is slowed.

1.7.1 Carbohydrate Binding Modules (CBM)

Cellulase enzymes from a number of fungal and bacterial sources are known contain a CBM as part of their architecture (Boraston et al., 2004). CBMs bind to carbohydrates, in this case cellulose, by aromatic residues and are attached to the catalytic domain of the enzyme by a linker (Figure 8), this effectively concentrates the enzyme onto the surface allowing for accelerated activity. The CBM binding also allows for progressive movement enabling the catalytic domain to carry out its function effectively (Jervis et al., 1997).
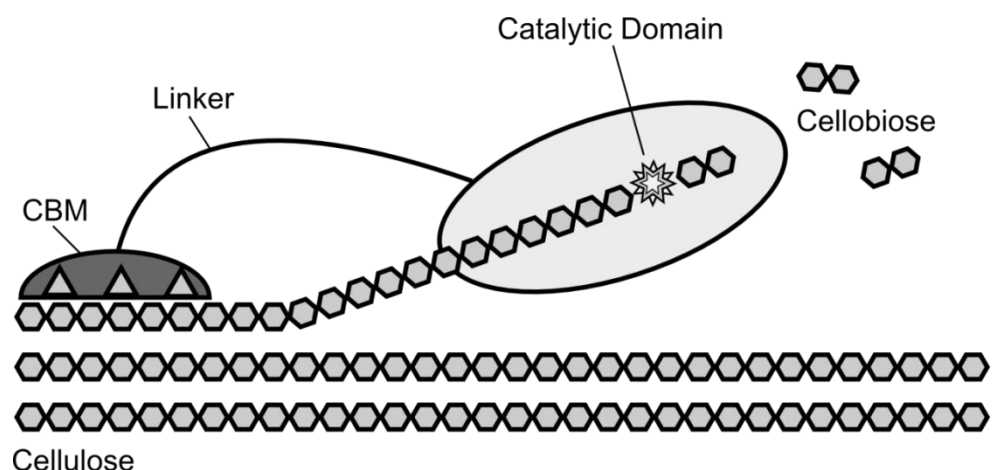
Figure 8. Carbohydrate binding module – adapted from Hildén and Johansson (2004)

1.7.2 Sources of cellulases

A large number of micro-organisms have been reported to produce cellulosic enzymes, largely they are fungi but some bacteria and actinomycetes are included in the list. Table 2 comprises a list of common cellulolytic micro-organisms with *Trichoderma reesei* being the most prevalent and well documented of these (Wyman, 1996).

Cellulolytic Micro-organism		
Fungi	Bacteria	Actinomycetes
Acremonium cellulolyticus	*Clostridium thermocellum*	*Streptomyces sp.*
Aspergillus acculeatus	*Ruminococcus albus*	*Thermoactinomyces sp.*
Aspergillus fumigatus	*Streptomyces sp.*	*Thermomonospora curvata*
Aspergillus niger		
Fusarium solani		
Penicillium funmiculosum		
Schizophyllum commune		
Sclerotium rolfsii		
Sporotrichum cellulophilum		
Talaromyces emersonii		
Thielavia terrestris		
Trichoderma koningii		
Trichoderma reesei		
Trichoderma viride		

Table 2. Common cellulolytic micro-organisms

1.7.3 Enzyme Nomenclature

In the 1950's the number of enzymes being discovered began to increase rapidly therefore necessitating a standard nomenclature system. The International Union of Biochemistry and Molecular Biology (IUBMB, formerly the International Union of Biochemistry) setup an International Commission on Enzymes in 1956, each enzyme is given an EC number based on its reaction separated into subclasses (Webb, 1992). Cellulase enzymes are classified by their reaction as in Table 3.

Enzyme Commission Number					Accepted Name
EC	3.	2.	1.	4	Cellulase (endo-cellulase)
EC	3.	2.	1.	21	Beta-glucosidase
EC	3.	2.	1.	91	1,4-β-cellobiosidase (non-reducing end)
EC	3.	2.	1.	176	1,4-β-cellobiosidase (reducing end)
	Hydrolases	Glycosylases	Hydrolysing O- and S-glycosyl compounds		

Table 3. Cellulase – Enzyme Commission numbers

In addition to this nomenclature method a further system was suggested, CAZy classification, whereby glycosyl-hydrolases are grouped into families based on the hydropathy profile of the amino acid sequence (Henrissat and Davies, 1997). Names are a combination of the enzyme substrate, family number and its order of discovery, for example the first discovered cellulase from family seven would be Cel7A. Although this method gives evolutionary information it doesn't give functional information as the IUBMB systems does (Hildén and Johansson, 2004).

Common Name	Common acronym	CAZy classification
Cellobiohydrolase-I	CBH-I	Cel7A
Cellobiohydrolase-II	CBH-II	Cel6A
Endo-glucanase-I	EG-I	Cel7B
Endo-glucanase-II	EG-II	Cel5A
Endo-glucanase-III	EG-III	Cel12A
Endo-glucanase-IV	EG-IV	Cel61A
Endo-glucanase-V	EG-V	Cel45A
Beta-glucosidase I	BGL-I	Cel3A
Beta-glucosidase II	BGL-II	Cel1A

Table 4. *T. reesei* cellulases (Fengel and Wegener, 1983)

1.7.4 Cellulase Activity

Quantities of enzyme can be expressed as per any other compound in molar amounts; however it is more usual for them to be measured in terms of enzyme activity. This activity is most frequently measured in Units (Mori and Barth, 1999) which are defined as 1 Unit (U) equals 1 µmol substrate converted per minute. As the Unit is expressed in minutes, which are themselves not an SI (Système international d'unités) unit an alternative, the katal, was postulated. The katal is defined as "the catalytic activity that will raise the rate of reaction by one mole per second in a specified assay system" (NREL, 2011), therefore carrying the units of mol/s. Practically the katal is too large for average use and so a more usual unit it the nanokatal (nkat), one unit can be calculated as 16.67 nkat (1 µmol/min = 1/60 µmol/s ~ 16.67 nkat).

In practical terms cellulase activity units are expressed in the production of glucose (final product) rather than the conversion of substrate, as cellulose has no precise molecular weight. Whilst there are a number of assays for cellulase activity the most commonly used are the Filter Paper Assay (FPA) and carboxymethly cellulose (CMCase) (Tsao, 1999). FPA uses Whatman No. 1 filter paper as a standard pure cellulose substrate and an amount of cellulase is added that will achieve exactly 2 mg of glucose product in one hour, this method can be found in more detail in §2.6 materials and methods (Ghose, 1987, NREL, 1996). The CMCase is a similar procedure using soluble carboxymethly cellulose as the substrate and aiming to release 0.5 mg glucose in 30 minutes (Ghose, 1987).

1.7.5 Additional enzymes

Commercial enzyme preparations also tend to contain additional enzymes in the preparation; xylanase (EC 3.2.1.8) being one of the most common and important (Fujii et al., 2009). Xylanase is instrumental in hydrolysing hemicellulose components, therefore aiding access for cellulase.

1.8 Acid Hydrolysis

The main rival to enzymatic hydrolysis of lignocellulose materials is the use of acid hydrolysis, although this technique is not utilised in this project a brief description is given here. Cellulose polymers are cleaved using either concentrated or dilute acid, to produce glucose monomers as a source for fermentation to bioalcohol, much the same as the matching enzyme process. Sulphuric acid is the most commonly used (Wyman, 1996) although many others such as hydrochloric have also been investigated. The process is generally sub-divided into two methodologies, either exploiting concentrated or dilute acid (Taherzadeh and Karimi, 2007). Concentrated acid has the advantage that it can be carried out at lower temperatures (~40°C); however it is necessary to have expensive equipment to resist its corrosive effects. Dilute acid has to be conducted at higher temperatures (~200°C) giving short residence times but has lower sugar conversions. Acid hydrolysis has the additional difficulties in that it requires the acid to be neutralised before moving onto the fermentation step, and unwanted inhibitors can be produced during the process.

1.9 Fermentation

The fermentation stage is where it is necessary to decide on the final desired bioalcohol output of the process. Obligate anaerobes such as *Clostridium* or *Methanogens* are able to produce butanol or methane respectively. Both of these are widely used, *Clostridium* in the acetone, butanol, ethanol (ABE) process (Ezeji et al., 2004) and *Methanogens* in anaerobic digestion (AD). This project however is focussed on facultatively anaerobic yeast, or more specifically *Saccharomyces cerevisiae* and its recognised production of ethanol. Yeasts are widely used in the brewing and baking industries and have been for thousands of years (Berry, 1982), and their metabolism, architecture and growth have been well documented.

Yeast have a typical single cell eukaryotic construction, with an outer cell wall containing internal, membrane enclosed, organelle structures (Walker, 1998) (Figure 9). An important structure in the cell is the mitochondrion; this is where aerobic energy is produced during normal respiration. In aerobic conditions

glucose is catabolised to pyruvate in the yeast cell via glycolysis. The pyruvate is in turn converted to acetyl coenzyme A (acetyl-CoA) which is utilised for energy production in the mitochondrion by way of the Krebs cycle (citric acid cycle) (Berry, 1982).
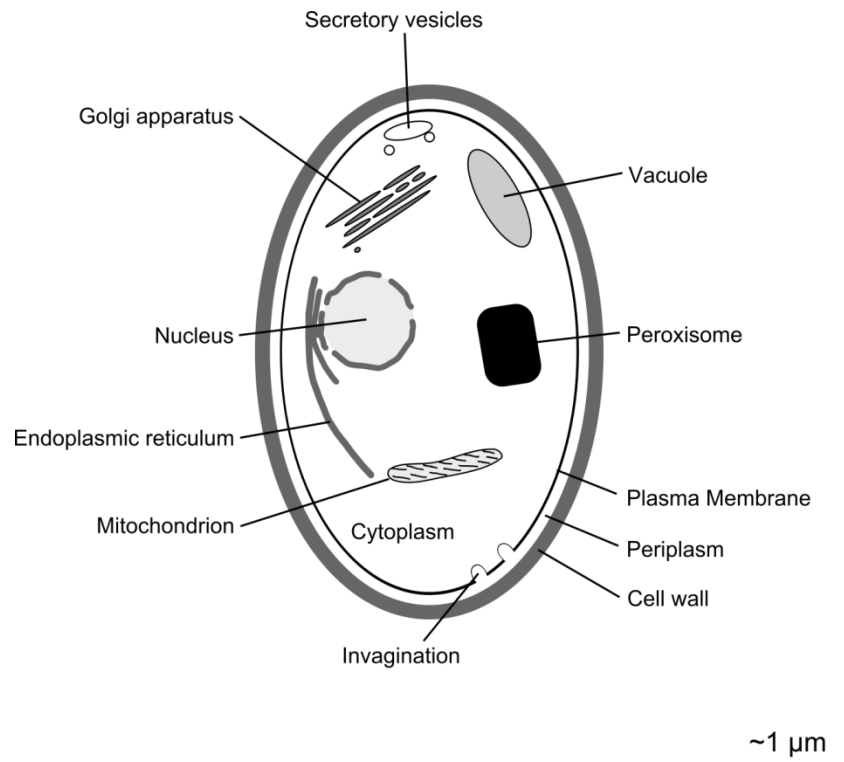
Figure 9. Yeast cell structure (Walker, 1998)

Conversely, in anaerobic conditions pyruvate from the glycolysis process is utilised through fermentation to produce ethanol, via the intermediary of acetaldehyde. Fermentation yields considerably less energy than that of aerobic respiration via the Krebs cycle, only 2 moles of adenosine triphosphate (ATP) are produced compared to 38 moles for every mole of glucose utilised aerobically (Lehninger et al., 2008). This leads to the phenomenon known as the Pasteur effect, whereby more glucose is consumed in anaerobic conditions as compared to an aerobic one (Strathern et al., 1981). Aerobically functioning yeast therefore have more propensity to increase cell biomass as compared to anaerobic ones. Fermentation is also known to occur in aerobic conditions where there are high levels of glucose available, the normal aerobic metabolic pathway is suppressed in preference to fermentation, known as the Crabtree effect (De Deken, 1966), it is postulated that

this is to make use of the anti-microbial effect of ethanol. If glucose is completely used up yeast are also then able to use any ethanol produced as their carbon source, in aerobic conditions ethanol can be reverted to acetaldehyde then to acetate and finally acetyl-CoA which is used in the Krebs cycle as discussed above (Berry, 1982), a simplified metabolic pathway for *Saccharomyces cerevisiae* is given in Figure 10.
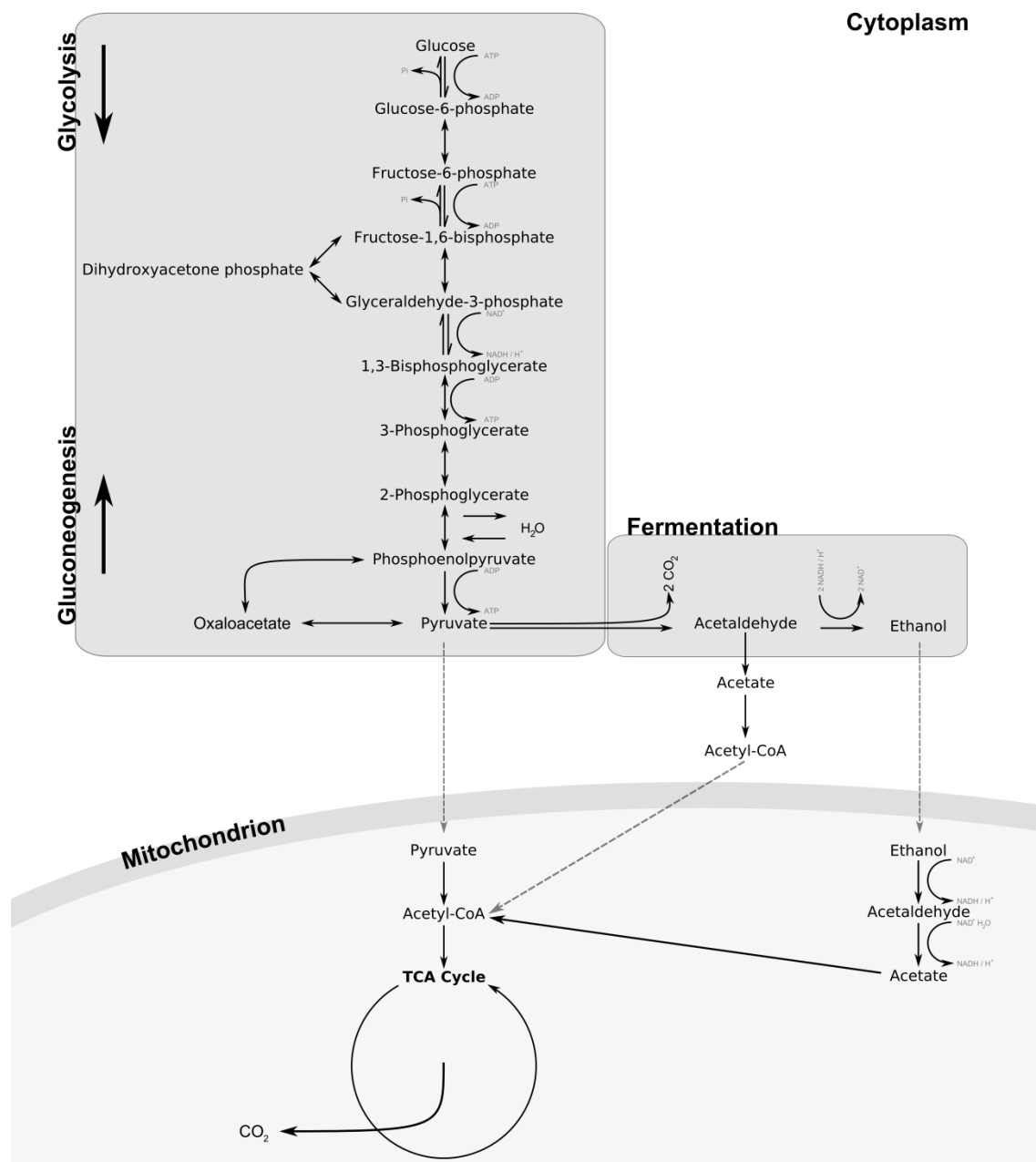
Figure 10. Simplified metabolism pathway for *Saccharomyces cerevisiae* compiled with data from www.biocyc.org (SRI International, 2011)

1.9.1 Bioalcohol fermentation methodologies

Bioalcohol fermentation is conducted in a number of different ways, the most basic of these being separate hydrolysis and fermentation (SHF). This method, as the name suggests, entails enzyme hydrolysis and fermentation being carried out in separate stages, with the output monosaccharides being fed to the fermenting organism after complete hydrolysis. This has the benefit of the individual stages being able to be carried out at their own optimum conditions (temperature, pH etc). Following on from SHF, the idea to improve the process by conducting the two stages together was proposed and was termed Simultaneous Saccharification and Fermentation (SSF) (Takagi, 1977). This system was found to give increased yields over SHF, this is due in part to reduction of end point inhibition of glucose on cellulase (Sun and Cheng, 2002). Moreover, production of alcohol coupled with healthy yeast cultures help prevent unwanted microbial contamination of the process, as per the Crabtree effect discussed earlier in this section. Additionally combining two vessels decreases initial plant cost with respect to industrialisation capital costs. A drawback of SSF is that one or both processes have to work at sub-optimal conditions due to them generally having dissimilar values.

Expansions on SSF methodology include: Semi-simultaneous Saccharification and fermentation (SSSF), Simultaneous Saccharification and Co-fermentation (SSCF) and Simultaneous Saccharification and extractive fermentation (SSEF). SSSF is much like standard SSF but includes a pre-fermentation hydrolysis at optimum conditions to allow high glucose concentrations for the initial yeast inoculum. SSCF provides a mixture of fermenting organisms allowing for both hexose and pentose sugars to be metabolised (Chandrakant and Bisaria, 1998). SSEF aims to remove the alcohol as it is produced by the micro-organism by enabling a constant flow of solvent (e.g. oleyl alcohol) to pass through the reaction chamber (Moritz and Duff, 1996).

A more recent idea is that of Consolidated Bioprocessing (CBP) which adds an extra layer of complexity by introducing a further micro-organism to the microbial community able to produce the appropriate enzymes at the same time as saccharification and fermentation are accomplished (Lynd et al., 2005).

1.9.2 Inhibitors

As discussed in §1.6 pre-treatment methodologies can often lead to fermentation inhibitors being formed. Figure 6 (page 30), highlighted the formation of 2-FA and 5-HMF from xylose and glucose respectively, formic and levulinic acids are also formed as breakdown products of these compounds (Meyer and Pedersen, 2010). Additionally acetic acid can be produced from hemicelluloses and lignin is known to breakdown into phenolic compounds (Bardet and Robert, 1985). These inhibit the fermentation process in different ways; weak acids can enter the cytosol of the yeast altering the pH therefore inhibiting growth, furfurals deactivate cell replication, and phenolic compounds affect the cell membrane disrupting transfer of compounds (Palmqvist and Hahn-Hägerdal, 2000). Table 5, summarises the inhibitory effects of a number of common compounds.

Inhibitor	Conc. (mg/mL)	Inhibition (%)	Reference
Furfural	4.0	79	(Palmqvist et al., 1999)
5-HMF	8.0	50	(Clark and Mackie, 1984)
Acetic acid	6.0	74	(Phowchinda et al., 1995)
Levulinic acid	40.0	50	(Clark and Mackie, 1984)
Vanillin	1.0	25	(Ando et al., 1986)
	1.3	50	(Clark and Mackie, 1984)
4-Hydroxybenzoic acid	1.0	30	(Ando et al., 1986)
Vanillic acid	1.0	2	(Ando et al., 1986)
	3.7	50	(Clark and Mackie, 1984)
4-Hydroxybenzaldehyde	1.0	72	(Ando et al., 1986)

Table 5. Inhibition of *Saccharomyces cerevisiae* ethanol fermentation by common inhibitory compounds adapted from Pienkos and Zhang (2009)

1.10 Bioalcohols

As briefly discussed in §1.9 any number of bioalcohols can become the end product of the process as glucose is a universal substrate for many fermentation processes. The most common bioalcohols produced are; biomethanol (CH_4O), bioethanol (C_2H_6O) and biobutanol ($C_4H_{10}O$). These fuels all have different

advantages and disadvantages, mostly related to their octane and energy density compositions.

1.10.1 Octane rating

Octane ratings define the ability of the fuel to resist self-ignition at high temperature and pressure, the larger the value the better the resistance. Octane is measured in two ways; Research Octane Number (RON) which simulates part throttle conditions (ATSM International, 2011b) and Motor Octane Number (MON) which is conducted at higher temperature and pressure to simulate full throttle conditions (ATSM International, 2011a). These two values can then be averaged to give an Anti-knock index (AKI) also known as a Pump Octane Number (PON).

1.10.2 Bioalcohol comparison

Higher octane numbers are more desirable as they enable engines to be operated at high pressures without fuels self-igniting and causing the engine to knock; this means that overall efficiency is greater. Energy density in a fuel is also desirable as it means that more energy per unit mass can be produced effectively allowing the engine to cover more distance for less fuel. A comparison of the three main bioalcohols is made against typical unleaded petroleum fuel in Table 6.

	Formula	Octane rating		Energy Density
		RON	MON	(kJ/kg)
Unleaded Petroleum	$C_{6.97}H_{14.02}$	95.0	85.0	42.60
Methanol	CH_4O	108.7	88.6	19.85
Ethanol	C_2H_6O	108.6	89.7	26.70
Butanol	$C_4H_{10}O$	96.0	78.0	33.10

Table 6. Bioalcohols comparison chart adapted from Eyidogan et al. (2010)

It can be seen that whilst methanol and ethanol have high octane ratings they suffer from low energy densities and in comparison butanol has better energy density but poorer octane rating. Methanol and ethanol benefit from cleaner burning, releasing less noxious particulates than their counterparts, but suffer from, in the case of methanol, toxicity and in both cases incompatibility with petrol

engines in their pure form due to their corrosiveness. Butanol however is almost directly compatibly with petrol engines and transportation infrastructure. The costs and benefits of each fuel have led to indecision within the industry regarding a definitive choice of bioalcohol for petrol replacement leading to an increase of flexible fuel car manufacture in recent years.

1.11 Distillation

Bioalcohol production by fermentation generates concentrations that are too poor to be used directly in any engine, it is therefore necessary to concentrate the liquor using the process of distillation. Distillation is a well-known procedure used in both industrial bioalcohol processes and also commercial alcohol manufacture, such as for whiskey and other spirits.
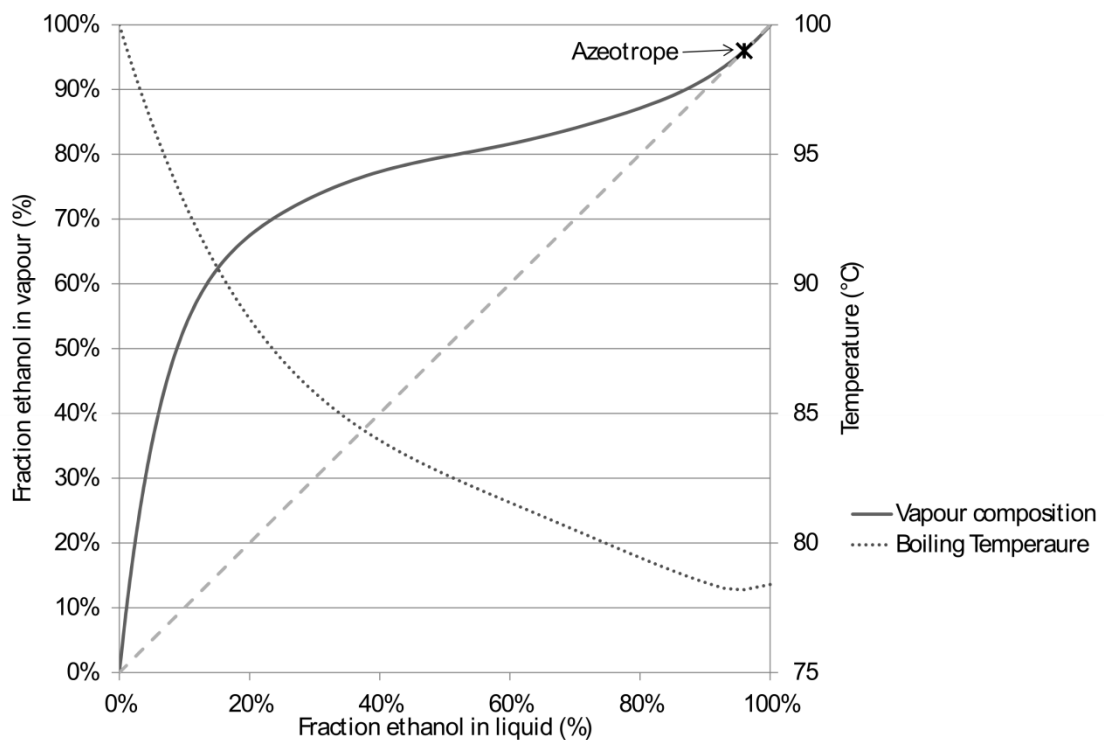
Figure 11. Liquid/vapour composition of ethanol distillation process, including azeotrope point

Distillation works on the principal that the vapour derived from boiling a solution differs in composition to the original solution (Hengstebeck, 1961). An example of the liquid/vapour compositions for ethanol can be seen in Figure 11, where the

vapour concentration crosses the point where liquid and vapour have identical compositions (dashed line) an azeotrope is formed. An azeotrope means that distillation alone is no longer able to increase the concentration of the solution; ethanol is known to form an azeotrope at 95.6% (v/v).

There are three main methods of distillation; differential, flash (or equilibrium) and rectification (Coulson et al., 1991). Differential is exemplified by a simple batch distillation where the solution is boiled in a still pot and the resulting vapour is removed and condensed immediately and is generally operated on a batch process. Flash involves heating the solution and then reducing the pressure as it passes into the still, therefore vaporising it. The vapour can then be condensed and removed from the still, this process is normally operated using a continuous basis. Finally rectification, probably the most widely used method, operates using a sectioned still where the vapour is condensed and re-vaporised several times in the course of a production run. The best example of this method is the factional still typically used in the petrochemical industry and is again generally run on a continuous basis.

While distillation will not form part of this project, from an integrated point of view, it is still important to take into consideration still design and operation to enable the best overall process economy. With this in mind it can be seen from Figure 12 that an important consideration with regard to steam usage, and therefore energy consumption, is the starting concentration of the fermentation liquor, with higher ethanol concentrations requiring less energy per volume to separate.

As mentioned above ethanol is known to form an azeotrope at 95.6% (v/v) this means that distillation alone will no longer yield higher concentrations of ethanol above this point. However there are a number of methods to increase the purity of the ethanol; drying with lime, molecular sieves, membranes, entrainer, and pressure reduction. The two most commonly used methods are entrainer and molecular sieves. Entrainer or azeotropic distillation evolves using an additional component (typically cyclohexane) which creates a three component azeotrope that boils at a temperature lower than any of the components in the mixture. The tertiary azeotrope requires more water than is available in the binary ethanol water

azeotrope and so ethanol is rejected and separates allowing it to be collected (Katzen et al., 2003). Molecular sieves use a bed of zeolite with a strong affinity to water and pore size smaller than ethanol (4.4 Å) but larger than water (2.8 Å), typically around 3 Å. Water is therefore attracted to the pores but ethanol is able to pass freely through the bed, leading to a pure ethanol product at the end of the process (Bibb Swain, 2003).
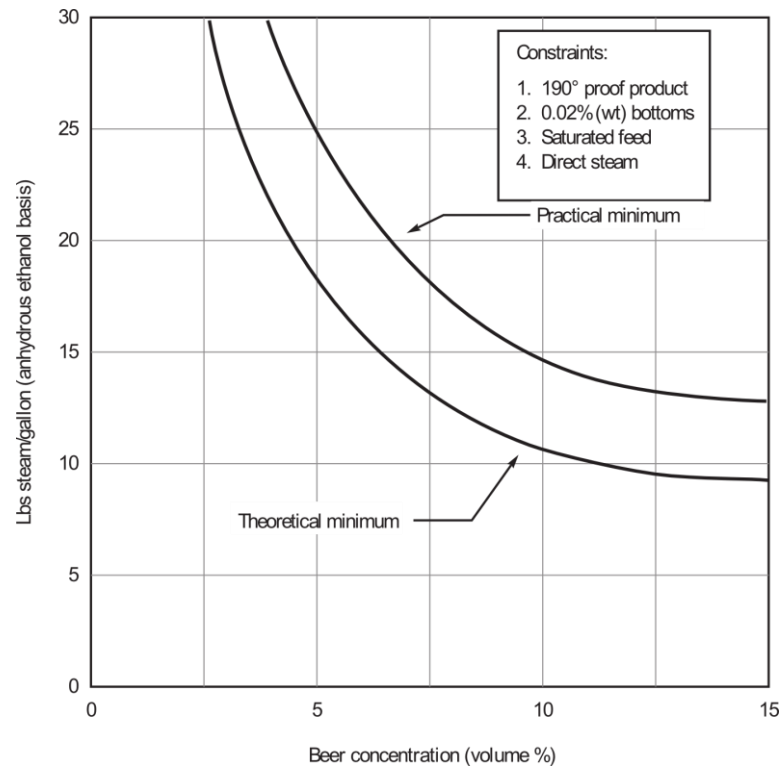
Figure 12. Distillation steam requirements (Katzen et al., 2003)

1.12 Co-products

The process of creating bioalcohol from waste will also produce a number of co-products that may also have economic significance, these may include compounds such as lignin and non-hydrolysed portions of the cellulose in the waste being compressed and used as solid fuels or building materials. There may also be other as yet undetermined co-products that could be reclaimed and utilised in a biorefinery approach that will help increase the economic viability of the process.

1.13 Aims of the research

The overall aim has been to improve the production of cellulosic ethanol from municipal solid waste with a focus on paper waste streams. This was broken down into key objectives to allow this to be an achievable goal.

1.13.1 Objective 1: Characterisation of waste

As MSW is a heterogeneous material thorough characterisation will be necessary to ascertain it's viability as a substrate for bioalcohol production. Little information is available on truly mixed MSW, with most literature utilising fractions of waste rather than as a whole, for example Li et al. (2007) where actual waste streams comprised of carrot and potato peelings, grass, paper and card. Ballesteros et al. (2010) looks at MSW after a steam pre-treatment, however again with such heterogeneity characterisation of an individual waste stream is still necessary.

With regard to paper substrates there are relatively few studies on exploiting solid paper based waste (Kuhad et al., 2010, Vynios et al., 2009, Wang et al., 2012, Wayman et al., 1992), with more focus on wet paper sludge (Banerjee, 2011, Kang et al., 2011, Linderoth, 1989) even though such substrates provide a good basis for developing the cellulose to ethanol process.

1.13.2 Objective 2: Optimisation of enzyme digestion

The use of enzymes to capitalise on cellulosic biomass sources has increased exponentially, due to the renewed interest in biofuels, with novel cellulase enzyme mixes now being formulated with improved sugar yield and reaction times. The efficacy of these mixtures with respect to innovative MSW/waste paper substrates, as opposed to more common ones (Singh et al., 2009), has yet to be thoroughly evaluated. It was therefore considered necessary to assess and optimise the combination and concentration of commercially available enzymes required to saccharify the greatest quantity of cellulose from MSW substrate; whilst overcoming the very great limitations caused by substrate concentration (see below).

1.13.3 Objective 3: Optimisation of Fermentation methodology

High ethanol production is necessary in order to maximise the overall efficacy of the process with regard to distillation energy efficiency as discussed in §1.11. It is therefore important to choose a yeast strain with high ethanol tolerance to allow production of these desired levels. The genus of *Saccharomyces* is widely used in the fermentation process and this was the starting point of experimentation, with the availability of the National Collection of Yeast Cultures (NCYC) at IFR allows for other strains to be evaluated rapidly.

1.13.4 Objective 4: Substrate concentration

In §1.11 the necessity for high ethanol concentrations in order to reduce the energy required for distillation is clear (Katzen et al., 2003). In order to achieve this requirement substrate concentrations at levels considerably above those currently utilised (Modenbach and Nokes, 2012) will be essential. It is therefore important to develop approaches and methodology that enables saccharification and latterly fermentation at high substrate concentration.

1.13.5 Objective 5: Integration and feasibility of overall process

The process as a whole should be continually evaluated with respect to pre-treatment, enzyme hydrolysis and fermentation in an integrated manner, taking into account products from one step that may have inhibitor effects on another. The feasibility of scaling the process to potential commercialisation was also a significant interest in this study.

2 General Materials and Methods

2.1 Chemicals and Reagents

A list of frequently used materials along with their suppliers is given in Table 7. All other chemicals and reagents used were of analytical grade and supplied by Sigma-Aldrich Ltd (UK) unless otherwise stated.

Material	Supplier
96 Deep well plate	Fisher Scientific UK Ltd, Loughborough, UK
96 well micro-titre reader plate	Fisher Scientific UK Ltd, Loughborough, UK
96 well PCR plates	Fisher Scientific UK Ltd, Loughborough, UK
Acroprep^{TM} 96 filter plate 0.2µm GHP	VWR International Ltd, Lutterworth, UK
Chromacol 300 µL glass vials	Essex Scientific Laboratory Supplies Ltd, Hadleigh, UK
Difco YM Media	Fisher Scientific UK Ltd, Loughborough, UK
GF/C filter paper	Fisher Scientific UK Ltd, Loughborough, UK
M-Real Evolve Copier paper	The Premier Group, Birmingham, UK
Screw cap tubes	Starlab (UK) Ltd, Milton Keynes, UK
Sigmacell® Cellulose, Type 20	Sigma Aldrich, Gillingham, UK
Sterilin universal containers	Fisher Scientific UK Ltd, Loughborough, UK
Thiomersal	Sigma Aldrich, Gillingham, UK
Whatman 0.2 µm PVDF syringe filter	Fisher Scientific UK Ltd, Loughborough, UK
Whatman No. 1 filter paper	Fisher Scientific UK Ltd, Loughborough, UK
Yeast Nitrogen Base	Formedium, Hunstanton, UK
Enzymes	
Accellerase$^®$ 1000	Genencor, Rochester, N.Y., USA
Accellerase$^®$ 1500	Genencor, Rochester, N.Y., USA
Acid Cellulase	NBS Biologicals, Huntingdon, UK
C013L	Biocatalysts Ltd, Cardiff, UK
Celluclast$^®$	Novozymes Corp, Bagsvaerd, Denmark
Depol^{TM} 740L	Biocatalysts Ltd, Cardiff, UK
Novozyme 188 (βG)	Novozymes Corp, Bagsvaerd, Denmark

Table 7. Frequently used materials and their supplier information

2.2 Gas Chromatography (GC)

2.2.1 Analytical Theory

GC analysis is used where desired analytes are able to be directly volatilised, or where they can be transformed into another form which can itself be volatilised. Samples are injected and immediately volatilised into a stream of inert gas (carrier gas or mobile phase), typically Nitrogen or Helium. The flow of gas forces the sample through a capillary column (stationary phase) which is designed to separate the compounds in the analyte. The stationary phase is a layer of liquid or polymer on a solid support which is chosen for its affinity to the desired compounds in the analyte. The compounds interact differently with the column causing their elution (or retention) times to differ and therefore be detected separately by a given detection method.

Figure 13 shows a schematic of a GC and includes a Flame Ionisation Detector (FID) which was used for the analyses in this project.

2.2.2 Flame Ionisation Detector (FID)

The FID pyrolyses the sample using a hydrogen flame, thus producing a stream of carbon ions. These ions can are then detected by oppositely charged electrodes where the ions induce a current. This current is amplified and the data can then be collected and analysed. FID is useful where the analyte is a carbohydrate and is therefore why it is used here. One drawback of FID is that it completely destroys the sample.
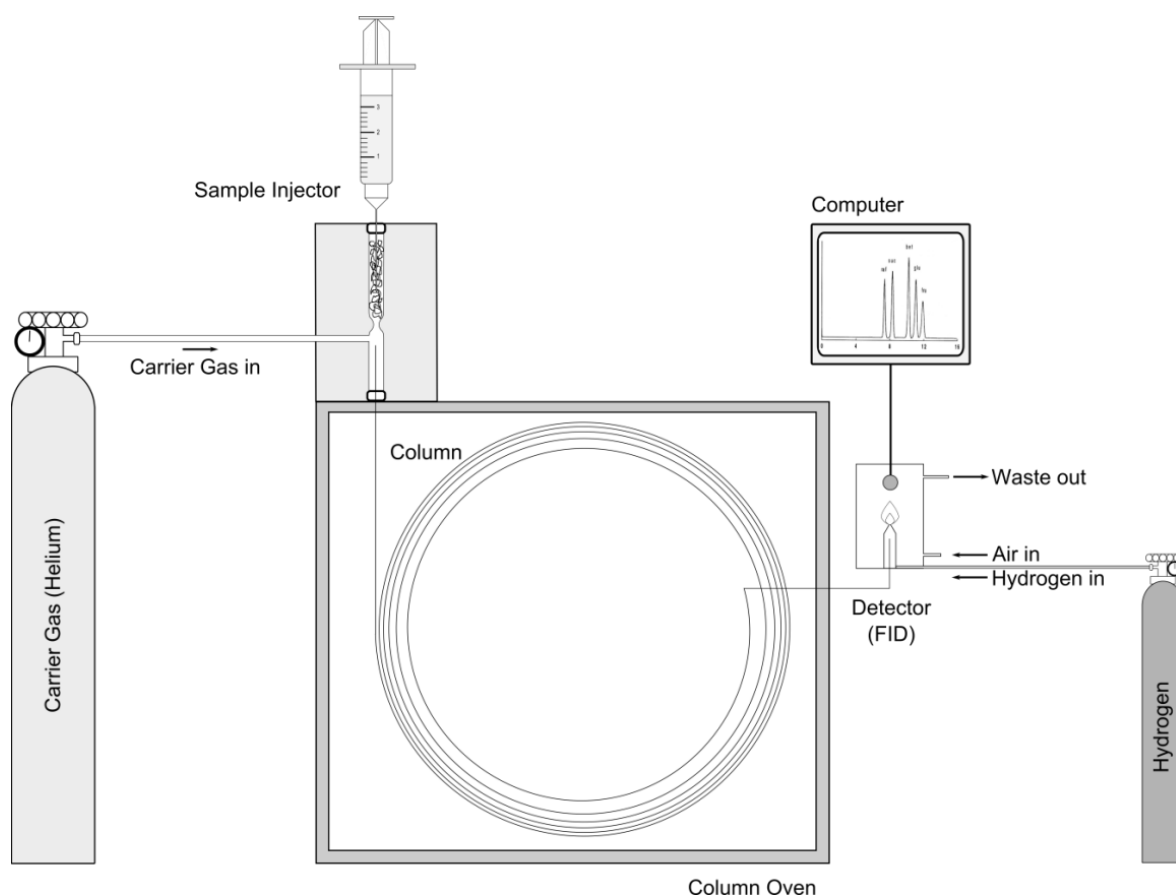
Figure 13. Gas Chromatograph Schematic – with flame ionisation detector

2.2.3 Analysis of carbohydrate by GC

This method was based on Blakeney et al (1983). Dried Solid residues samples (2-4 mg) were hydrolysed to monosaccharides using an adapted Saeman hydrolysis method (Saeman et al., 1945), 200 µL 72% (w/w) H_2SO_4 at room temperature for 3 hours (with occasional stirring) followed by dilution to 1 mol/L H_2SO_4 by addition of 2.2 mL ultrapure water then incubated at 100°C for 2.5 hours. 0.5 mL of sample was taken after the first hour at 100°C for performing uronic acid analysis, see section § 2.9.1. Samples were cooled on ice and 200 µL 1 mg/mL 2-deoxyglucose (2-DOG) was added to act as an internal standard. 1 mL of sample was transferred to a clean tube and the H_2SO_4 was neutralised with 300 µL NH_3 and verified to be pH 8-9 with universal indicator paper. A 150 mg/mL solution of $NaBH_4$ was made in 3 mol/L NH_3, 100 µL was added to all samples and incubated for 1 hour at 30°C to reduce the carbohydrate samples. 200 µL acetic acid was then added over ice to destroy any excess $NaBH_4$. 300 µL of sample, 450 µL 1-

methylimidazole and 3 mL acetic anhydride were combined in a clean tube and incubated at 30°C for 30 minutes to acetylate the sample. 3.5 mL ultrapure water was added on ice to destroy any excess acetic anhydride and 3 mL dichloromethane (DCM) added to enable a solvent extraction. The non-organic layer was aspirated, then a further 3 mL ultrapure water was used to wash the organic phase twice more, aspirating the non-organic layer each time. Samples were then dried in a sample concentrator (Bibby Scientific Limited, Stone, UK) under a stream of nitrogen. Samples were reconstituted in 1 mL acetone and then transferred to glass vials for GC analysis using a Perkin-Elmer Autosystem XL (Perkin Elmer, Seer Green, UK) and a RTX-225 (Restek, Bellefonte, USA) column. GC method can be found in Appendix A. An alternative sugars analysis method involved using only 1 mol/L H_2SO_4 to determine the quantity of non-cellulosic carbohydrate. The method was essentially the same but missed the 72% (w/w) H_2SO_4 step just performing the 1 mol/L hydrolysis for 2.5 hours at 100°C and continuing from there. In both cases standards were made by accurately weighing anhydrous sugars (glucose, xylose, fucose, rhamnose, arabinose, galactose and mannose) and adding 2-DOG internal standard in the same proportions as the samples. Dilutions were made to create a standard curve that encompasses the likely sample concentrations and this was run at the same time as the samples.

2.3 High Performance Liquid Chromatography (HPLC)

2.3.1 Analytical Theory

HPLC involves similar principals to GC utilising mobile and stationary phases to separate compounds from a given sample, a schematic can be seen in Figure 14. HPLC is divided into two types, normal phase and reverse phase, reverse phase being the most commonly used. The difference between then being with normal phase it is the silica packed column that is polar and the solvent is not, and reverse phase is the opposite with the column having C_8 or C_{18} covalently bonded to the silica to make it non polar. Typical mobile phase in the case of reverse phase is an aqueous or organic liquid, for example, water, dilute acid, methanol or acetonitrile. This is moved through the system via a highly controlled pump which is able to give a constant pressure and flow rate. The stationary phase is chosen based on compound that are to be separated. Separation occurs based on the

polarity of the compound and it's affinity to the column chosen. Once it has passed through the column it can then be detected with an appropriate detector, the most common of which; Refractive Index (RI), Diode Array (DAD) and Photo Diode Array (PDA) are described below.
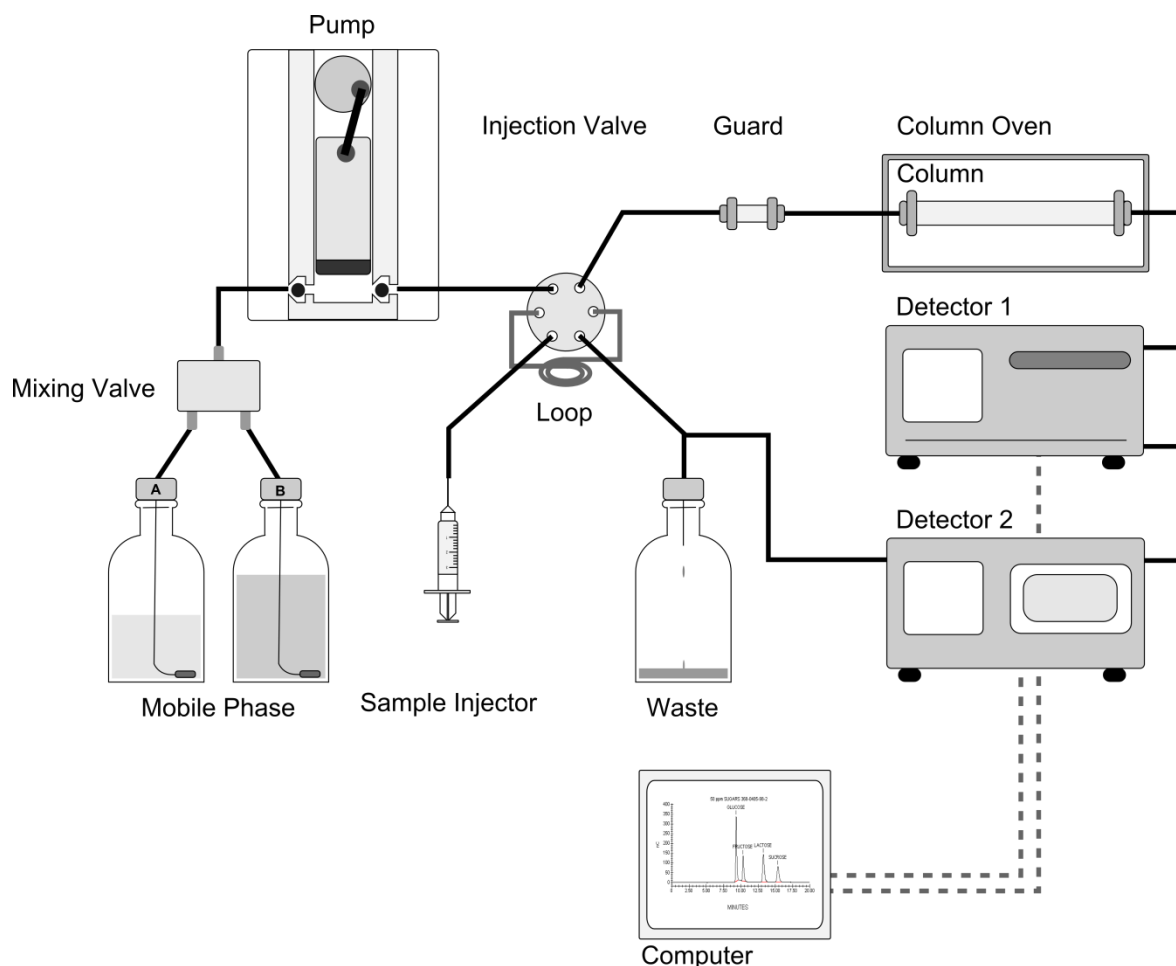
Figure 14. High Performance Liquid Chromatograph schematic

2.3.2 Diode-array detector (DAD)

The DAD is the most commonly used Ultraviolet (UV) or Ultraviolet and visible light (UV-VIS) detector. Light is passed through a flow cell and then is dispersed on passing through a diffraction grating. The amount of dispersed light for a range of wavelengths is then able to be detected in the detector; a diagram can be seen in Figure 15. This enables complete absorbance spectra to be recorded for each time sampling point. The DAD is therefore able to produce both, a chromatogram for a given wavelength, where the sample is expected to absorb strongly, and also

spectra for each time point. This means that the purity of each peak can be investigated to ascertain problems such as co-elution of multiple compounds.
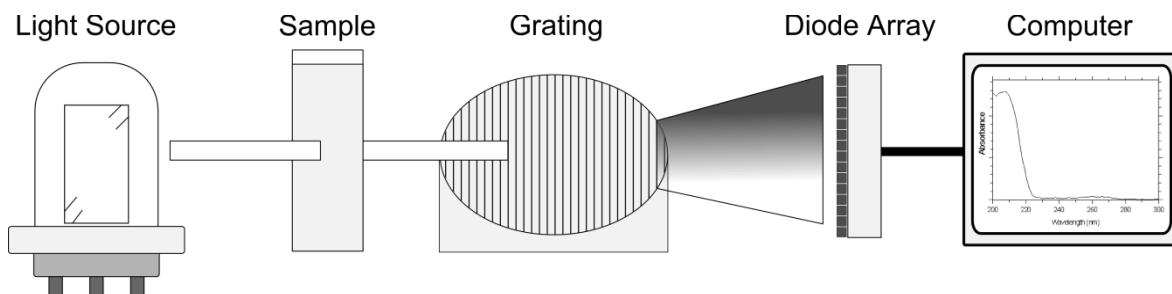
Figure 15. Diode-array detector diagram

2.3.3 Refractive Index (RI) Detector

Another common detector utilises the different refractive indices of materials. The RI detector works first by equilibrating a split flow cell with the currently used mobile phase. Once this is complete the reference side is kept static and the sample is passed through the opposite side. The difference in refractive index causes light passed through the cell to be deflected and this change can be measured. An example diagram can be found below (Figure 16).
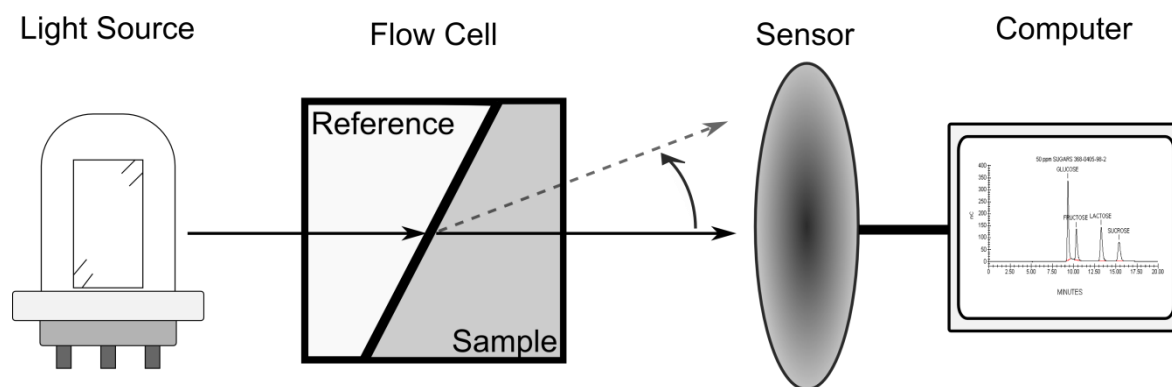
Figure 16. Refractive Index detector diagram

2.3.4 HPLC general sample preparation

Unless otherwise stated HPLC samples were boiled for 10 minutes in a water bath to denature any residual enzymes and/or yeast. Residual solids were then removed by centrifugation at 13,000 rpm for 5 minutes. Supernatant was filtered

using 0.2 µm PVDF syringe filters into 300 µL Chromacol glass vials, and capped securely.

2.3.5 Analysis of carbohydrate by HPLC

Whilst similar to the GC version of this method, the HPLC version's sample preparation was substantially faster and was based on a Nation Renewable Energy Laboratory (NREL) procedure (NREL, 2011). 10-15 mg sample was weighed out into glass tubes with screw top lids. 200 µL 72% (w/w) H_2SO_4 was added to all samples and incubated for 3 hours at room temperature. Acid was diluted to 1 mol/L by the addition of 2.2 mL ultrapure water and incubated again at 100°C for 2.5 hours. An internal standard of *myo*-inositol was made at a concentration of 10 mg/mL, 100 µL was added to all samples. Samples were carefully neutralised over ice with a 2 mol/L $CaCO_3$ solution, under constant stirring to keep it homogenous. Samples were filtered through AcroPrep^{TM} 0.2 µm GHP Membrane 96 Well Filter Plates in a centrifuge (Eppendorf, UK) at 500 rpm for 10 minutes into a 96 deep well collection plate. The plate was sealed and loaded directly onto a Series 200 LC instrument (Perkin Elmer, Seer Green, UK) equipped with a refractive index detector. The analyses were carried out using an Aminex HPX-87P carbohydrate analysis column (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) with matching guard columns operating at 65°C with ultrapure water as mobile phase at a flow rate of 0.6 mL/min. Standard curves of anhydrous sugars were produced, but a partial disadvantage of this method when compared to the GC version was that some of the carbohydrates co-elute. Glucose and 2-DOG (12.5 minutes), galactose and rhamnose (14.4 minutes), and arabinose and fucose (15.51 minutes) meaning that 2-DOG cannot be used as an internal standard in this case. An alternative internal standard of *myo*-inositol (cyclohexane-1,2,3,4,5,6-hexol) was therefore used for this method as it elutes after 33 minutes. HPLC method can be found in Appendix B.

2.3.6 Dissolved carbohydrate by HPLC

Concentration of dissolved carbohydrates were directly analysed using the HPLC method described above §2.3.5 and sample preparation as in §2.3.4.

2.3.7 Organic acids/inhibitors by HPLC

Levels of organic acids were analysed by HPLC using the Series 200 LC instrument equipped with both a refractive index detector and photodiode array detector reading at 210 nm. An Aminex HPX-87H organic acid analysis column (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) and matching guard operating at 65°C with 0.005 mol/L H_2SO_4 as mobile phase at a flow rate of 0.6 mL/min. HPLC method can be seen in Appendix C. Standards of known inhibitors (5-Hydroxymethylfurfural (5-HMF), 2-Furaldehyde (2-FA), Acetic Acid, Formic Acid) were made at known concentrations and samples are prepared as in §2.3.4.

2.3.8 Size-exclusion chromatography (SEC)

SEC separation is based solely on molecular size and there is no interaction by the analyte with the column (Mori and Barth, 1999). The column has a precise array of pore sizes, smaller molecules are able to penetrate these and therefore access a greater volume of the column thus increasing their elution time. SEC is useful as it can be used to separate a complex assortment of samples exclusively based on molecular size.

Molecular mass profiles of hydrolysed samples were produced by HPLC-SEC using a serial array of: TSK Gel G8000, TSK Gel 4000 and TSK Gel 3000 columns (Tosoh Bioscience, Tokyo, Japan). A Series 200 LC instrument equipped with a refractive index detector was used. 0.2 mol/L Sodium Nitrate ($NaNO_3$) with 0.05% (w/v) sodium azide constituted the mobile phase and was maintained at a 0.5 mL/min isocratic flow rate at 35°C. A polysaccharide calibration kit (Polymer Laboratories, Church Stretton, UK) containing polysaccharides ranging from molecular masses of 1,660,000 to 180 (glucose) with the addition of cellobiose was used to create a calibration curve. HPLC-SEC method can be found in Appendix D.

2.4 Fourier transform infrared (FT-IR) spectroscopy

Infrared (IR) radiation is passed through a sample; a proportion of this radiation is absorbed in the molecular bonds of the compound leading to unique transmission

spectra. The original spectrum is converted by computer from time domain to frequency domain using a mathematical technique called Fourier transform, this allows a spectrum of frequencies to be easily analysed. FT-IR is widely used as it is a fast and non-destructive technique, a schematic can be seen in Figure 17.
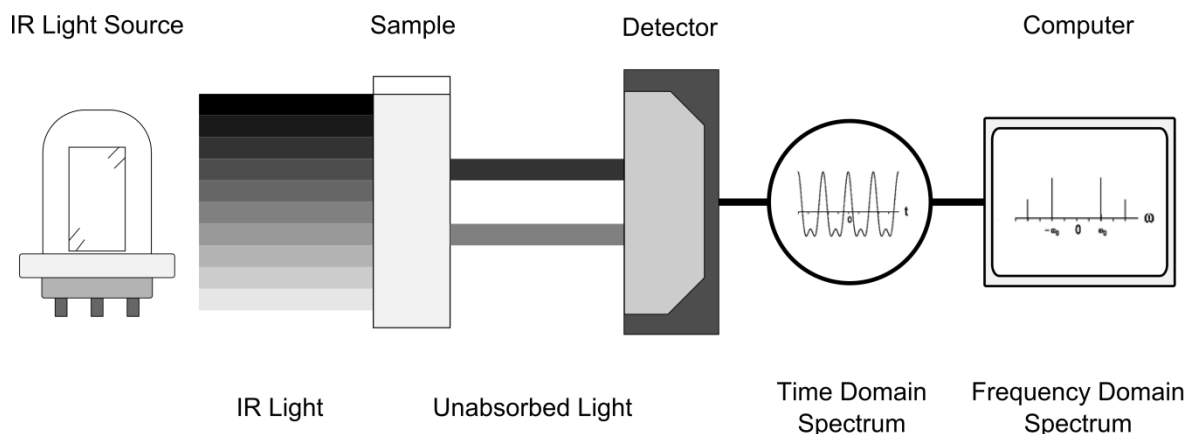
Figure 17. FT-IR schematic

2.4.1 FT-IR Method

A Bio-Rad 175 C FTS spectrophotometer (Bio-Rad Laboratories, Hemel Hempstead, UK) was used for experimentation it was equipped with an MCT detector and Golden Gate single refection diamond ATR sampling accessory; samples were measured in triplicate over a range of 800-4000 cm^{-1} with a resolution of 4 cm^{-1}. Air was used as a background and 64 scans were taken for each spectrum. Final spectra were averaged and normalised but no other treatment was carried out.

2.5 Steam explosion

Steam explosion is a pre-treatment method used to increase the accessibility of ligno-cellulosic materials to enzymatic hydrolysis, as discussed in §1.6.5. The apparatus utilises high pressure steam and fast operating valves to produce this effect, see Figure 18 for diagram. Sample was fed in through a funnel at the top of the system into a reaction chamber that is sealed by valves at either end, high pressure steam (max 3 MPa, approx 230°C) is then used to heat the system to desired temperature/pressure for required residence time. Once conditions have

been met pressure is realised instantaneously and the sample explodes into the neighbouring cyclone which separates it from the steam fraction. The steam then goes on to be water cooled and filtered through charcoal before being vented to atmosphere.
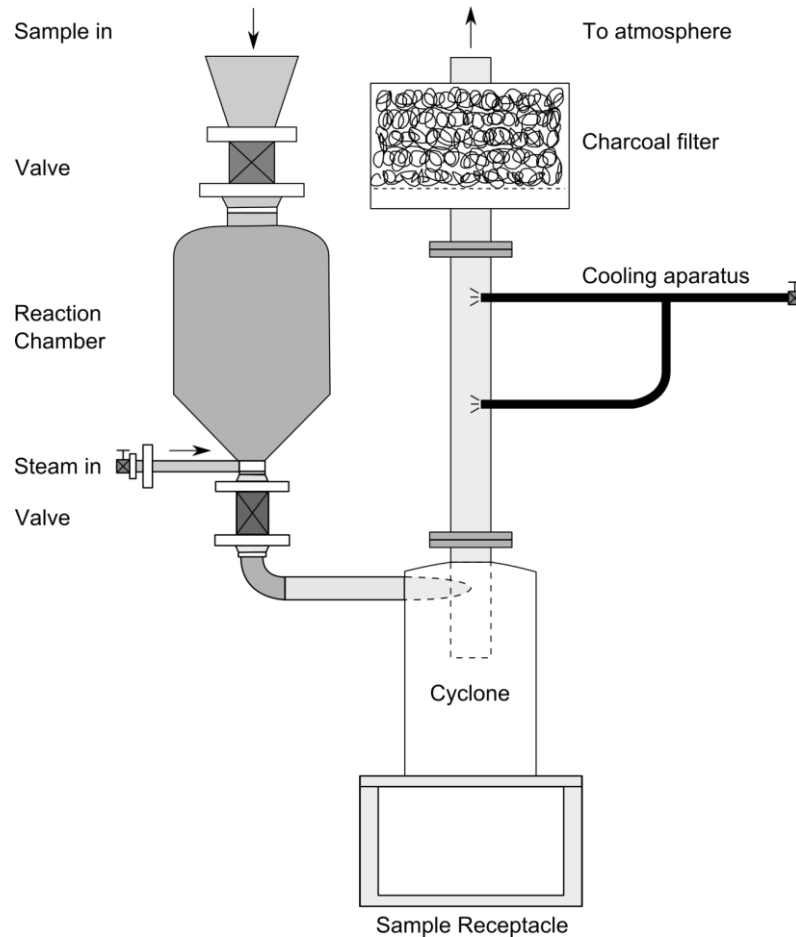
Figure 18. Steam explosion equipment schematic

As steam explosion is carried out with discreet temperature and residence time factors, it can be hard to compare samples where both variables have been altered. To alleviate this, a severity factor (SF) can be calculated from the variables using Equation 1 below, where SF is severity factor, *t* is residence time in minutes and T is temperature where 100°C is taken to be unity and the system is assumed to follow first order kinetics and obey to Arrhenius law (Overend et al., 1987).

$$SF = log_{10} \; t.exp^{\frac{T-100}{14.75}}$$ (1)

Equation 1. Severity factor

This allows the variables to be plotted on one continuum rather than as discrete instances.

2.6 Filter Paper Unit (FPU) determination method

The National Renewable Energy Laboratory (NREL) Filter paper assay for cellulase activity (NREL, 1996) based on the work of Ghose (Ghose, 1987) was used to quantify the standard activity of all the enzymes used.

Strips (1 x 6 cm) of Whatman No. 1 filter paper (50 mg) were rolled and inserted into test tubes as substrate for the assay. 0.1 mol/L sodium acetate (NaOAc) buffer (pH 5.0) was used as standard buffer solution throughout this project and so was also used here. Several dilutions of the enzyme to be assayed were made in this buffer with a target of bracketing a concentration enabling the release of 2.0 mg glucose in 60 minutes. For each set of assays the following tubes were prepared (Table 8), solutions were pre-equilibrated to 50°C.

A stock of 10 mg/mL glucose was made and dilutions were made as per Table 9.

	Buffer (ml)	Diluted Enzyme (ml)	Filter Paper Strip (50 mg)
Reagent Blank	1.5	-	-
Substrate Control	1.5	-	+
Enzyme Control*	1.0	0.5	-
Assay*	1.0	0.5	+

* one for each enzyme dilution

Table 8. FPU Assay tubes

Glucose Stock (mL)	Buffer (mL)	mg/mL	mg/0.5 mL
1	0.5	6.67	3.35
1	1	5	2.5
1	2	3.33	1.65
1	4	2	1

Table 9. FPU calibration standards

0.5 mL of each standard was added to 1 mL buffer and all samples and standards were incubated for exactly 60 minutes, adding 3.0 mL DNS reagent at the end to terminate the assay. Tubes were then boiled for 5 minutes in a water bath to develop colour (see §2.9.3 for more information on the DNS procedure) then cooled on ice and centrifuged (3,000 rpm) to remove solid residue. 200 µL of supernatant was then transferred to a micro titre plate and read at 540 nm in an LT-4000 micro plate reader (Labtech, UK)

The sample data were plotted against the glucose standard curve with enzyme blank subtracted to give a quantification of glucose released. The logarithm of the enzyme dilution factor was plotted against the glucose calculated from the standard curves enabling the concentration at which 2.0 mg of glucose would have been liberated to be determined. The FPU of the sample was then calculated with the following equation (Equation 2).

$$Filter\ Paper\ Units = 0.37 * Enzmye\ Dilution\ Factor\ \ units/mL \qquad (2)$$

Equation 2. FPU

The 0.37 factor comes from FPU having the units of µmol [glucose]/minute (Equation 3)

$$0.37 = \frac{2mg\ (Glucose)}{0.180\ (mg\ mol\ glucose\ )*0.5\ mL\ (Volume)*60\ minutes(incubation\ time)} \tag{3}$$

Equation 3. FPU factor

2.7   Yeast preparation

Unless otherwise stated the yeast used throughout this project was *Saccharomyces cerevisiae,* strain number NCYC 2826 (National Collection of Yeast Cultures (NCYC), Norwich, UK). The strain was grown from a slope by inoculation into 1 L of Difco, Yeast and Mould (YM) broth: 0.3% (w/v) Yeast Extract, 0.3% (w/v) Malt Extract, 0.5% (w/v) Peptone and 1% (w/v) Dextrose and allowed to grow over the period of ≥3 days at 25°C. The temperature was then reduced to 4°C and the yeast was allowed to settle. YM media was decanted off and the yeast cells are then reconstituted in 500 mL of yeast nitrogen base prior to inoculation into the reaction vessel. Cell count readings were taken to find the total viable count prior to inoculation into hydrolysate, using a NucleoCounter® YC-100™ (ChemoMetec, Denmark)

2.8   Estimation of ethanol production by measurement of carbon dioxide production from yeast fermentation

Ethanol was produced from glucose following the basic stoichiometry as seen in Equation 4, with just over half (51.1% w/w) converted, with the rest being released as carbon dioxide.

$$\begin{array}{c} C_6H_{12}O_6 \\ Glucose \\ (180\ g/mol) \end{array} \rightarrow \begin{array}{c} 2CO_2 \\ Carbon\ Dioxide \\ (44\ g/mol) \\ 48.8\% \end{array} + \begin{array}{c} 2C_2H_6O \\ Ethanol \\ (46\ g/mol) \\ 51.1\% \end{array} \tag{4}$$
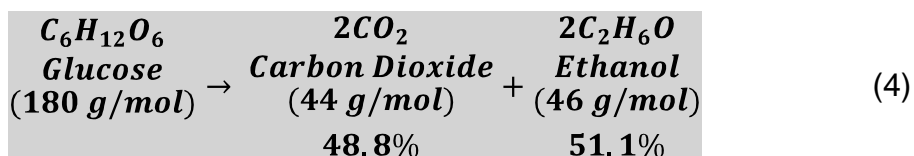
Equation 4. Basic glucose to ethanol stoichiometry

From the volume of carbon dioxide produced, it was therefore possible to calculate the corresponding quantity of ethanol evolved at standard temperature and pressure. Equation 5 determines the volume of gas evolved for every 1% (v/v) ethanol produced based on a total working volume of 5 L, the volume of a mole of

carbon dioxide being 22.4 L/mol (at standard temperature and pressure) and the density of ethanol being 789 g/L.

$$Volume\ CO_2\ L\ = \frac{22.4\ L\ mol\ \times(0.01*5)\ L\ \times789\ (g\ L)}{46\ (g\ mol)} = 19.21\ L \qquad (5)$$

Equation 5. Volume of Carbon Dioxide

Carbon dioxide is measured using a GFM17 mass flow meter (Aalborg®, US) which was attached to an appropriate reaction vessel and data logged using Orchestrator (Measurement Systems Ltd (MSL), Newbury, UK).

2.9   Colourimetric assays

Colourimetric assay are simple methods where given reactions result in products that have an optical absorbance at specific wavelengths of light. This enables reactants to be quantified simply using a spectrophotometer or more practically, for multiple samples, a micro plate reader.

2.9.1   Uronic acid assay

Uronic acids were quantified by method adapted from Blumenkr (1973). 1.2 mL 25 mmol/L sodium tetraborate in concentrated H_2SO_4 was added to acid-washed test tubes and cooled on ice. 0.2 mL of uronic acid standard or sample was added and mixed, tops of tubes were covered with glass balls and heated at 100°C for 10 min in a boiling water bath. Tubes were cooled on ice and once cold 20 µL of 0.15% (w/v) 3-phenyl phenol in 0.5% (w/v) NaOH was added to three replicates and 20 µL 0.5% (w/v) NaOH to a fourth as a reagent blank and mixed. Tubes were developed in the dark for 30 minutes at room temperature. 200 µL of each sample was transferred into micro-titration plate absorbance at 490 nm was measured in a plate reader. The absorbance of the reagent blank was subtracted upon calculation from standard curve.

2.9.2 Glucose oxidase/peroxidise (GOPOD) assay

The GOPOD assay kit (Megazyme, Bray, Ireland) allows for a specific quantification of D-glucose by colourimetric assay. The kit works by utilising two specific enzymes to produce quinoneimine dye quantitatively from D-glucose, see Equation 6 and Equation 7.

$$\text{D-Glucose} + O_2 + H_2O \xrightarrow{\text{(glucose oxidase)}} \text{D-gluconate} + H_2O_2 \tag{6}$$
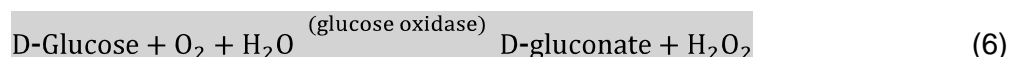
Equation 6. GOPOD Equation A

$$2H_2O_2 + \text{p-hydroxybenzoic acid} + \text{4-aminoantipyrine} \xrightarrow{\text{(peroxidase)}} \text{quinoneimine dye} + 4H_2O \tag{7}$$
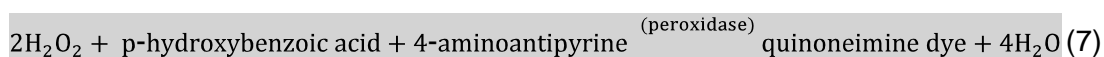
Equation 7. GOPOD Equation B

GOPOD reagent was made up according to instructions in the Megazyme assay kit. The GOPOD method was limited by the amount of 4-aminoantipyrine (0.08 mg/mL) meaning the maximum glucose usable per assay can be calculated in Equation 8.

$$2 * \frac{0.08 (g\ L)}{203\ (g\ mol)} * 600 * 10^{-6}\ l\ * 180\ (g\ mol) * \frac{1}{20*10^{-6}\ L} = 4.26\ g\ L \tag{8}$$

Equation 8. GOPOD maximum glucose concentration

Therefore a low glucose concentration standard curve of 0-5 mg/mL anhydrous D-glucose was made with ten dilutions. If the sample was likely to be outside this range then it was necessary to dilute the sample to bring it into range.

20 µL sample/standard was transferred into 2 mL tubes (eppendorf, UK) with addition of 600 µL of GOPOD reagent, vortexed thoroughly. Tubes are then incubated at 50°C for 20 min, then 200 µL transferred into a 96 well reader plate ensuring there was a standard curve on each plate used. Finally read at 510 nm in a micro plate reader. Glucose was calculated from the standard curve minus reagent blanks.

2.9.3 Dinitrosalicylic acid (DNS) total reducing sugars assay

The DNS method is regularly used to estimate the concentration of reducing sugars in hydrolysis liquors. Unlike the GOPOD method detailed in § 2.9.2 it is not glucose dependant and reacts with any reducing sugar (Equation 9), making the method broader but less specific in its scope, and providing an alternative to more in depth, but time-consuming methods such as GC or HPLC.

$$3,5\text{-dinitrosalicylic acid} + \text{reducing sugar} \rightarrow 3\text{-amino-5-nitrosalic acid} \qquad (9)$$

Equation 9. DNS Equation

The traditional method postulated by Sumner (1921) and updated by Miller (1959) was centred around a number of simple procedural steps; mixing dinitrosalicylic acid reagent with a sample, heating to catalyse the reduction reaction (Equation 9), and measuring the visible absorbance of the reaction products (3-amino-5-nitrosalic acid).

Initial experimentation in this thesis was conducted using the traditional procedure at millilitre scale, DNS reagent was prepared, 1% (w/v) 3,5-dinitrosalicylic acid, 30% (w/v) sodium potassium tartrate and 0.4 mol/L NaOH. 300 µL sample was added to 300 µL DNS reagent solution, this was then boiled in a water bath for 5 minutes to develop the colour. Samples are cooled on ice and centrifuged at 13,000 rpm for 5 minutes to remove any particulates. A 200 µL aliquot was transferred to a 96 well reader plate and read at 540 nm in a micro plate reader. The results were quantified against a standards curve of appropriate sugar.

With the introduction of a liquid handling robot (Tecan, Switzerland) part way through the project the DNS method was updated to allow multiplexing and therefore increased assay speed. Automation of this assay had been previously pursued (Miyazaki et al., 2006, King et al., 2009, Shankar et al., 2009, Goncalves et al., 2010, Song et al., 2010, Navarro et al., 2010) but these attempts to scale down the method whilst improving liquid transfer times often left heating and cooling times high (> 5 min) or even in some cases lengthened (10 minutes) (Goncalves et al. 2010). All heat the sample at 95-100°C, 10 min to ensure full

colouration (Miyazaki et al. 2006; Shankar et al. 2009, Song et al. 2010, Navarro et *al.* 2010) but extended heating regimes can potentially cause inaccuracies via evaporation (Navarro et al., 2010).

Poor standardization of the DNS method is compounded by the seemingly arbitrary use wavelengths for final quantification, for example as low as 490 nm (Xu et al., 2010) to as high as 580 nm (Iandolo et al., 2011). Furthermore, dilutions of hydrolysates (King et al., 2009, Goncalves et al., 2010) are sometimes necessary to bring the reaction products within range of detection.

With these factors in mind, a number of short experiments were undertaken to enable the semi-automation of this method and to standardise the reading wavelength, heating regime and sample to DNS ratio (Wood et al., 2012).

2.9.4 Optimising reaction volumes

Traditional sample-to-DNS ratios (50% sample/DNS) necessitates extensive dilution of high concentration hydrolysates to bring them into a readable range. This dilution of sample can introduce error and extend assay time by adding an additional liquid transfer step. Moreover, accurate dilutions require *a priori* knowledge of the neutral sugar concentration in the hydrolysate.

A sixty-point D-glucose calibration curve (0-29.5 mg/mL) was analysed using varying quantities of sample to DNS (1, 5, 10, 20, 30, 40, and 50% sample/DNS made up to 180 µL, working volume of plates used) to establish if lower sample volumes could be used to eliminated the need for previous sample dilutions. Solutions were heated in 96 well PCR plates using a thermocycler (Biometra, Germany) for 5 min at 100°C and quantified at 540 nm.

At relatively low glucose concentrations 5% sample/DNS, greatest linearity and resolution was achieved ($y = 0.0919x - 0.065$, $r^2 = 0.9993$, Figure 19). Larger sample volumes improved resolution but impaired linearity over this range (0-30 mg/mL); the opposite was true for lower sample volumes. For this reason, 5% sample/DNS was selected for further optimisation.
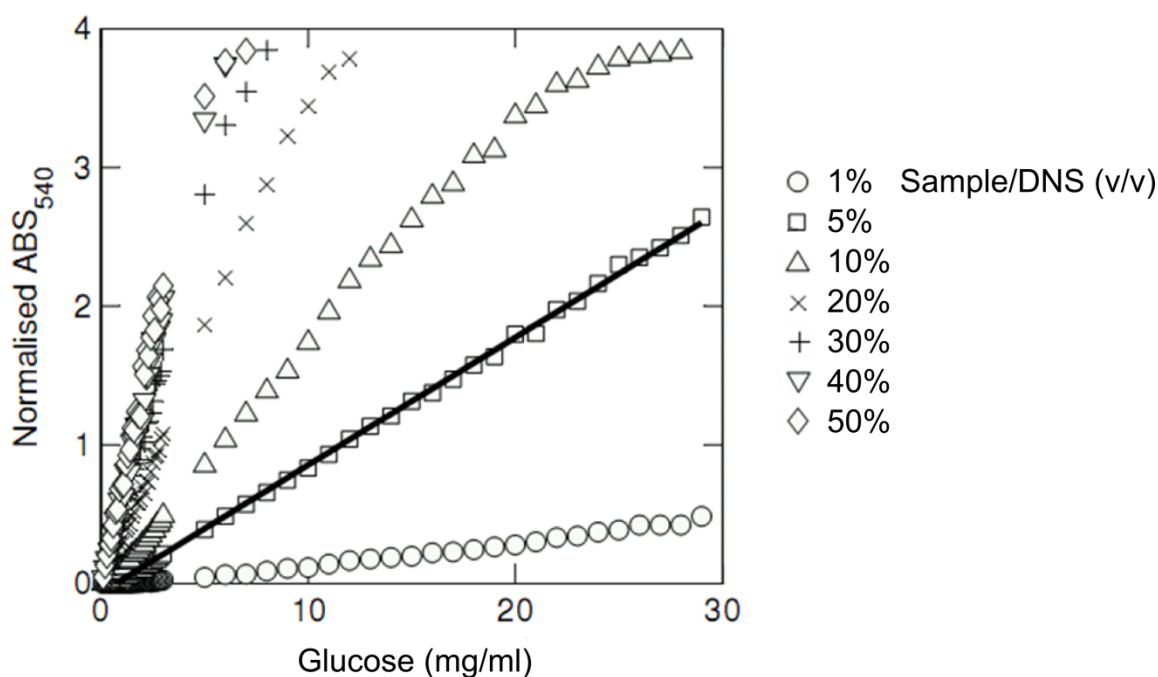
Figure 19. The effect of differing ratios of sample to DNS on the absorbance of the reaction products after heating at 100°C, 5 min. Reaction volumes containing 5% sample/DNS were selected as they displayed a linear calibration curve (black line, y = 0.0919x - 0.065, r^2 = 0.9993) over this range (0-29.5 mg/mL n = 60)

2.9.5 Optimising temperature and timing regimes

A further calibration curve (0-29.5 mg/mL, 5% sample/DNS) was tested at a variety of temperatures and incubation times (Figure 20) in a thermocycler (Biometra, Germany). Heating at 100°C, 1 min (2 min 23 seconds total time) was found to be sufficient to fully colour the DNS, significantly faster than previous methods (Goncalves et al., 2010, King et al., 2009, Miyazaki et al., 2006, Shankar et al., 2009, Song et al., 2010, Navarro et al., 2010). Lower temperatures can also be used and attain linear calibration curves (r^2 > 0.99) however longer incubation periods are needed (5, 2 and 1 min for 70, 80, and 90°C respectively) and may be used in cases where evaporative loss is a problem – i.e. when pierceable adhesive sealing mats are used (Navarro et al., 2010).
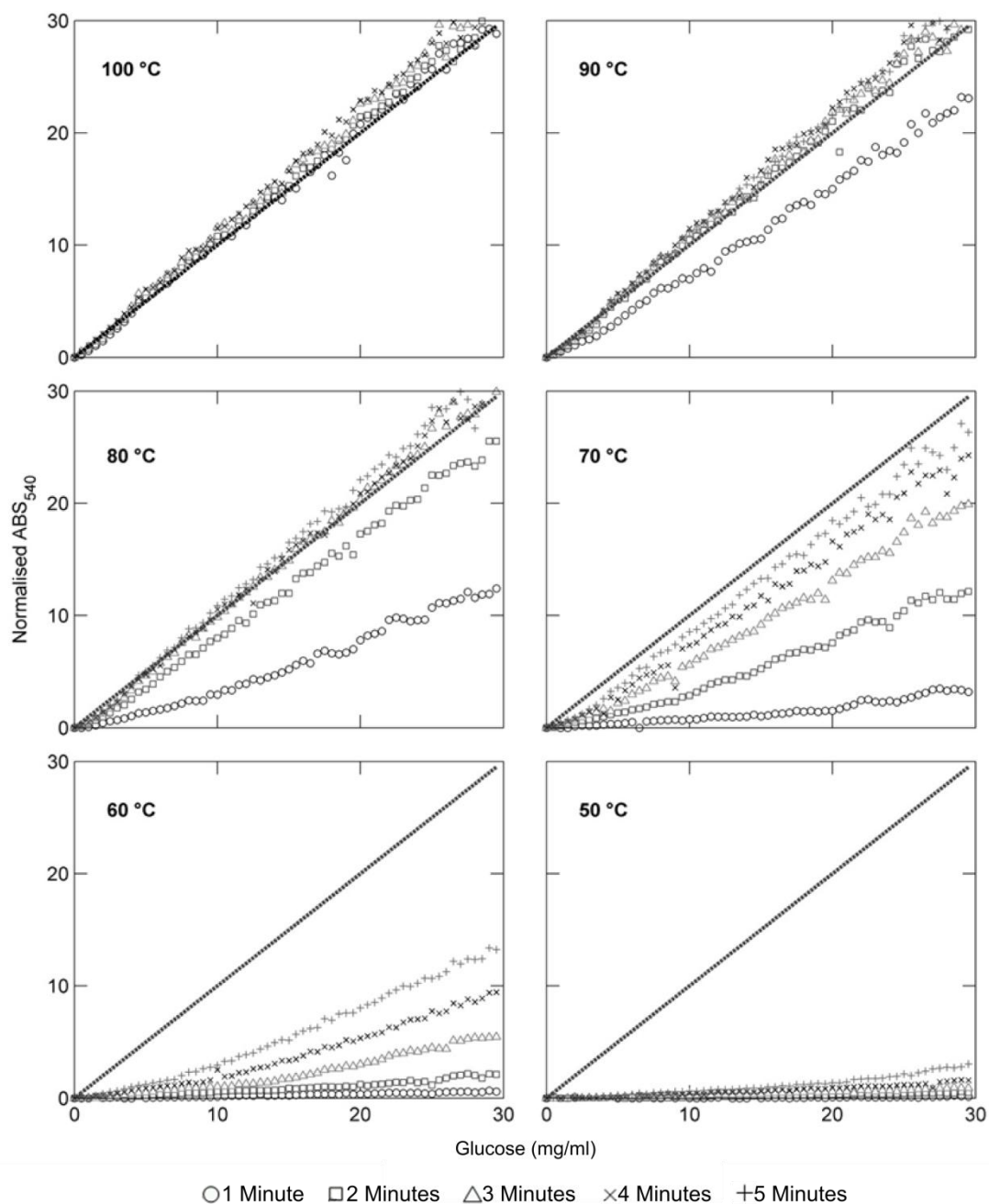
Figure 20. Optimisation of reagent heating regimes. The dashed line is indicative of full colouration (100°C, 5 min). Abortion was quantified (540 nm) and values have been expressed relative to full coloration (x = y)

2.9.6 Reading at different wavelengths

As a range of wavelengths are currently used to quantify the end product of the DNS reaction two overlapping calibration curves (0-29.5 and 25-100 mg/mL) were generated (5% sample/DNS, heated 100°C, 1 min) and analysed at varying wavelengths to ascertain the optimum reading wavelength (Figure 21).

Reading at 575 nm gave optimum linearity (r^2 = 0.9999857) but poorer resolution (slope = 0.0244798) when lower glucose concentrations are used (0-29.5 mg/mL, n = 60). Reading at 520 nm gave optimum resolution (slope Abs_{520} = 0.133689) but linearity was impaired (r^2 = 0.993796). Wavelengths between 540-605 nm all gain linear calibration curves (r^2 > 0.9999) and are therefore suitable wavelengths to use when sample concentration is < 30 mg/mL. The best compromise between linearity and resolution is achieved when reading at 540 nm (slope = 0.079916, r^2 = 0.999926851) when samples contain < 30 mg/mL. However, when reading at 540 nm, the calibration curve becomes non-linear at sample concentrations > 35 mg/mL therefore in these cases, higher wavelengths should be used.

At higher glucose concentrations (30-100 mg/mL, n = 10) optimum linearity was obtained at 600 nm (r^2 = 0.99851) but again, resolution is reduced (slope Abs_{600} = 0.0135). Reading at 580 nm gives the greatest possible resolution (slope Abs_{580} = 0.0228) while maintaining a linear calibration curve over this range (r^2 > 0.998).

Therefore, it is suggested that samples of unknown concentrations should be run with two calibration curves covering 0-25 mg/mL and 25-100 mg/mL on the same plate. The former calibration curve should be read at 540 nm and the latter at 580 nm and an appropriate wavelength selected for the sample depending on its absorbance.

Although intermediate wavelengths could be used, reading at two wavelengths ensures the best resolution and linearity is achieved when analysing a particular sample.
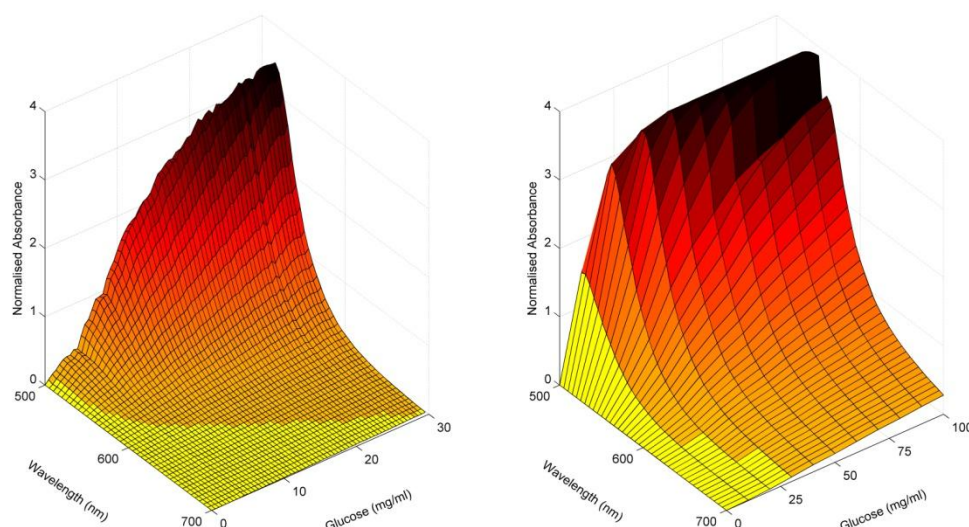
Figure 21. Optimisation of DNS reading wavelength

2.9.7   Validation using a complex substrate

Enzymatically saccharified (10% (w/v) substrate, 5% (v/v) Accellerase® 1500, 18 h, 50°C) steam exploded wheat straw (10 min at 200°C, 210°C and 220°C) liquors were analysed using the optimised method (5% sample/DNS heated at 100°C, 1 min, read at 540 nm). This was compared to the original DNS method following Miller (1959) – adding DNS reagent (3 mL) to a 10 x diluted sample (3 mL), boiling at 100°C, 5 min, and reading at 575 nm.

Hydrolysates from enzymatically saccharified, steam-exploded wheat straw were analysed following the refined method (5% sample/DNS → 100°C, 1 min → read at 540 nm) to demonstrate that it can be used to analyse complex substrates (Table 10). The refined method estimated then mean neutral sugar content of all hydrolysates of a particular treatment within ± 2 mg/mL with 95% confidence, a slight improvement on the original manual method (± 3 mg/mL). This suggests that the variation in neutral sugar content between independent digestions is ≥ that of either method.

However, within a single replicate, the refined method consistently outperforms the original (Table 10). The mean neutral sugar concentration found in each

independent replicate can normally be estimated within ± 0.44 mg/mL with 95% confidence, compared to ± 2.41 mg/mL when the original method is used. The circa five fold improvement in precision is most likely caused by the removal of superfluous dilution steps.

Temp (°C)	Multiplexed method (current study)		Manual Method (Miller 1959)	
	μ	S.D.	μ	S.D.
200				
Rep 1	14.49	0.184	16.28	0.736
Rep 2	13.84	0.076	15.80	0.551
Rep 3	16.06	0.200	16.01	1.174
Total	14.79	1.000	16.03	0.774
210				
Rep 1	14.78	0.305	16.34	1.109
Rep 2	15.31	0.335	17.80	0.448
Rep 3	16.58	0.192	16.77	1.936
Total	15.56	0.839	16.97	1.310
220				
Rep 1	14.91	0.451	16.76	1.806
Rep 2	15.85	0.198	17.64	1.431
Rep 3	16.24	0.019	17.44	1.634
Total	15.67	0.642	17.28	1.469

Table 10. Digestion liquors of enzymatically hydrolysed wheat straw pre-treated at varying temperatures

Therefore the use of 5% sample/DNS, to analyse hydrolysates containing 0-100 mg/mL reducing sugars was used. A thermocycler is used to heat the samples (100°C, 1 min) before quantification and reading is conducted at 540 nm for samples containing 0-25 mg/mL or alternatively at 580 nm for samples containing 25-100 mg/mL neutral sugars. This method is significantly faster, more precise and requires fewer dilution steps than other currently used manual or automated methods and is therefore used in preference to the traditional method where possible in the project.

3 Characterisation of municipal solid waste (MSW) and related waste paper streams

As previously discussed in §1.1 MSW is a profoundly heterogeneous material, therefore reducing sugar and uronic acid composition analyses were carried out to characterise the waste and determine it's possible use as a substrate for bioalcohol utilisation. Comparative analyses were also conducted on solid copier paper. GC sugars analysis by 1 mol/L H_2SO_4 provides data of non-cellulosic sugars and 72% (w/w) H_2SO_4 produces data encompassing all available sugar in the substrate, therefore making it possible to calculate the proportion of reducing sugar from a cellulosic source. Microscopy was carried out on these substrates to provide further compositional information.

3.1 Materials and Methods

3.1.1 Substrates

A quantity of MSW was secured from the Biffa waste site in Leicester (Biffa Recycling Centre, Leicester, UK); it consisted of black bag waste that had been macerated in a ball mill. The process, shown in Figure 22, involved preliminary sorting of waste to remove recyclable paper and card, the remaining waste was then loaded into the ball mill. Metals and plastics were removed after this process leaving an organic residue, which was washed with the eluent going to anaerobic digestion (AD) and the remainder directed to landfill. This final fibrous organic residue that would otherwise have been destined for landfill was the original substrate used in this study.

Additionally M-Real Evolve Office 80 g/m^2 copier paper was used as the standard paper type throughout this study.

3.1.2 Alcohol Insoluble Residue (AIR)

1 L of 70% (v/v) ethanol was added to 207.33 g of fibrous organic material described in §3.1.1 to make it biologically safe then homogenised with an Ultra-Turrax® homogeniser (IKA-Werke GmbH and Co. KG, Staufen, Germany). The homogenised sample was then boiled in a water bath for 5 minutes and filtered

through nylon mesh, this process was repeated twice more with 300 mL of ethanol. A final boil, wash and filtering was carried out using 300 mL acetone, then the sample was spread out onto a tray and allowed to dry in a fume cupboard until constant weight was achieved.

3.1.3 Copier paper preparation

M-Real Evolve paper was prepared using an Impega hole punch (Lyreco, Telford, UK) to give 6 mm diameter paper circles.

3.1.4 Analyses

GC sugars and uronic acid are conducted as described in §2.2.3 and §2.9.1. Additionally starch is assayed using a Megazyme starch testing kit (Megazyme, Bray, Ireland) following the standard operating procedure described therein.
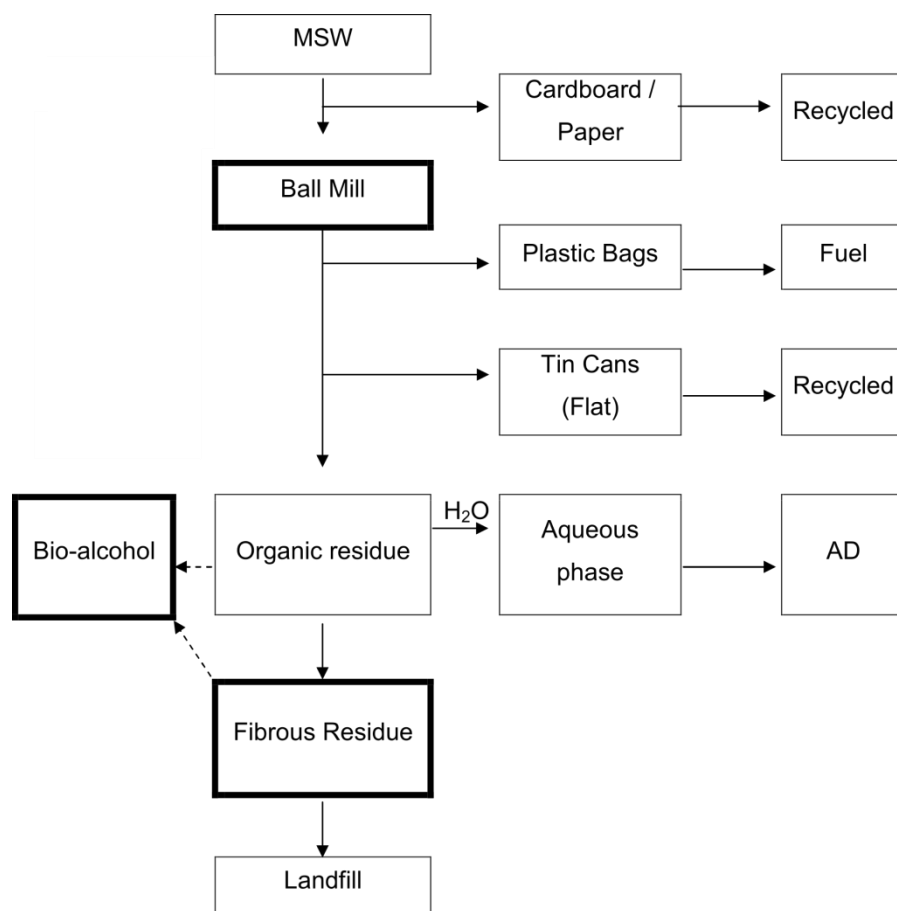
Figure 22. Biffa MSW – Ball mill flow chart

3.2 Results and discussion

The initial weight of the Biffa MSW sample was taken as 207.33 g, this sample was then treated as in method for AIR §3.1.2, constant dry weight of the sample was measured as 81.9 g. Therefore 60.5% (w/w) was determined as either moisture content or alcohol soluble fraction. The dried sample was then separated into waste types, results can be seen in Table 11. The biomass fraction was then ground in IKA-A10 mill (IKA-Werke GmbH and Co. KG, Staufen, Germany) to attempt to reduce the fibre size, the ground sample was then passed through a 1 mm mesh, this process yielded a fibrous waste fraction (FW: 69% w/w) and a particulate waste fraction (PW: 31% w/w).

Component name	Percentage (w/w) (wet weight)	Percentage (w/w) (dry weight)
Water / Alcohol Soluble Fraction	60.5	-
Biomass	34.2	86.7
Plastic	1.1	2.8
Metal	0.1	0.2
Paper	0.1	0.2
Wood / Large Plant Material	0.3	0.7
Foam / Sponge	< 0.1	0.1
Glass	0.8	2.1
Stone	1.0	2.5
Printed Circuit Board (PCB)	0.1	0.3
Soil / Grit / Particulate Glass	1.7	4.3

Table 11. Biffa waste – fractional composition

FW and PW samples were then assayed for reducing sugar content (GC Sugars), and uronic acid, Figure 23 shows that there was non-cellulosic sugar present in FW material. This equates to approximately 9.8% (w/w total monomeric sugars) of total dry mass. This was likely to come from food and plant sugars, and starch in the case of glucose (5.75% w/w), such as would occur in processed food waste and hemicelluloses. When concentrated acid (72% w/w H_2SO_4) hydrolysis was completed on the FW material (Figure 23) 48.3% (w/w) of the total mass is hydrolysed to sugars, 38.9% (w/w) of that being glucose, this shows that there is a

high percentage of cellulosic material in the waste (33.2% w/w), which can hypothetically be hydrolysed by an enzyme saccharification processes.
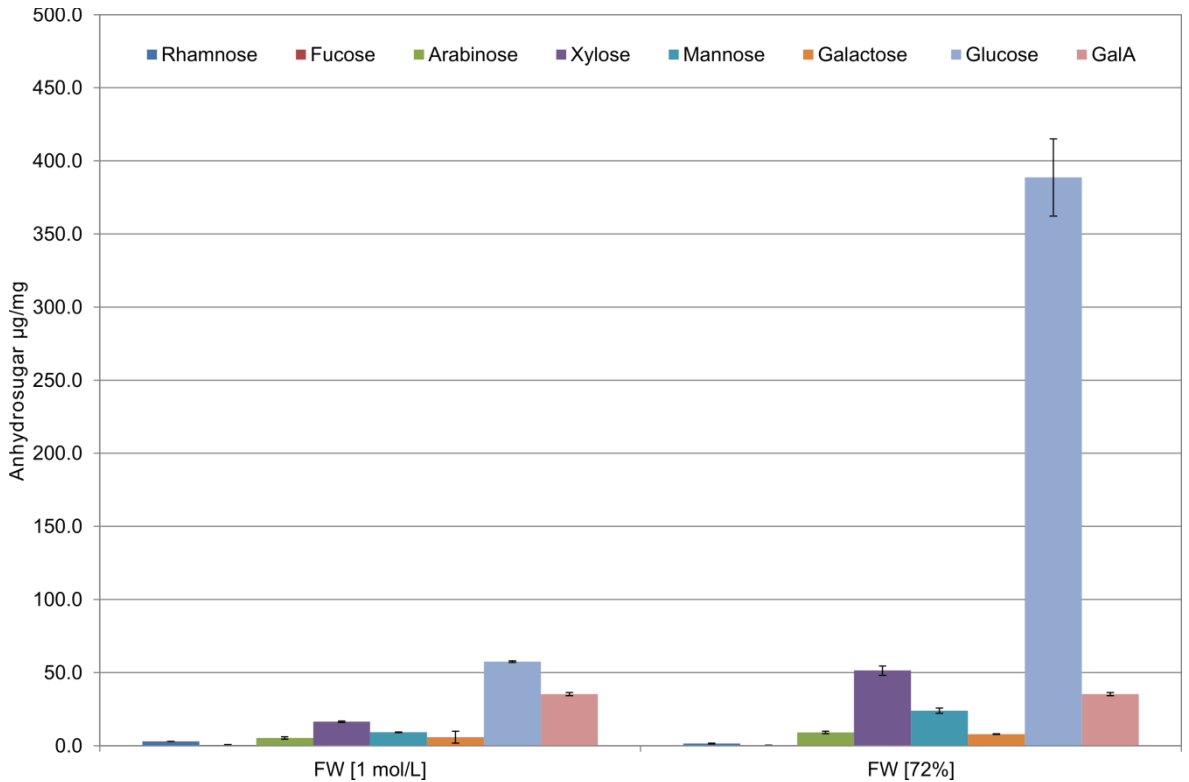
Figure 23. Biffa waste – fibrous fraction – sugars analysis

Figure 24, shows matching data from sample PW. This shows considerably less sugar released both in 1 mol/L H_2SO_4 (3.0% w/w) and 72% (w/w) H_2SO_4 (10.6% w/w) hydrolyses, therefore 6.3% (w/w) cellulosic content available. This low content can most aptly be explained by any glass and grit still within the sample being concentrated in this fraction due to its method of collection. The cellulosic content of the original AIR can therefore be calculated as 21.6% (w/w) from the proportions of the fractions and cellulose there within.
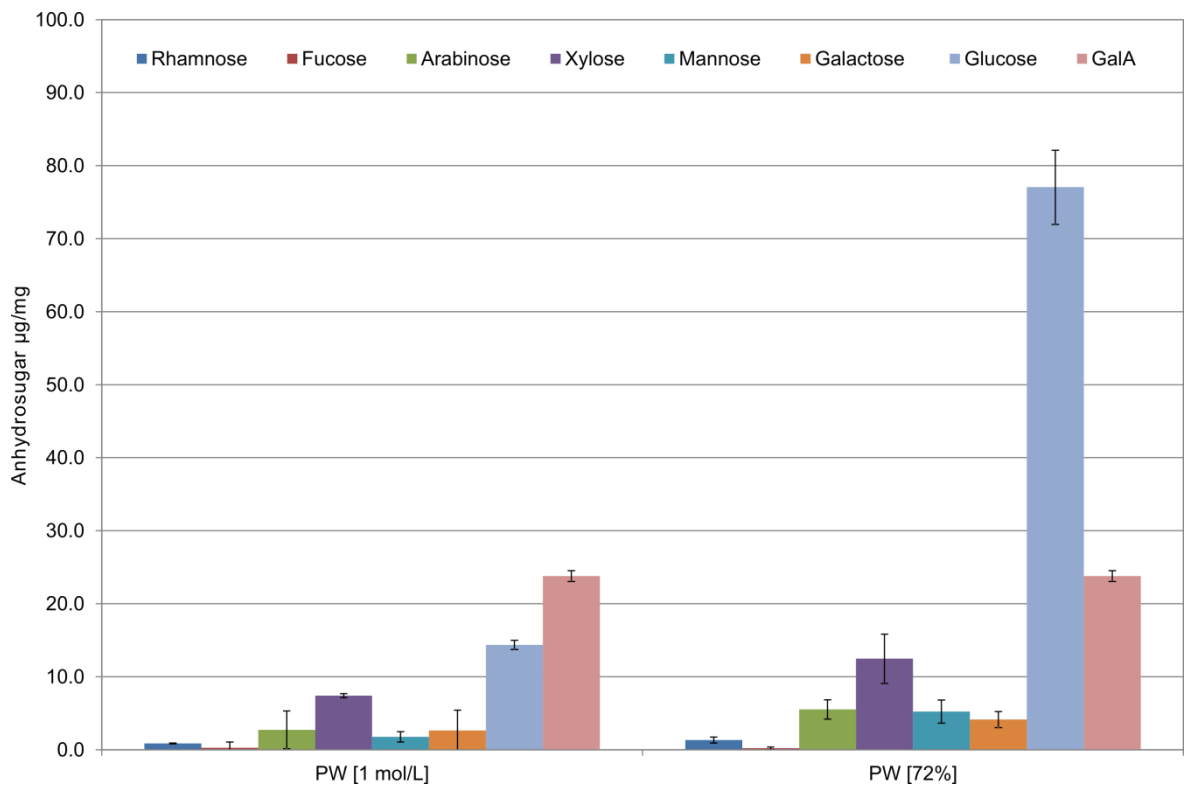
Figure 24. Biffa waste – particulate fraction – sugars analysis

Figure 25 shows the same acid hydrolysis steps carried out on copier paper (CP). 11.1% (w/w) is hydrolysed to sugars with 1 mol/L H_2SO_4 as compared to 62.0% (w/w) when 72% (w/w) H_2SO_4 is used. This shows that 46.34% (w/w) glucose is cellulosic in origin with the other 4.1% (w/w) deriving from starch as corroborated by Megazyme starch assay (4.1% w/w). It is also notable that the xylose released by 1 mol/L acid treatment was less than that after 72% (w/w) treatment, this suggests that a significant proportion of the xylan was locked into the crystalline microfibril, and not accessible to dilute acid hydrolysis. This high level of cellulose makes paper a very good candidate for a feedstock of the waste to biofuel process. It is also believed that other kinds of paper have more cellulosic material present than office grade copier paper, which includes a large proportion of clay or $CaCO_3$ (approx 30-40% w/w), whereas tissue and card do not. Paper manufacturing companies are required to produce a "paper profile" that gives details of the make up of the paper; the paper profile for M-Real can be seen in Appendix E (page 174) and shows that it is made entirely from recovered pulp.
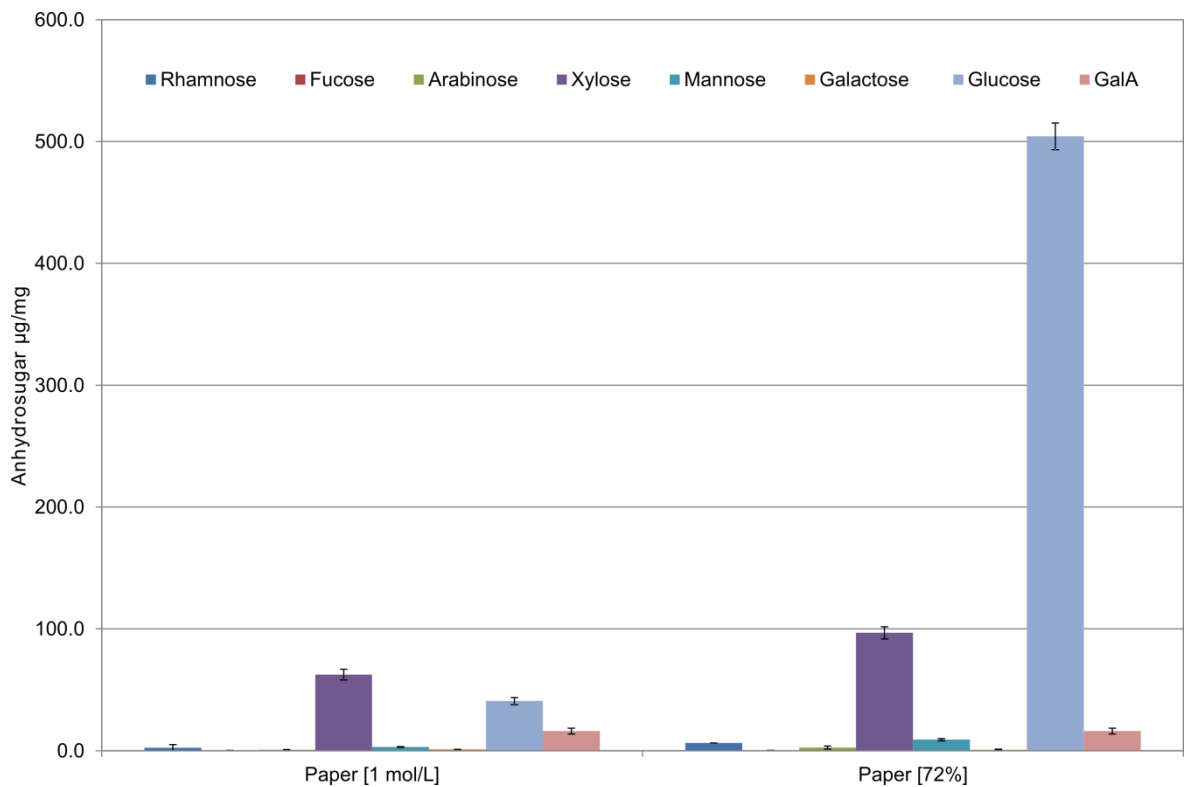
Figure 25. Copier paper – sugars analysis

Small quantities of the three samples (CP, FW and PW) were studied using an Olympus BX60 brightfield microscope (Olympus, Japan) to ascertain their composition; the results can be seen in Figure 26. The difference between FW and PW samples can be clearly seen, with PW predominantly consisting of particulate matter, mostly likely sand, soil and glass, with little fibrous material. The FW sample conversely is made up from mainly fibrous material with small quantities of particulate material; this is expected due the method of producing the two samples. The CP samples can be seen to consist of principally cellulosic fibres and clumps of particulate matter can also be seen, which would comprise of the added fillers (Kaolin and $CaCO_3$). The similarity between FW and CP samples is evident, suggesting that the FW sample is comprised largely of plant fibres similar to, and maybe in some cases the same as paper.

Magnification

| 4x | 10x | 20x |
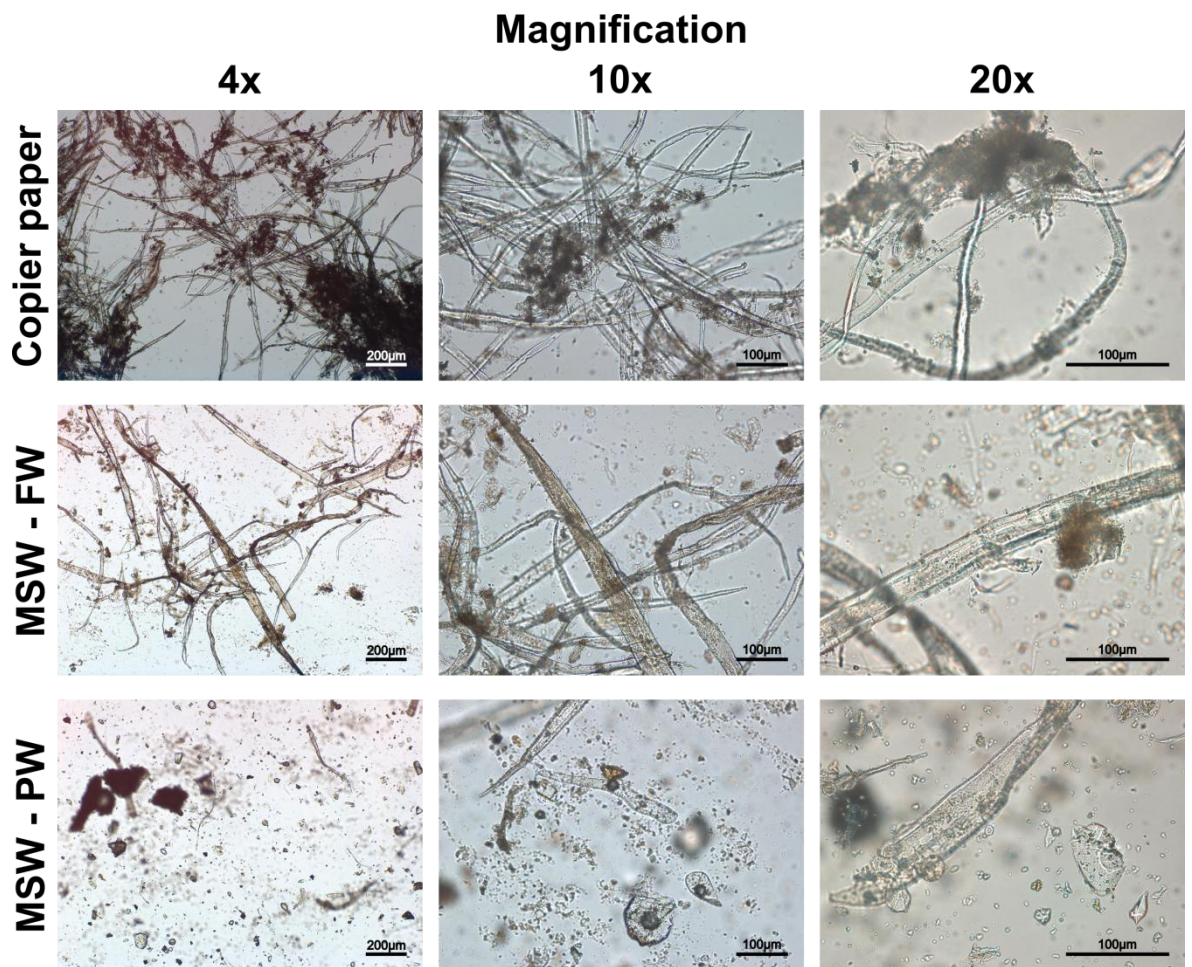
Figure 26. MSW and paper micrographs

3.3   Conclusion

Both FW and CP contain significant cellulosic and sugar rich components and therefore would make a useful source material for the waste to biofuel process. These wastes would otherwise likely be sent to landfill and therefore this process would not only alleviate the monetary cost of this but also produce a saleable product from an otherwise unwanted source feedstock.

The final content of fermentable sugars from the substrate obviously will play an important role as to the use of MSW or waster paper/card as a substrate for the final biofuel process, but there is also the question of what other components are contained within the waste that may affect the process whether negatively or positively. There are likely to be a number of other chemicals, such as inks, glues and microbial breakdown products, within the MSW that are not determined by the

analyses carried out in this section. For this reason and due to the results of the microscopy highlighting the similarity in plant fibre content within FW and CP samples, waste paper was chosen as the initial substrate as it is more easily defined; with the processing history of the source able to be followed more simply than a mixed waste stream such as MSW. Furthermore the requirement of MSW having to be made microbially safe before investigation carried out means that the substrate may be unnaturally altered by any sterilisation step. Using this substrate would give an indication of the possibility of utilising real MSW substrates in the future.

4 Optimisation of the enzymatic hydrolysis of cellulose

Chapter 3 highlighted the suitability of CP as a substrate, therefore a range of commercial cellulase preparations and conditions were studied in order to optimise conditions for enzymatic hydrolyses using solid ink free copier paper as cellulosic substrate.

This chapter also discusses the problems and possible solutions to optimise the enzyme hydrolysis section of second generation biofuel production, by addressing some of the common operational problems highlighted by recent work, such as end product inhibition and "solids effects" where high substrate concentrations used to increase the sugar yield reduce the conversion of cellulose (Kristensen et al., 2009). The overall aim is to identify factors increasing the yield of fermentable sugar and to thus optimise the enzyme mixtures used.

Additionally experimentation was carried out on the same range of enzymes to ascertain the effects of alcohols on their hydrolysis action. This is important in regard to the integrated approach to the system with the shift toward SSF systems over SHF becoming more prevalent in a laboratory setting. It is therefore important to observe the effect of common bioalcohols on the enzymatic system as SSF means that these enzymes will come into contact with alcohols in this scenario.

4.1 Materials and Methods

The classic DNS method (§2.9.3), HPLC dissolved carbohydrate (§2.3.6), HPLC SEC (§2.3.8) and GC Solid Sugars (§2.2.3) analyses are used in this chapter.

4.1.1 Materials

Five commercially available fungal cellulase mixtures, Acid Cellulase, *Trichoderma reesei*; Accellerase® 1000, *Trichoderma reesei*; Celluclast®, *Trichoderma reesei*; Depol™ 740L, *Humicola sp.*; and C013L, *Trichoderma sp.*, were chosen for this study on the basis of their high cellulase activity and, in the case of Depol™ 740L, its additional useful cell wall degrading side activities. These five enzyme preparations were used "as provided" in all experiments without any desalting or

other purification steps, thus reflecting practical usage potential in an industrial setting. The substrates used in this study were traceable sources of paper or cellulose: Whatman No. 1 filter paper, pure Sigmacell® Cellulose type 20 and M-Real Evolve Office 80 g/m^2 copier paper.

4.1.2 Standard enzyme digestions

Comparative enzyme digestions consisted of substrate (6 mm diameter punched circles of M-Real Evolve copier paper) at 2.75% (w/v). Cellulases were added to an excess of the level indicated by the supplier to a concentration of 0.5 units (µmol glucose released/min) per mL. This equated to between 35 and 60 U/mL (where defined by the suppliers' labels). Incubations were carried out with or without the addition of βG (2.5 U/mL, which equates to 42 nano katal/mL; Novozyme 188) in 100 mmol/L sodium acetate buffer (pH 5.0) in a total volume of 18 mL. The National Renewable Energy Laboratory (NREL) Filter Paper Assay (FPU) for cellulase activity (NREL, 1996) based on the work of Ghose (Ghose, 1987) was used to quantify the standard activity, see §2.6. Where required, thiomersal (mercury based antimicrobial) was added to the solution at 0.01% (w/v) to prevent microbial contamination. Thiomersal is an antifungal agent and was chosen because at least part of the contamination was believed to be originating from the enzyme preparation itself. The assays were carried out in 30 mL universal containers and were allowed to roll freely on a tray in a thermo-circulating incubator (Gerhardt, Brackley, UK) set to 50°C and 120 rpm. Regular samples were taken over a time course of 72 hours and free reducing group generation determined.

4.1.3 Substrate Concentration

The effects on substrate concentration were tested using the Accellerase® 1000 preparation only. Substrate concentration was calculated on a weight per weight basis. Samples had a standard enzyme/substrate loading of 20 FPU/g of substrate made up to 20 g total in a 30 mL Sterilin® universal container with 100 mM Sodium Acetate buffer (pH 5.0). Substrate concentrations of up to 50% (w/w) were included. Thiomersal was added as described above. Samples were then allowed

to roll freely on a tray in a thermo-circulating incubator set to 50°C at 120 rpm for 7-14 days to allow maximum digestion.

4.1.4 Step-wise addition of substrate

Stepwise additions of an extra 5% (w/w) substrate were added to selected samples to simulate a continuous process. Initial substrate concentrations ranged from 5-15% (w/w) to which additional 5% (w/w) aliquots were added after 24 and 48 hours to give total final substrate concentrations of 15, 20 and 25% (w/w). This allowed assessment of the digestion capacity after complete (or near complete) digestion of a first batch of substrate.

4.1.5 Filtration of residual solids

Residual solids from enzymatic assays were filtered on pre-weighed glass fibre filter paper using a 3 piece vacuum filter (Fisher Scientific, UK). Samples were washed with 100 mL ultrapure water then dried in an oven at 40°C until a constant dry weight was achieved. The percentage digestion could then be calculated from the residual weight and the known original weight. The quantity of recalcitrant material was calculated from sugars analysis data (Table 12) and corroborated by publically available "paper profiles" (see appendix E), indicating the quantities of kaolin filler and calcium carbonate.

4.1.6 Enzyme-substrate ratio

0.5 g copier paper substrate was digested in a total volume of 20 mL (2.5% w/v) with the following concentrations of Accellerase® 1000 enzyme: 40, 20, 10, 5 and 1 FPU/g of substrate. Samples were prepared in 30 mL Sterilin® universal containers and allowed to roll freely whilst incubating at 50°C in a thermo-circulating incubator set to 120 rpm. Samples were taken at regular intervals and assayed for reducing sugars using the DNS method outlined above.

4.1.7 Alcohol Inhibition

Four of the cellulase mixtures from §4.1.1 (Acid Cellulase; Accellerase® 1000; Depol™ 740L and C013L) were assayed in the presence of increasing

concentrations (1-10% v/v) of methanol, ethanol, propan-2-ol and n-butanol for the hydrolysis of pure Sigmacell® Cellulose (Type 20) and Whatman No. 1 filter paper circles (6 mm diameter). These alcohols were chosen as they are the most widely accepted petro-diesel substitutes/additions and therefore the most probable to be produced in an SSF process. Assays were performed at the enzymes optimum conditions, with either 250 µL of 10 mg/mL Sigmacell® in 0.1 mol/L sodium acetate buffer (pH 5.0) or one 6 mm diameter circle of Whatman No. 1 filter paper as the substrate, in a total volume of 250 µL. 6 cm^2 of Whatman No. 1 filter paper is equivalent to 50 mg of cellulose (Mandels et al., 1976) therefore a 6 mm diameter circle would contain 2.356 mg of cellulose, comparable to the 2.5 mg present in 250 µL of Sigmacell® solution used. Assays were incubated using a bespoke rotating incubator set to 50°C and 10 rpm. Samples were fully inverted on each rotation, thus providing adequate mixing at this speed. Samples were removed after 30 minutes and assayed immediately with DNS reagent. The 0% (v/v) sample was used as a control in each case, from which the percentage inhibition/activation could be calculated as a percentage increase/decrease in release of total reducing sugars.

4.2 Results and Discussion

4.2.1 Paper composition

From chapter 3, M-Real copier paper was found to have the following composition: 5.01% (w/w) moisture, 4.1% (w/w) starch, 46% (w/w) cellulose, 11.9% (w/w) Hemicellulose, 1% (w/w) Lignin and 33% (w/w) kaolin/calcium carbonate comparable to other literature analyses (Wang et al., 2012). The paper is made from recovered fibre and as such it is not possible to determine the origin of the cellulose fibre. The sugar composition is shown in Table 12. The main component is glucose (over 50%), 4.1% of which was released in 1 mol/L H_2SO_4 hydrolysis alone. This methodology indicates that the glucose was mainly cellulosic in origin. The only other significant sugar component comprised xylose (97 µg/mg).

Enzyme	βG	[H$_2$SO$_4$]†	Individual Monosaccharides (µg anhydrosugar /mg copier paper)							Total (µg/mg)
			Rha	Fuc	Ara	Xyl	Man	Gal	Glu	
None*‡	**-**	**72% (w/w)**	**9.4**	**0.2**	**2.5**	**96.7**	**9.0**	**0.8**	**504.2**	**622.9**
None*	-	1 mol/L	2.4	0.1	0.8	62.5	3.1	1.0	40.8	110.7
Depol™ 740L	-	72% (w/w)	2.1	0.1	2.5	36.4	14.5	1.1	434.7	491.3
Depol™ 740L	+	72% (w/w)	1.4	0.4	1.9	23.1	13.1	1.4	323.4	364.7
Accellerase®	-	72% (w/w)	0.8	0.1	2.7	32.1	11.7	2.0	278.5	327.9
Accellerase®	+	72% (w/w)	0.6	0.2	1.1	7.7	11.7	1.7	86.1	109.1
C013L	-	72% (w/w)	1.8	0.0	2.7	46.6	9.9	0.8	339.3	401.1
C013L	+	72% (w/w)	1.7	0.1	2.0	22.5	10.6	1.3	209.5	247.5
Acid Cellulase	-	72% (w/w)	1.4	0.1	1.8	58.0	11.3	0.6	344.7	417.9
Acid Cellulase	+	72% (w/w)	3.1	0.2	1.2	20.9	10.6	1.2	185.8	222.9
Celluclast®	-	72% (w/w)	0.9	0.0	1.0	27.4	16.0	1.2	215.5	262.0
Celluclast®	+	72% (w/w)	0.1	0.0	0.7	5.7	8.7	1.6	56.9	73.7

* Undigested paper

‡ 72% (w/w) H$_2$SO$_4$ hydrolysis with no enzymatic digestion indicates total carbohydrate in substrate

† Concentration of sulphuric acid used (see §2.2.3)

Table 12. Sugars analysis of paper and post-digestion insoluble residues

4.2.2 Enzymatic saccharification

In order to maximise the digestion of the paper substrate, a high initial dose of enzyme was used (at least double the recommended amount of Accellerase® 1000 or equivalent). Digestion was investigated with a range of enzymes added to a concentration of 0.5 units (μmol glucose released /min) per mL, with and without additional excess βG (2.5 U/mL) to ensure hydrolysis of cellobiose, an inhibitor of cellulose hydrolysis (Gruno et al., 2004). The initial studies (Figure 27-31) demonstrated that the addition of βG resulted in up to a three-fold increase in the sugars released over 12 hours. However a sharp decline was observed after this time. Microscopic investigations indicated that this was due to microbial metabolic activity. To address this, an antimicrobial agent, thiomersal (0.01% w/v), was included in further assays (Figure 32-36). Samples were incubated for 144 hours and the supernatants were analysed by HPLC to provide information on the release of glucose, xylose and cellobiose in the presence and absence of βG.
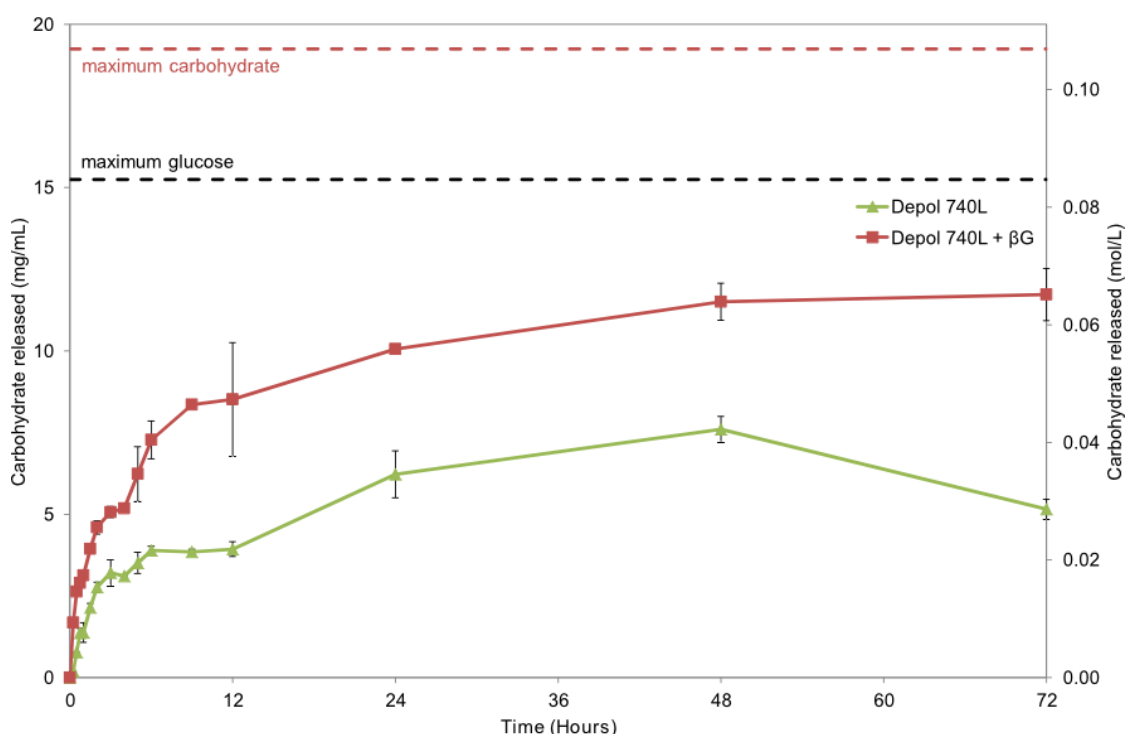
Figure 27. DNS reducing sugar assay – Depol^{TM} 740 with and without βG, theoretical maximum glucose (15.2 mg/mL) and total carbohydrate (19.3 mg/mL) shown as dashed horizontal lines, calculated against a standard curve of glucose
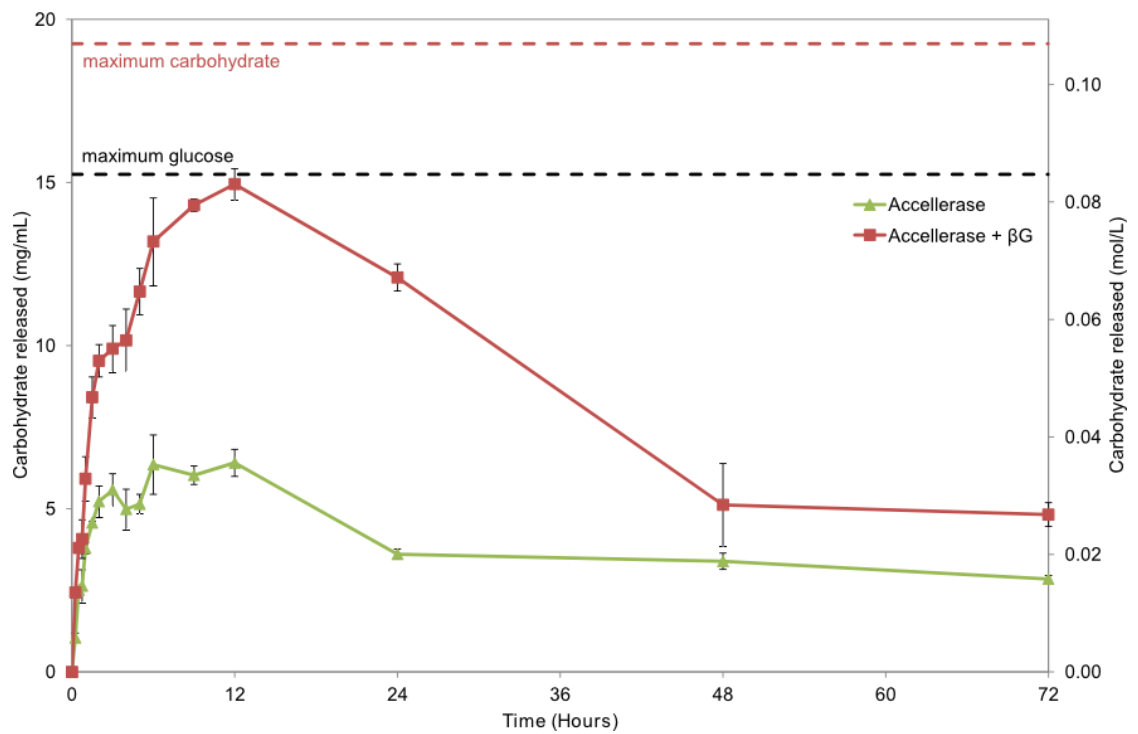
Figure 28. DNS Reducing sugar assay – Accellerase® 1000 with and without βG, theoretical maximum glucose (15.2 mg/mL) and total carbohydrate (19.3 mg/mL) shown as dashed horizontal lines, calculated against a standard curve of glucose
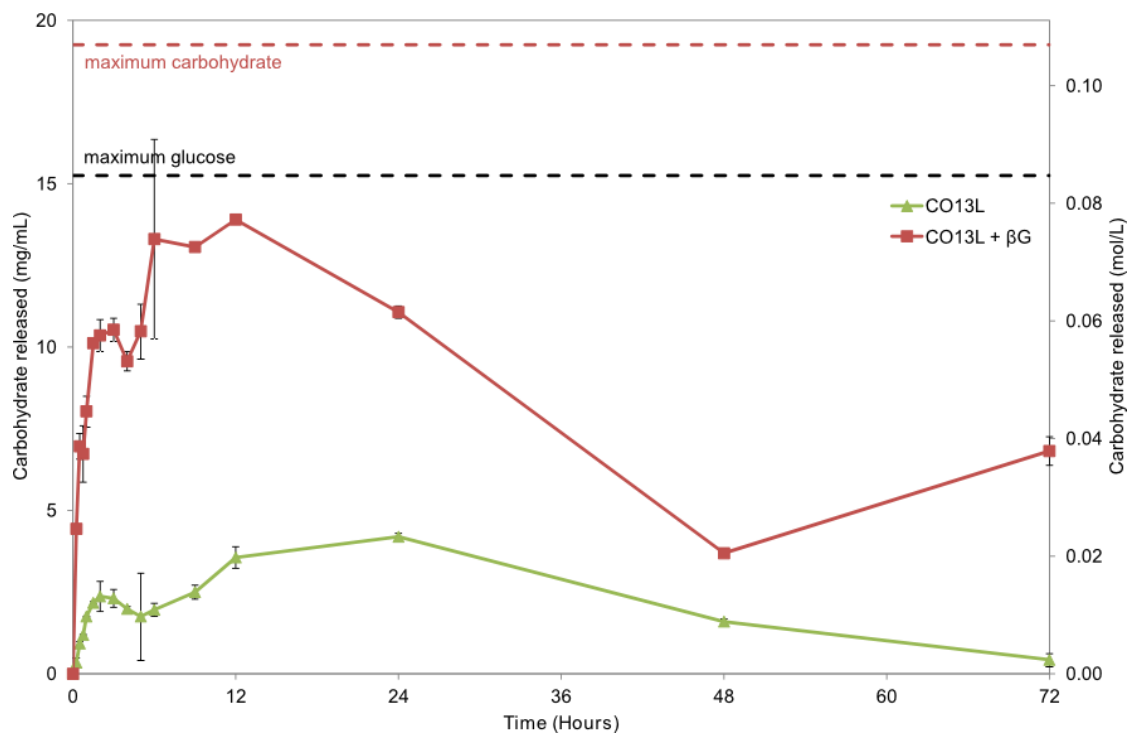
Figure 29. DNS Reducing sugar assay – C013L with and without βG, theoretical maximum glucose (15.2 mg/mL) and total carbohydrate (19.3 mg/mL) shown as dashed horizontal lines, calculated against a standard curve of glucose
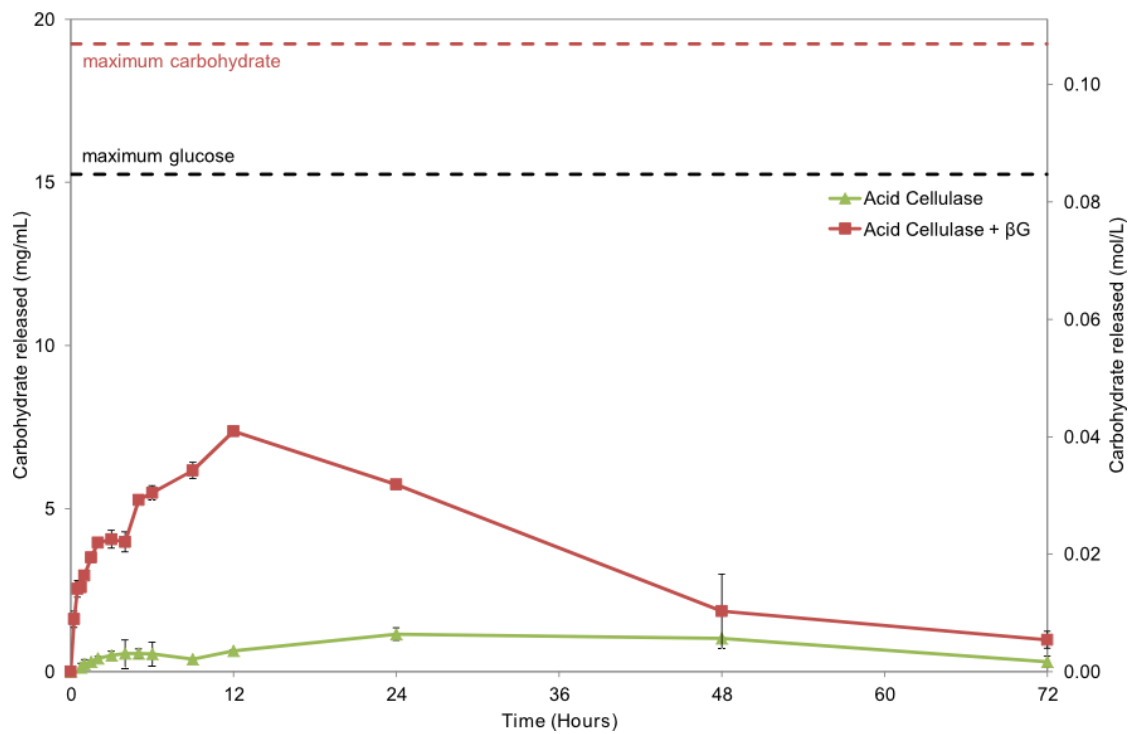
Figure 30. DNS Reducing sugar assay – Acid Cellulase with and without βG, theoretical maximum glucose (15.2 mg/mL) and total carbohydrate (19.3 mg/mL) shown as dashed horizontal lines, calculated against a standard curve of glucose
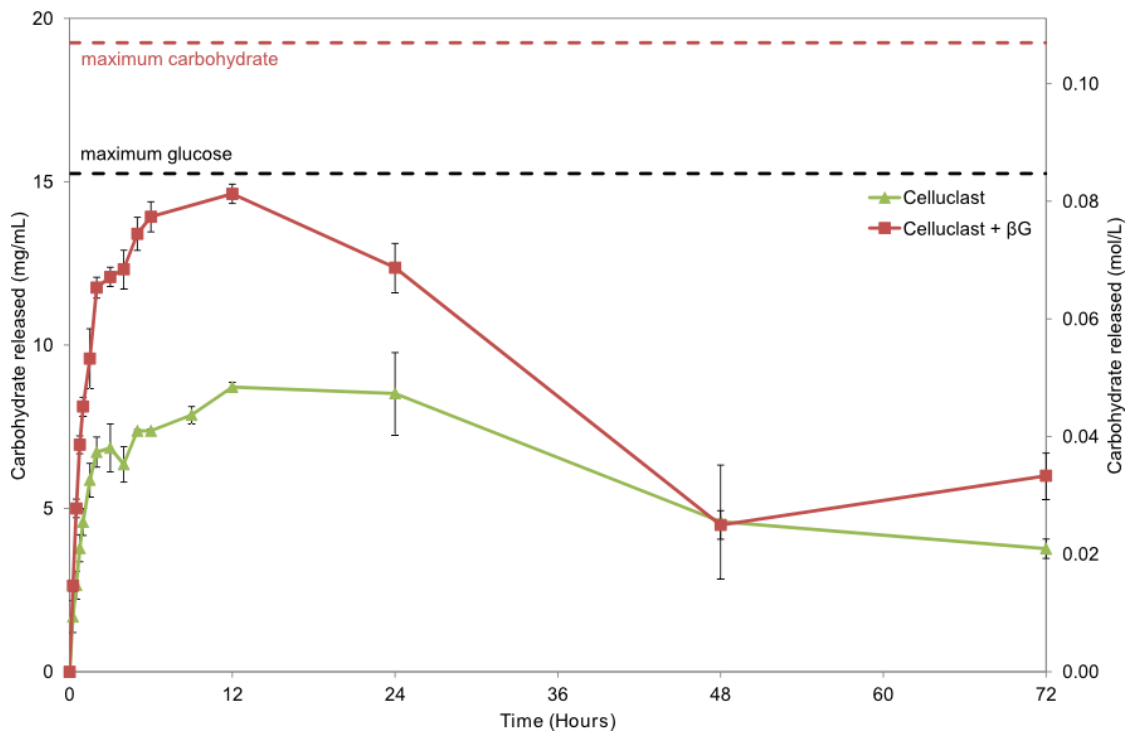
Figure 31. DNS Reducing sugar assay – Celluclast[®] with and without βG, theoretical maximum glucose (15.2 mg/mL) and total carbohydrate (19.3 mg/mL) shown as dashed horizontal lines, calculated against a standard curve of glucose

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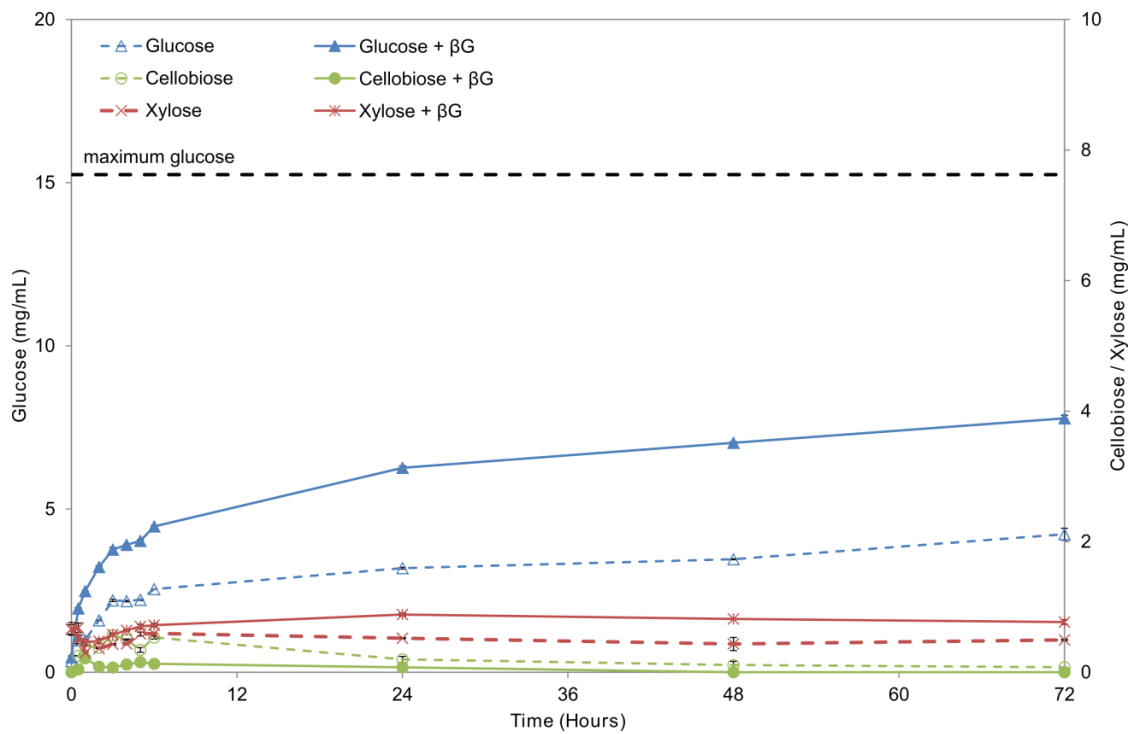
Figure 32. HPLC analysis of Depol^{TM} 740L repeat with thiomersal addition, theoretical maximum (15.2 mg/mL) shown as dashed horizontal line
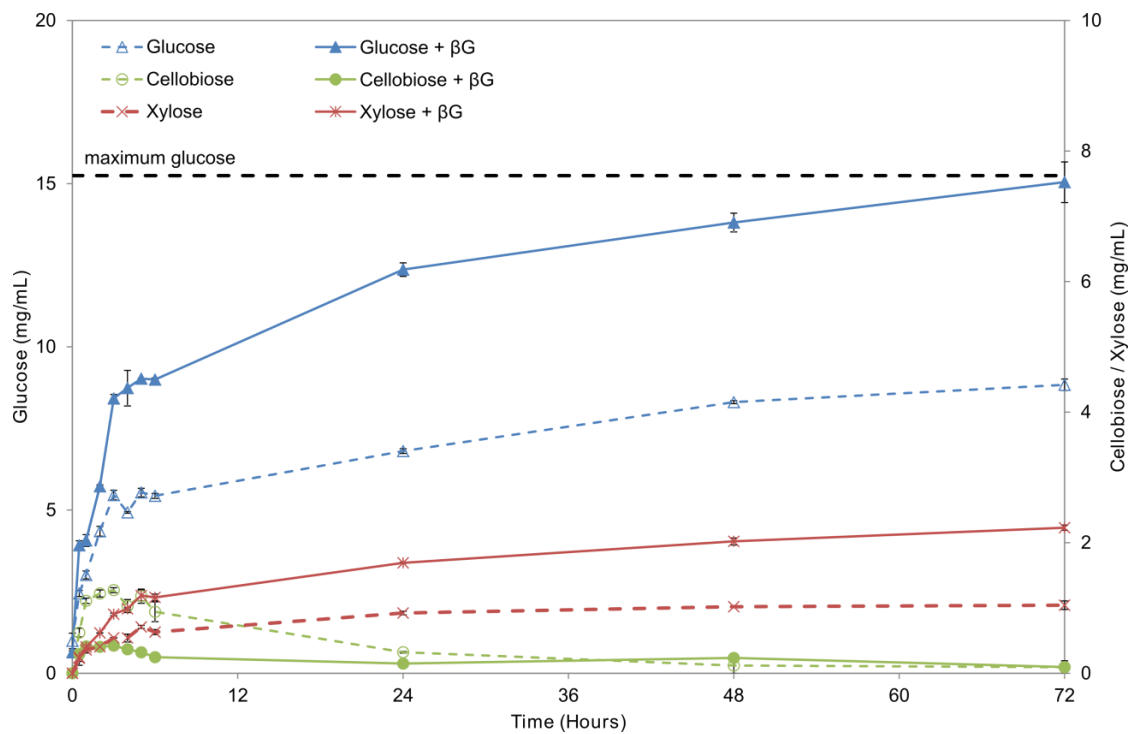
Figure 33. HPLC analysis of Accellerase® 1000 repeat with thiomersal addition, theoretical maximum (15.2 mg/mL) shown as dashed horizontal line
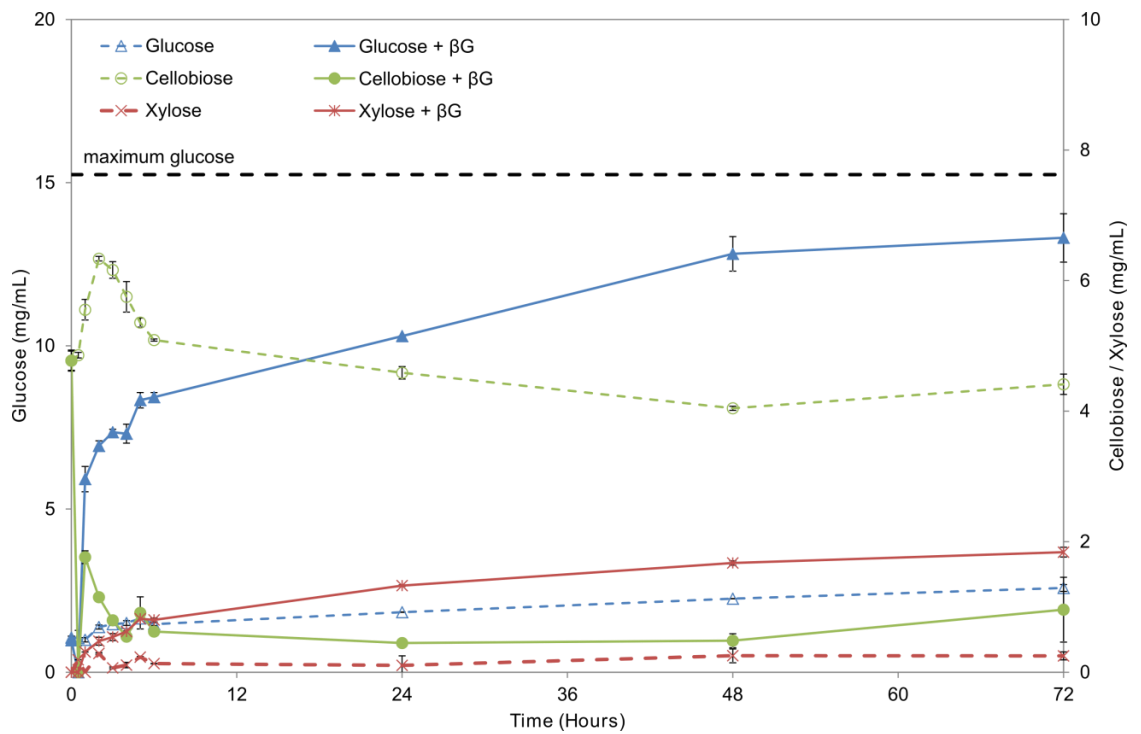
Figure 34. HPLC analysis of C013L repeat with thiomersal addition, theoretical maximum (15.2 mg/mL) shown as dashed horizontal line
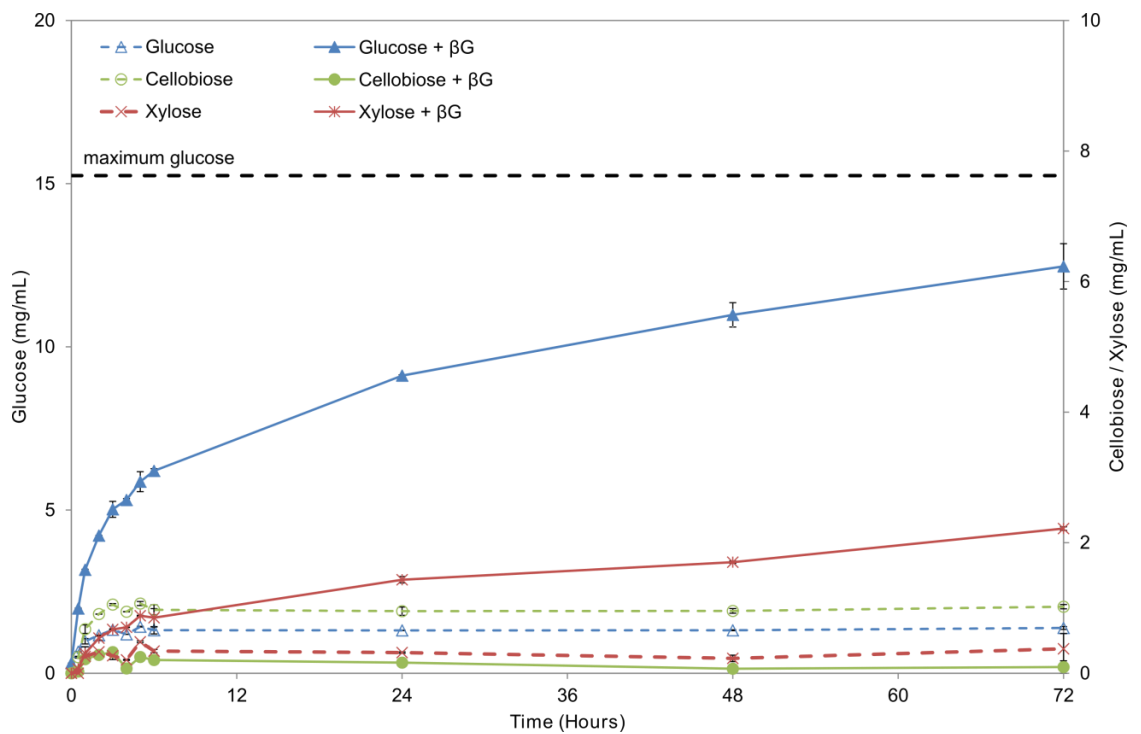
Figure 35. HPLC analysis of Acid Cellulase repeat with thiomersal addition, theoretical maximum (15.2 mg/mL) shown as dashed horizontal line
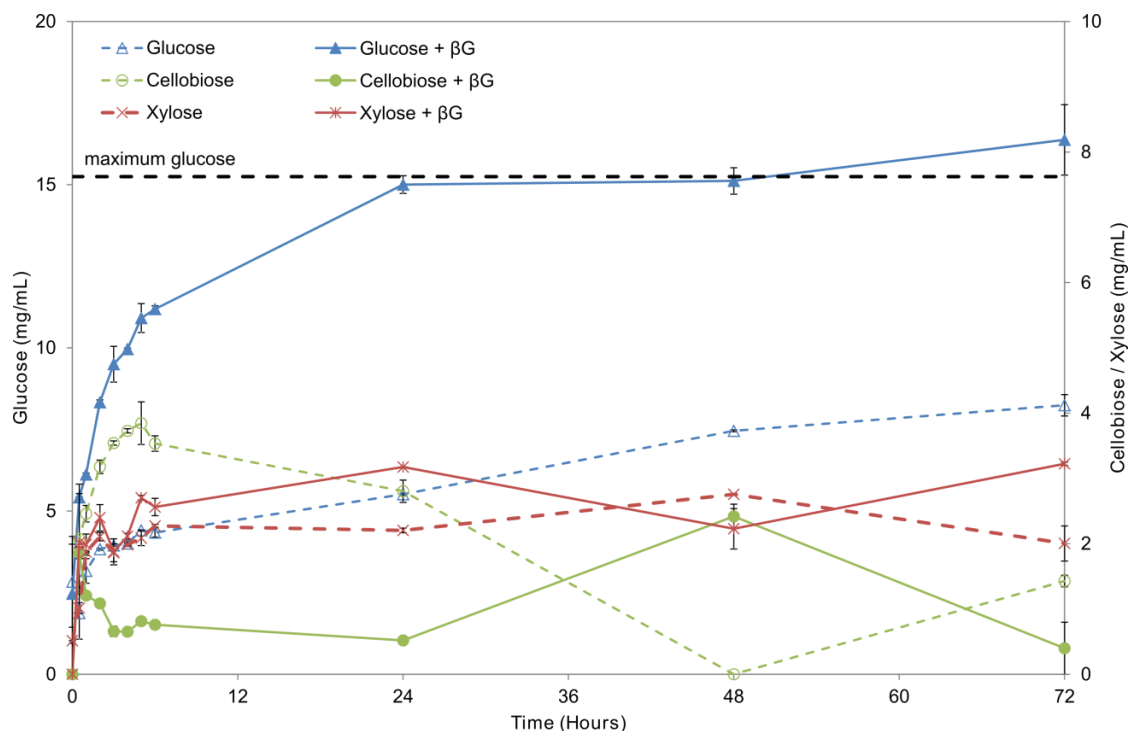
Figure 36. HPLC analysis of Celluclast® repeat with thiomersal addition, theoretical maximum (15.2 mg/mL) shown as dashed horizontal line

The results showed that although similar and excess levels were used, the different enzyme preparations gave very different hydrolysis profiles. With no additional βG, glucose production tailed off after 6 hours and remained low, achieving 19% and 10% (w/w) of theoretical maximum for C013L and Acid Cellulase (Figure 29 and Figure 30). For Accellerase® 1000, Depol™ 740L and Celluclast® digestion continued and was most effective with Accellerase® 1000 and Celluclast®, achieving similar levels after 72 hours (Figure 28 and Figure 31). The levels of cellobiose (measured by HPLC; Figure 37) varied widely and gave no clear pattern in relation to the extent of digestion. For example cellobiose was generally very low in the Accellerase® 1000 digest (Figure 28) whilst it was initially high for Celluclast® and then dropped slowly during the incubation period (Figure 31). In contrast, addition of extra βG had a general significant impact on digestion. In all cases except for Depol™ 740L the extent of glucose release over 144 hours was increased considerably to about 14 mg/mL and xylose to 5-6 mg/mL. This was accompanied by a substantial decrease in the levels of cellobiose. These changes are also reflected in the initial rates of change of both free glucose and cellobiose (Table 13). In all cases, addition of βG reduced the initial rate of

increase in cellobiose, and increased considerably the initial rate of glucose release. The combined release of cellobiose and glucose when no βG is added is significantly smaller than the extent of glucose produced when βG is added, 52% (w/w) in the case of C013L (Figure 34). This shows that βG is preventing the inhibition of the other cellulases by cellobiose and increasing the degree of hydrolysis and not merely hydrolysing the already available cellobiose. Interestingly, in the presence of added βG, the initial rates of glucose release for Accellerase$^{®}$ 1000 and Celluclast$^{®}$ were similar. The βG-related increase in xylose release is probably due to the enhanced accessibility by xylanases due to the improved digestibility of cellulose (with which the xylans presumably interact closely). Nevertheless, it is possible that cellobiose can inhibit endoxylanases and its digestion with added βG removed this inhibition as found by Lo Leggio and Pickersgill (1999) or that the βG itself contained xylanases.

Enzyme	βG	Initial Rates (mg/min)	
		Cellobiose	Glucose
Depol^{TM} 740L	-	0.53	1.38
Depol^{TM} 740L	+	0.09	3.21
Accellerase$^{®}$	-	1.32	3.13
Accellerase$^{®}$	+	0.62	6.98
C013L	-	0.46	0.04
C013L	+	-2.99	5.45
Acid Cellulase	-	0.52	1.06
Acid Cellulase	+	0.06	3.57
Celluclast$^{®}$	-	4.39	0.54
Celluclast$^{®}$	+	3.94	6.40

Table 13. Initial rates (30 minutes) of cellobiose and glucose production during hydrolysis of copier paper with a range of cellulases

The levels of undigestible material remaining (including inorganic components; Figure 38) were consistent with the release of glucose as modulated by the addition of βG. Overall, the most effective digestion was achieved utilising Celluclast$^{®}$ with additional βG, however due to high initial levels of carbohydrate present in the enzyme preparation (Figure 36) and the cost implications of using this enzyme, Accellerase$^{®}$ 1000 supplemented with βG was selected for further

study, itself hydrolysing/solubilising 99.27% (w/w) of the carbohydrate. This is a significant improvement over recent literature results using newspaper (Kuhad et al., 2010) and waste paper (Vynios et al., 2009) and also comparable to levels achieved by Marques (2008) although that study used recycled paper sludge as opposed to solid copier paper. The digestion was also at comparable to levels found in another study on office printer paper (Chen et al., 2011) with additional enzyme dosing and pH buffering overcoming the necessity of extra processing needed to remove the calcium carbonate therein described. The relative benefits of each method would require further consideration with regard to processing and enzyme loading costs.
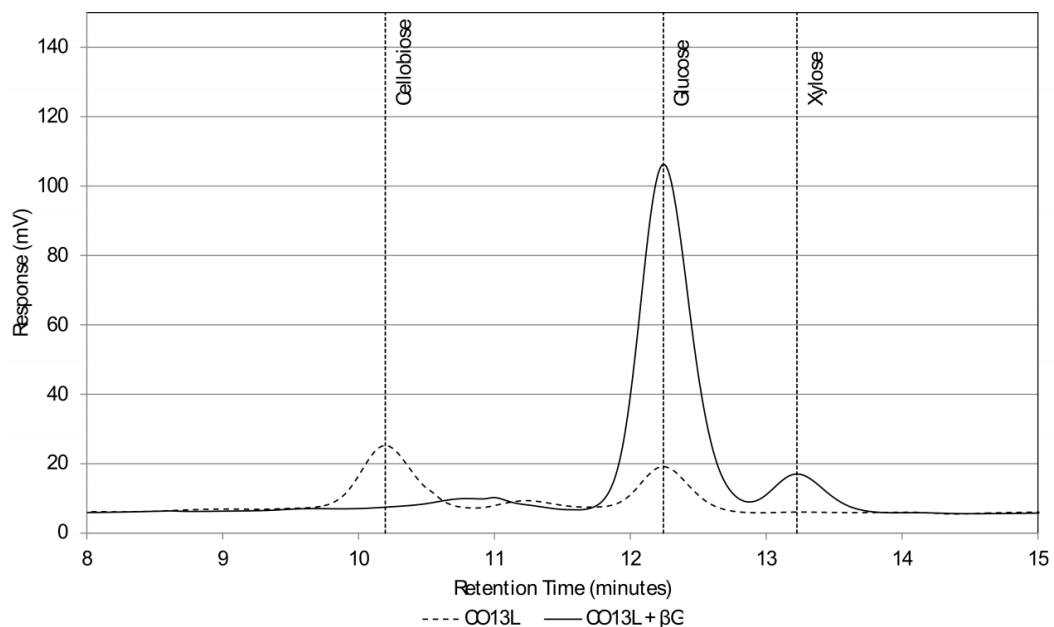
Figure 37. HPLC Molecular Mass analysis of products of digestion with cellulose C013L with (solid line) and without (dotted line) additional βG after 144 hours of digestion

Representative microscopic visualisation of the dried recalcitrant materials is shown in Figure 39 and Figure 40 again highlighting the importance of added βG. The residue remaining after digestion with Acid Cellulase and additional βG is shown in Figure 40 and is devoid of any structured cellulose. The particulate material consists of the residual kaolin ($Al_2Si_2O_5(OH)_4$) and calcium carbonate ($CaCO_3$) which is added to copier paper as a filler during manufacture (Bundy and Ishley, 1991). In contrast, the residue remaining after digestion with only Acid Cellulase contained much undegraded cellular fibres (Figure 39).
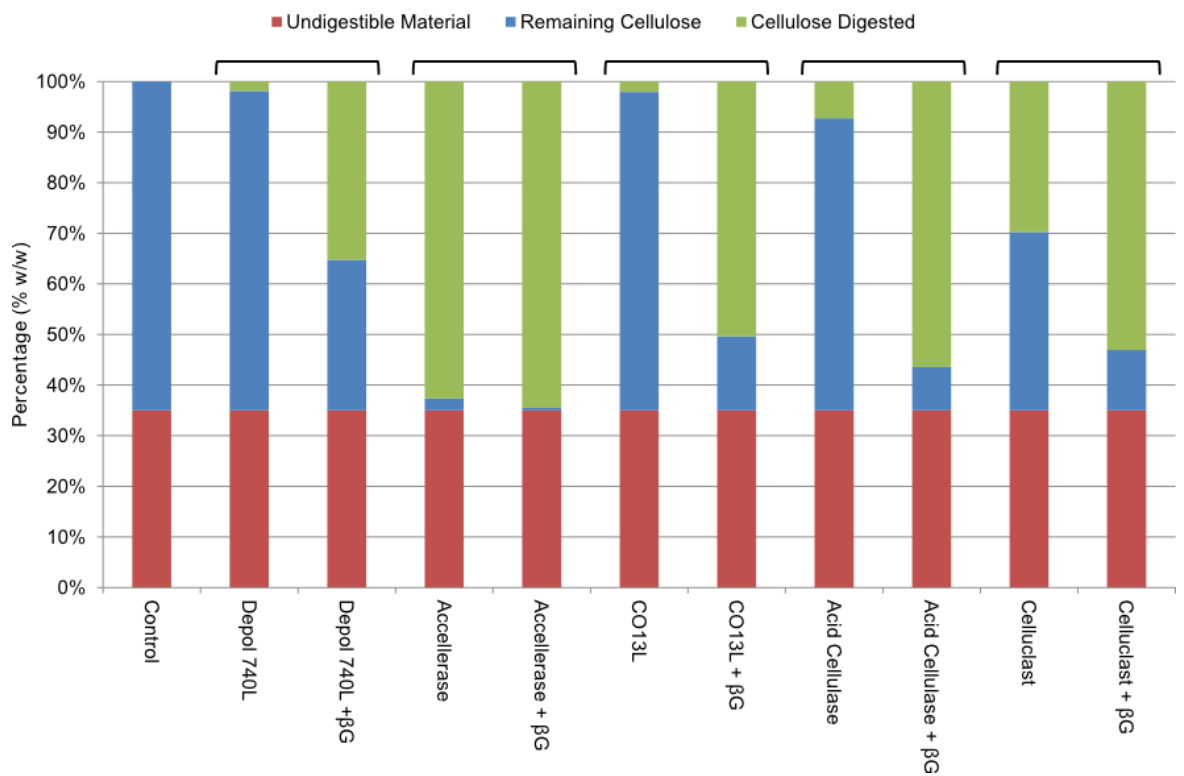
Figure 38. Digested weights remaining with and without the addition of βG, percentage of original material
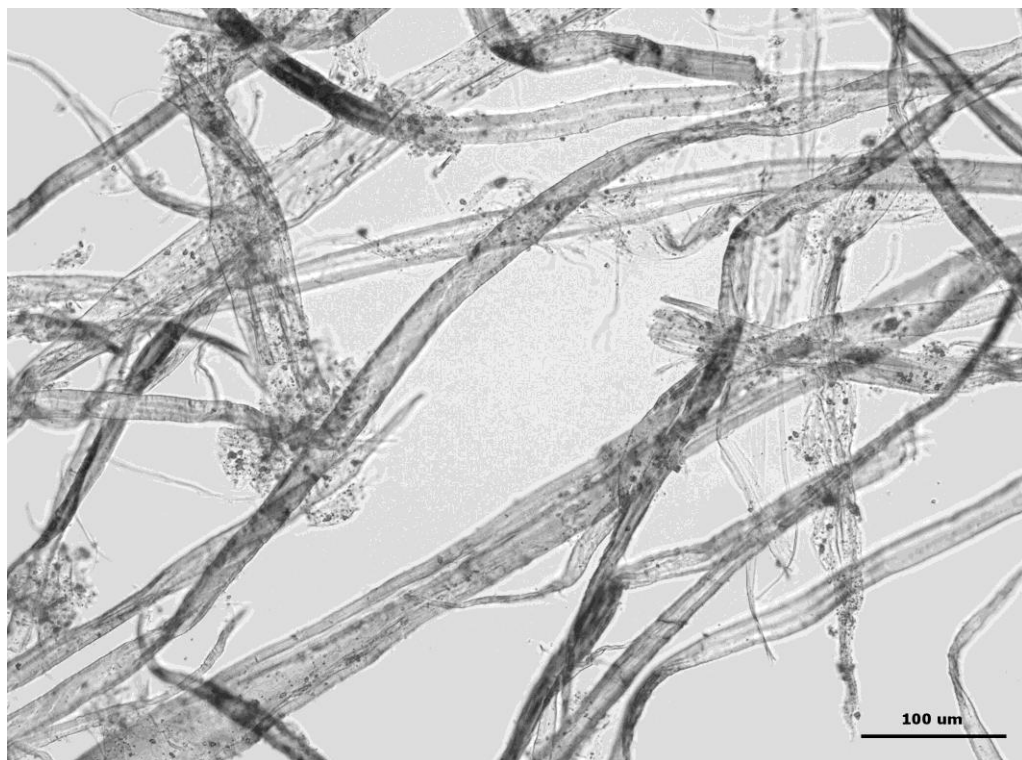
Figure 39. Micrograph of Acid Cellulase digestion
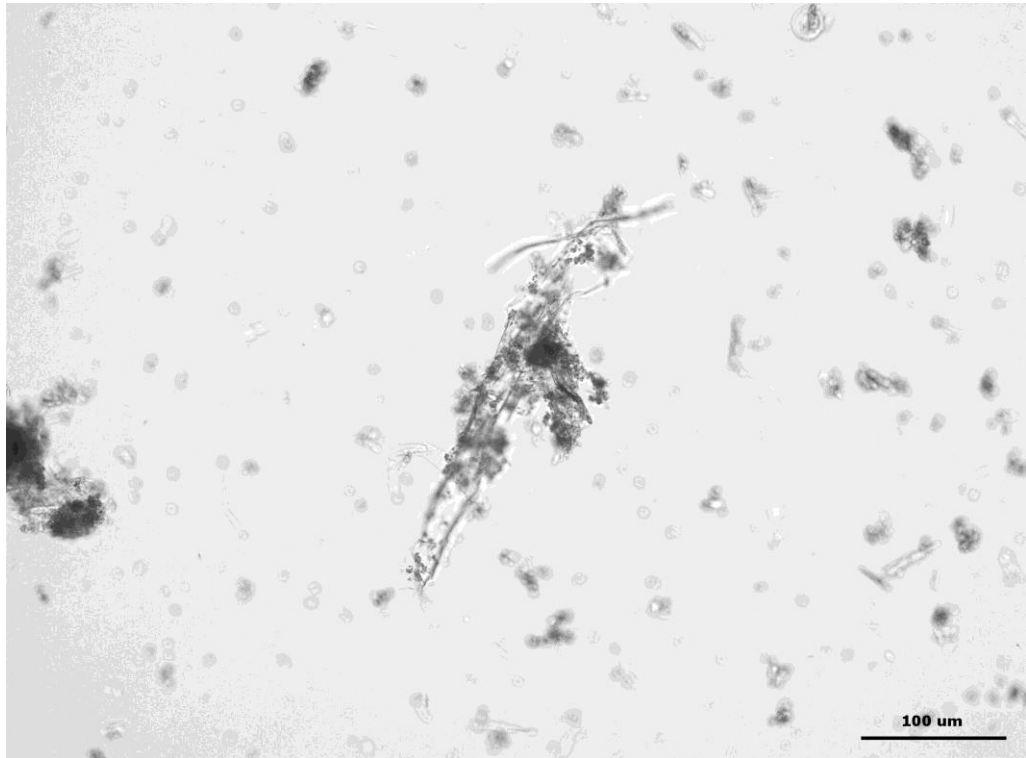
Figure 40. Micrograph of Acid Cellulase with additional βG digestion

4.2.3 Enzyme loading

On the basis of the results above, more detailed digestion studies were carried out with substrate (2.5%, w/v) and Accellerase® 1000 at a range (1-40 FPU/g of substrate) of concentrations with and without additional excess βG (2.5 U/mL). The results (Figure 41) again showed that βG has a significant impact on the initial rates and final glucose yield. However it also demonstrated that at cellulase loadings of below 20 FPU/g of substrate, the final plateau yield of glucose decreased. On the basis of this result, suggesting that an enzyme concentration of greater than 10 and less than or equal to 20 FPU/g of substrate was the optimal for digestion, an enzyme loading of 20 FPU/g of substrate was chosen for further evaluation.
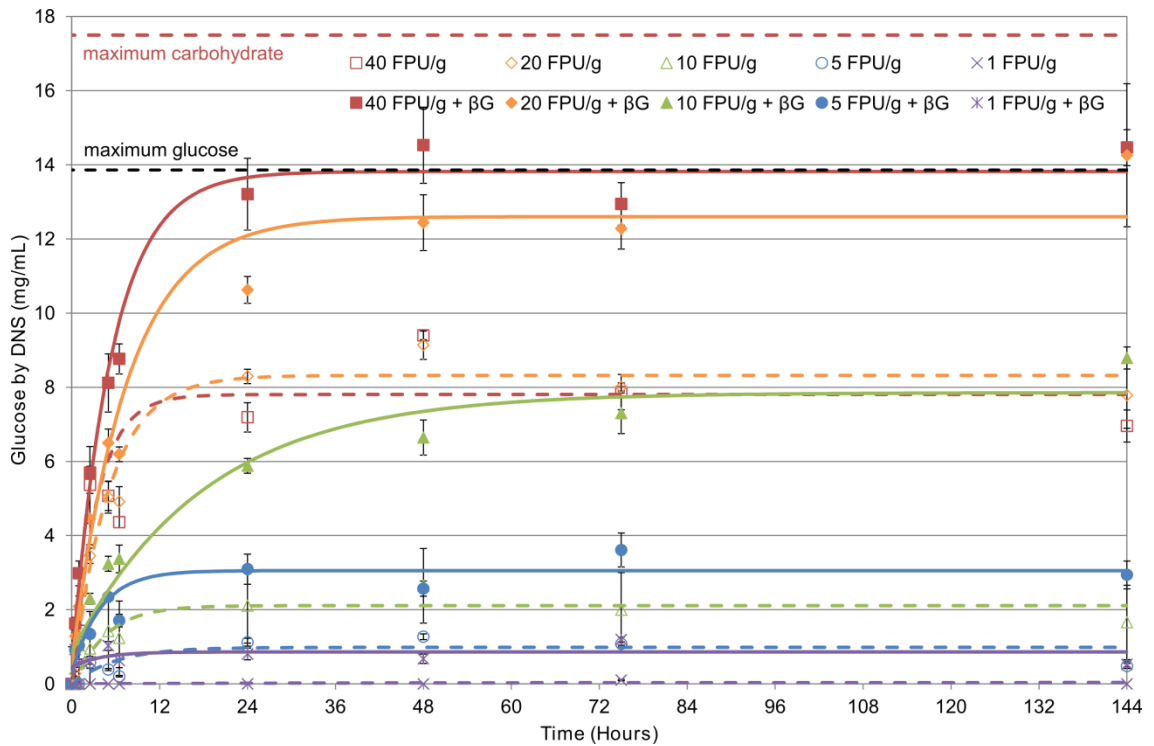
Figure 41. Effect of enzyme loading on the hydrolysis of paper, theoretical maximum glucose (13.9 mg/mL) and total carbohydrate (17.5 mg/mL) shown as dashed horizontal lines

4.2.4 Substrate Concentration

Initial digestions (above) involved paper substrate in the region of 2.5% (w/v). This would limit the potential concentration of glucose realised through hydrolysis to a maximum of approximately 1.25% (w/v) of the total paper substrate. In order to assess the potential of increasing the final glucose concentration, the impact of paper substrate concentration on digestion was investigated (Figure 42). The bars in the histogram provide data on the degree of digestion (left-hand Y axis) and the diamond points show the potential maximum concentration of sugars inferred from that degradation (right-hand Y-axis). Square points (where present) provide an estimate of free sugars as measured by the DNS procedure.
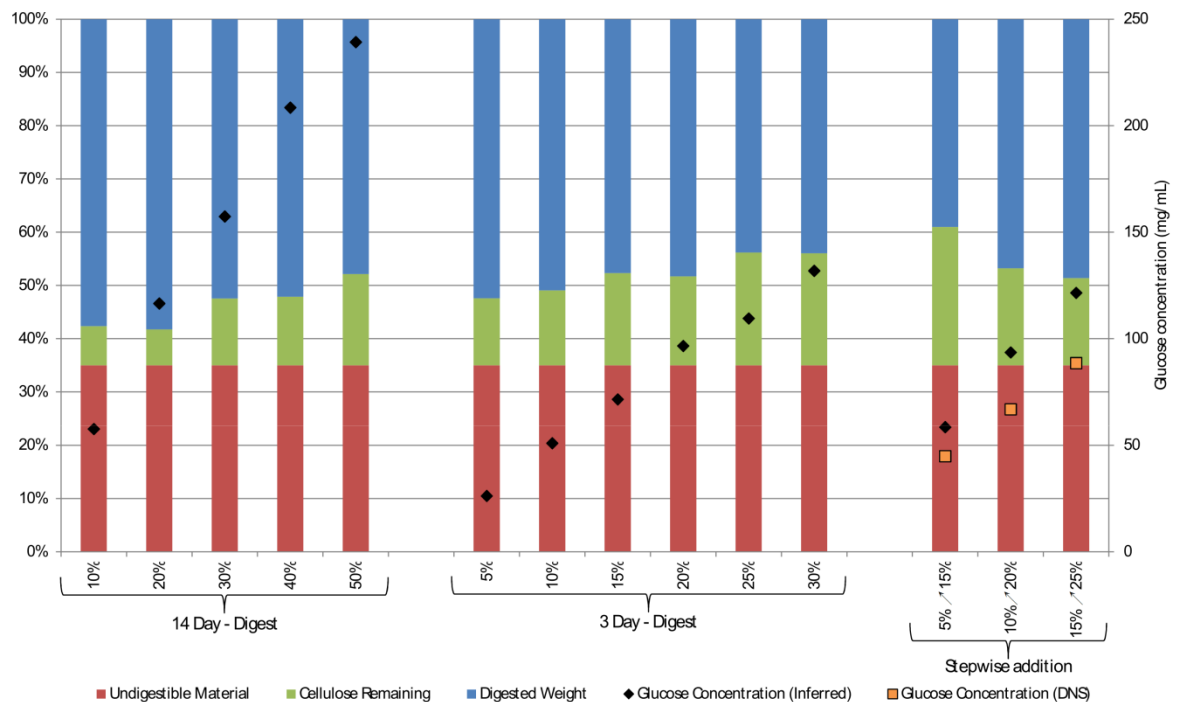
Figure 42. Effect of substrate loading, either as single addition or as multiple additions, on the degree of digestion and on final glucose concentrations

Sequential addition of substrate (with no further addition of enzyme) was performed to evaluate whether the concentration of released glucose could be increased incrementally as a semi-continuous process. The results (Figure 42 right hand side) show that the sequential addition of paper (initial enzyme concentration Accellerase® 1000 without βG supplementation was 20 FPU/g of substrate) facilitated an increase in the actual concentration of sugar released to nearly 90 mg/mL (approximately 0.5 mol/L). The concentration of sugar inferred from the recovery of recalcitrant residue was a little higher at about 115 mg/mL (0.63 mol/L). This difference is likely to be due to the presence of undigested cellobiose in the soluble fraction. The addition of substrate meant that the final enzyme:substrate ratio decreased to between 12 FPU/g of substrate where final substrate concentration was 25% (w/w), and 6.7 FPU/g of substrate where final substrate concentration was 15% (w/w). These substrate loading percentages show an improvement over current literature where 2-11% (w/v) is used (Banerjee, 2011, Peng and Chen, 2011, Prasetyo et al., 2011).

Hydrolysis reactions were also carried out over 3 days at substrate loadings of up to 30% (w/w). The enzyme loadings were all 20 FPU/g of substrate. The results show that the extent of hydrolysis was lower at higher substrate concentrations, but enabled a potentially higher concentration of glucose to be achieved (up to 130 mg/mL). Interestingly, the initial high substrate loading gave slightly lower inferred sugar yields as compared with the sequential addition approach (as well as using more enzyme). This is probably because the batch additions liquefied more rapidly than the single addition and facilitated greater mixing and a higher hydrolysis rate during the digestion period.

Higher substrate loadings of up to 50% (w/w) could also be effectively hydrolysed over 14 days (Figure 41), resulting in a potential sugar concentration of over 240 mg/mL. Initially the paper was essentially a wet solid, but eventually became liquefied. This level of glucose in the hydrolysate is approximately a factor of 10 larger than that reported in most literature in this area (Marques et al., 2008) although 10 days is probably too long from a commercial perspective.

It is often the case with ligno-cellulosic substrates, that the substrate concentration is generally inversely related to the percentage of hydrolysis achieved, even when the enzyme/substrate concentration is kept the same This is termed "solids effect"; (Kristensen et al., 2009). However, the results in this study indicate that for copier paper, the effect is not as pronounced as it is with more lignified materials. The presence of lignin and hemicelluloses may therefore be contributing factors of this effect (Kristensen et al., 2009). It is possible that the low levels of hemicellulose in the paper (Table 12) also exerted further effect.

4.2.5 Alcohol Inhibition

Alcohol Inhibition has been noted in SSF before (Bezerra and Dias, 2005, Ooshima et al., 1985). In Figures 43, 44, 45, 46 and 48, inhibition is measured as the percentage change of reducing sugar release, measured by DNS, compared to the 0% (v/v) sample i.e. in the absence of alcohol.

4.2.6 General Inhibition

Over an alcohol concentration range of 0-10% (v/v), a common trend was observed in which increasing the concentration of alcohol resulted in an increase in the degree of inhibition of the measured activity. The results demonstrate that concentrations above approximately 3% (v/v) alcohol, which would be readily achieved during fermentation, have a significant inhibitory effect on the action of the saccharification and would therefore diminish the SSF process as these concentrations increase. This is similar to the finding of Bezerra (2005) and Ooshima (1985) where alcohol inhibited the activity of the cellulase in SSF due to denaturing of the enzyme at higher concentrations.

4.2.7 Activation at low alcohol concentrations

It can also be seen (Figure 44) that there is an activation effect on hydrolytic activity at alcohol concentrations of approximately 1-3% (v/v). The effect is less pronounced in Figure 43, Figure 45, and Figure 46 but even where there is no actual positive effect there is a visible inflection on the graphs again at ~2% (v/v), giving less than the expected linearly proportional inhibition.

With the exception of the high activation of Depol[TM] 740L with Sigmacell[®] and ethanol (Figure 44) there is a general trend of reduction in activation as alcohol chain length increases. It is also worth noting that in general C013L and Depol[TM] 740L have similar reactions to alcohol as do Accellerase[®] 1000 and Acid cellulase. This may be due to the fact the both C013L and Depol[TM] 740L are both produced by the same supplier (Biocatalysts) and therefore most likely have similar purification steps carried out on them. This can be seen in that C013L and Depol[TM] 740L are substantially less opaque than either Accellerase[®] 1000 or Acid cellulase.
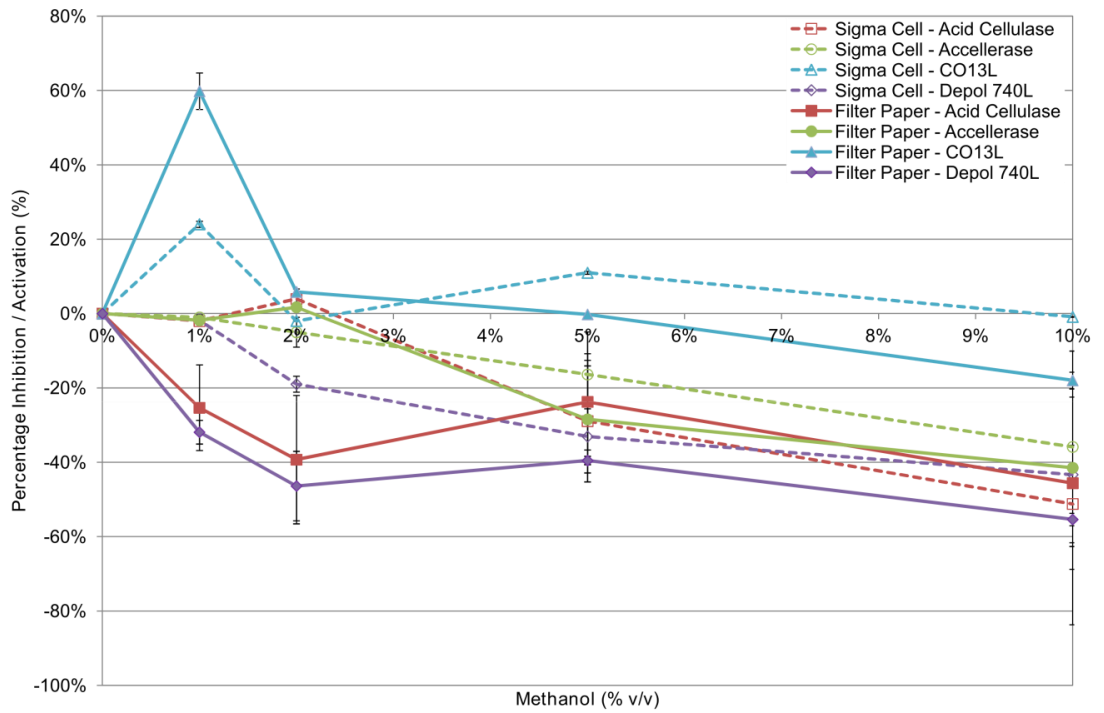
Figure 43. Alcohol inhibition/activation – methanol
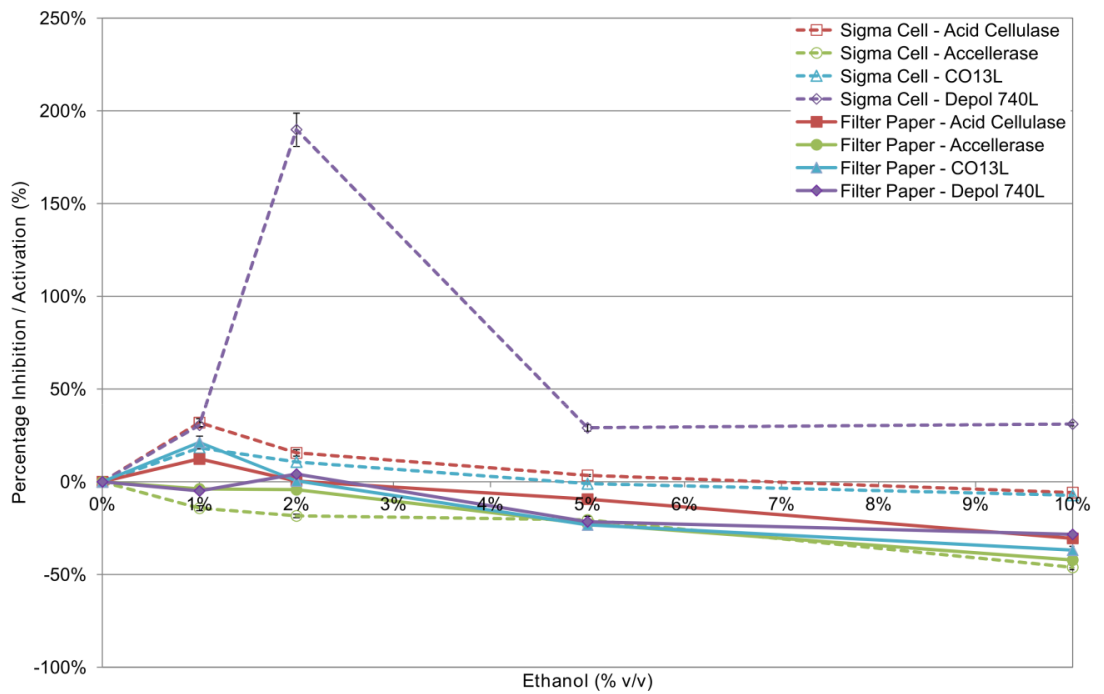
Figure 44. Alcohol inhibition/activation – ethanol
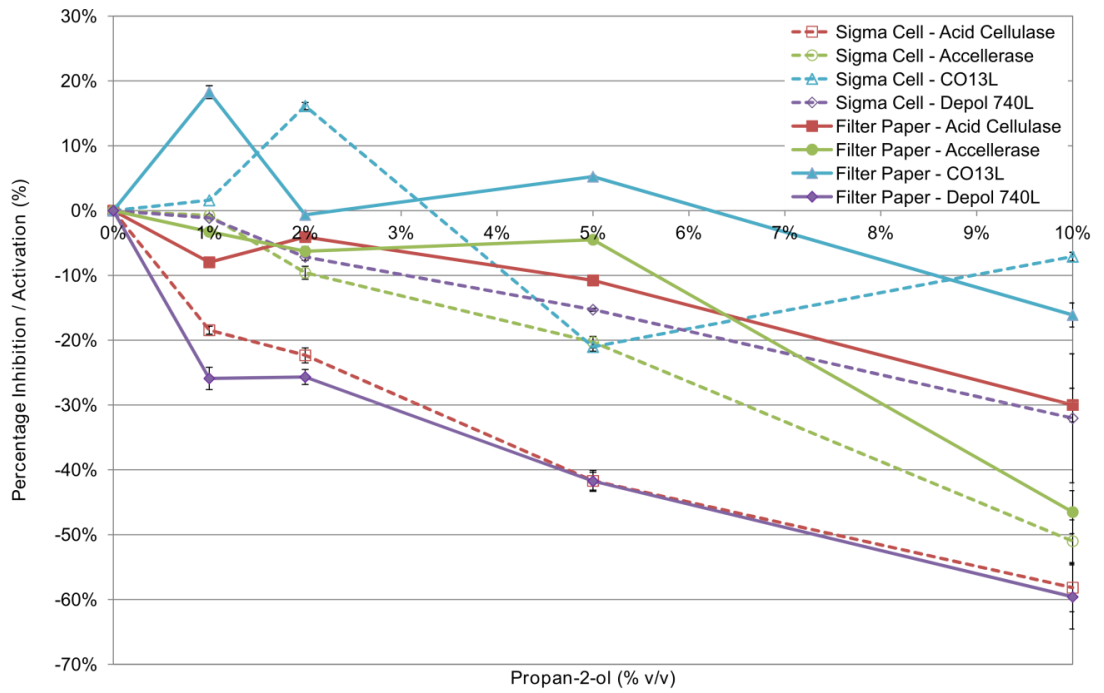
Figure 45. Alcohol inhibition/activation – propan-2-ol
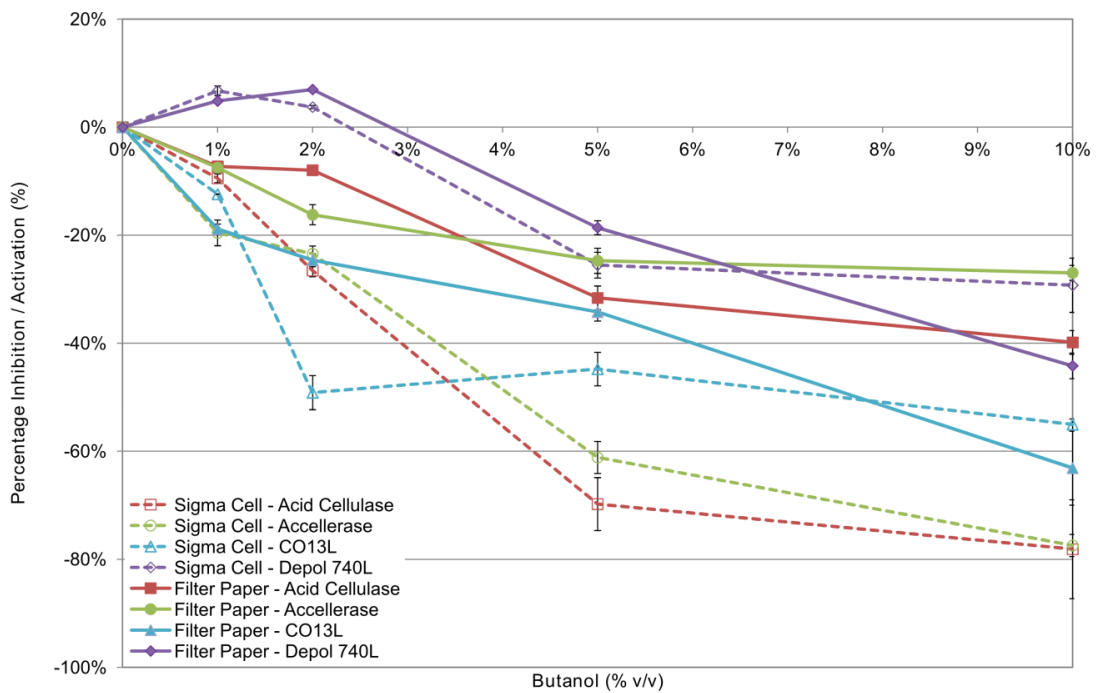
Figure 46. Alcohol inhibition/activation – butanol
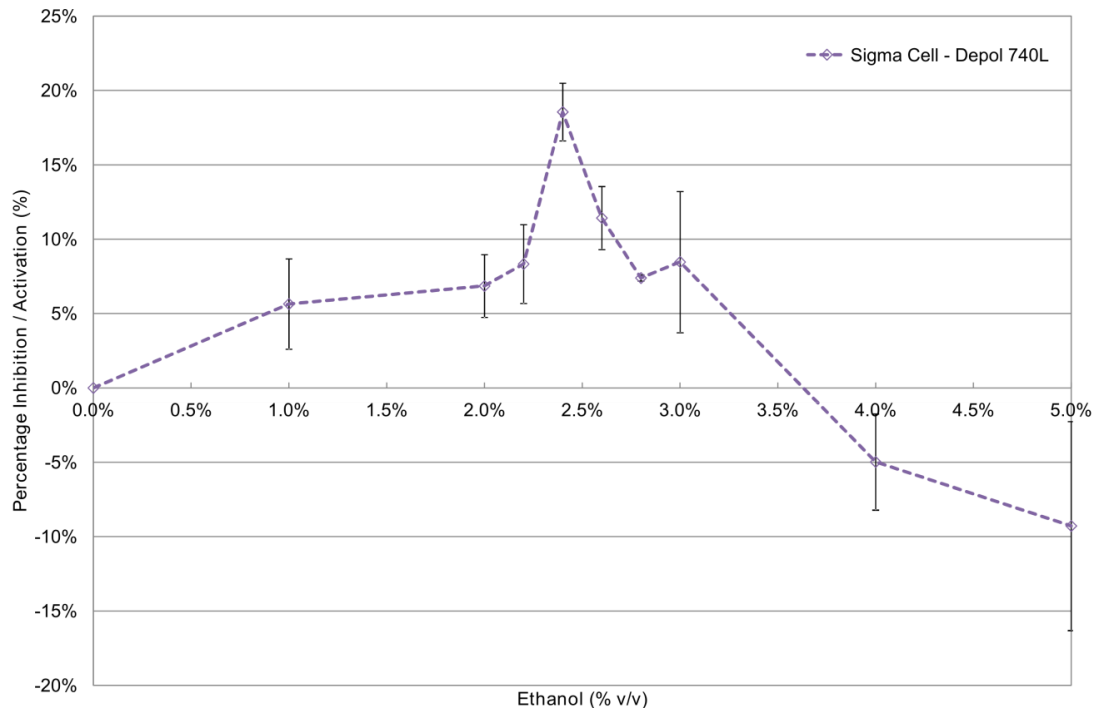
Figure 47. Alcohol inhibition/activation – Optimisation with Depol^{TM} 740L

In order to shed more light on the impact of alcohols at low concentrations, a detailed study was carried out to evaluate more precisely the concentration at which alcohols might have the greatest stimulatory effect. As ethanol was found to give the greatest effect on Sigmacell$^{®}$ these were chosen as the inhibitor and substrate. Depol^{TM} 740L was used as the enzyme as it showed the greatest activation effect. Sample points were taken between 1 and 5% ethanol (v/v) and the results are shown in Figure 47. Within these parameters ethanol has an optimal activation effect at 2.4% (v/v). Above 4% (v/v) it again starts to have a negative effect on the hydrolysis. The activation effect may be more prevalent in Depol^{TM} 740L due to the fact that it is purified from *Humicola sp.* as opposed to *Trichoderma sp.* in the case of the other enzymes used therefore having a slightly different cellulase hydrolysis system (Schülein, 1997), furthermore Depol^{TM} 740L yields the lowest actual concentration at 0% (v/v) meaning that smaller fluctuations yield higher percentage activations. The mechanistic basis of this effect is discussed further below.
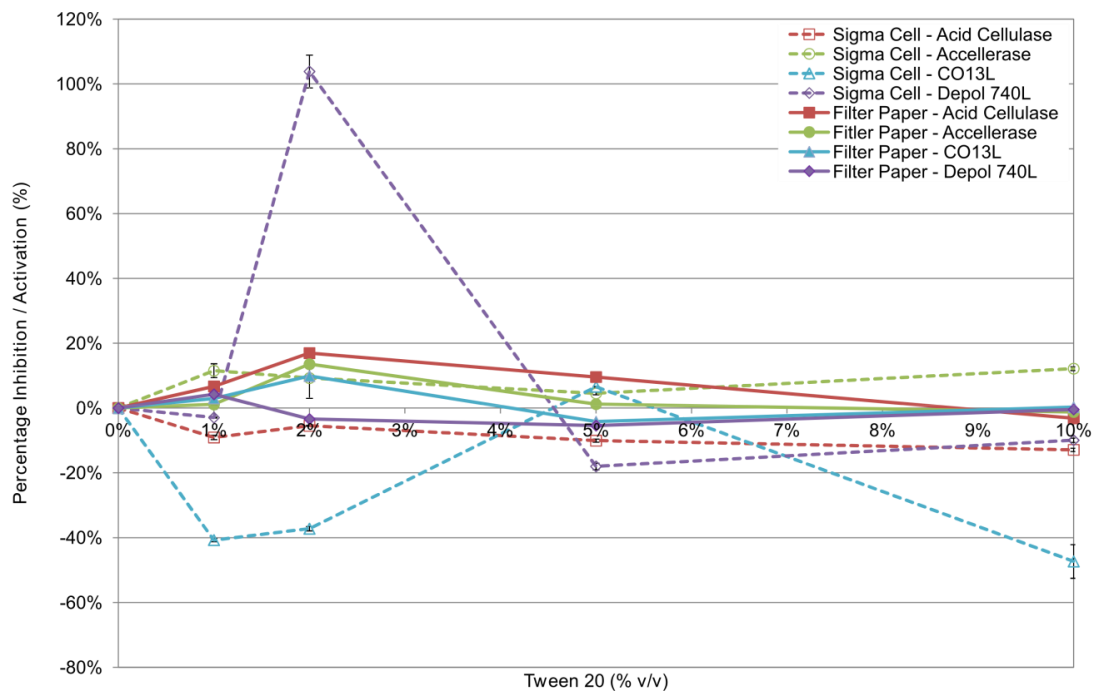
Figure 48. Alcohol inhibition/activation – Tween[®]

4.2.8   Solubility and detergency

It is possible that the activation effect of alcohol at low concentrations is due to an increase in substrate solubility, and/or an increase in the accessibility of the enzyme to the substrate, thereby increasing the efficiency of saccharification. This hypothesis was tested using a proprietary detergent, Tween[®] 20 (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany), in place of alcohol. When Tween[®] 20 was used (Figure 48), the detergent provided similar results to those seen with ethanol (Figure 44), Depol[TM] 740L again was affected further than other enzymes reasserting the probability that the different enzyme system (*Humicola sp*) is effected more. Therefore, this would seem to suggest that a hydrophobicity or protein solubilisation effect may well underlie the stimulation of enzyme saccharification.

As commercial cellulase is made up of a number of sub-enzymes, repeat experiments were carried out using sub-enzyme specific substrates to ascertain if the alcohol was activating any sub-enzymes in particular, for example using Azo-CM-Cellulose to test the effects on endo-cellulases. However these assays

showed no positive effect on any of the sub-enzyme groups at low levels of alcohol. Nonetheless all the specific substrates are water soluble whereas the substrates previously used have been either totally or partially insoluble. Therefore another set of assays was carried out using carboxymethylcellulose (CMC) which is a soluble form of cellulose as a substrate, Depol[TM] 740L and ethanol with conditions equivalent to prior experiments. These assays also showed no notable activation of the cellulases at low concentrations of alcohol. These data lead to the hypothesis that CBMs on the cellulase may be more readily able to attach and detach from the cellulose surface in the presences of small quantities of alcohol, due to the detergency effect (as per Tween[®] 20) therefore reducing non-productive binding. When levels of alcohol are increased the CBMs may then detach too readily or be unable to attach at all meaning that the cellulase is considerably less productive. This hypothesis would explain why activation is greater in insoluble substrates such as filter paper where cellulases are more likely to become blocked by perpendicular and entangled groups of cellulose fibres within the substrate than a more soluble substrate.(McLean et al., 2002).

4.3 Conclusion

The results show that for the effective enzymatic hydrolysis of copier paper, single-step quantitative hydrolysis was achieved with loadings of Accellerase[®] 1000 greater than 10 FPU/g of substrate (20 FPU/g of substrate being the optimum tried) in the presence of additional βG and an anti-microbial agent. βG not only increased the extent of digestion, but also the initial rate of digestion. Although the solids effect was witnessed with the copier paper substrate it was overcome if sufficient levels of enzyme were present. This enabled sequential additions of paper to be made, degrading nearly all the cellulose fibres. As a result, the final enzyme loading could be effectively reduced whilst facilitating high glucose concentrations. This provides suitable information for developing scaled-up processing approaches which are the subject of further studies.

Low concentrations of Tween[®] 20 and in some cases ethanol has great potential as an additive stimulating the hydrolysis action of some cellulase preparations, enabling increased glucose yield. Solid, insoluble substrates such as

lignocellulose appear to benefit most from this phenomenon, possibly due to the alleviation of non-productive binding allowing CBMs to attach/detach easily from the cellulose surfaces. It should also be noted that alcohol concentration can have a profoundly negative effect on the cellulase activity when concentrations exceed approximately 3-4% (v/v), with inhibition climbing to 50-60% (compared to no alcohol addition) in some cases.

5 The effects of steam explosion pre-treatment on saccharification of paper cellulose

As discussed in §1.6 pre-treatment is an important part of the lignocellulosic bioalcohol process enabling the opening up of the cellulose structure (reducing crystallinity) and therefore increasing digestibility. Chapter 4 sought to optimise enzyme digestion of copier paper in the absence of any pre-treatment. However thermophysical pre-treatment of the substrate might be expected to improve the digestion either by allowing the use of less enzyme or increasing the speed of digestion (Ewanick and Bura, 2010). As one of the preferred pre-treatment processes is steam explosion (§1.6.5), it has been chosen for the study using copier paper as a substrate as there has been little previous work in this area. In this study, effect of pre-treatment intensity, as defined by severity factor, has been evaluated in relation to the digestibility of copier paper cellulose with Accellerase® 1500 and co-fermentation by *S.cerevisiae.*

Comparisons have also been made with filter paper as a source of cellulose and also wheat straw as a standard substrate for bioalcohol production. The levels of inhibitors produced at high severities were studied along with their possible reduction by the addition of copier paper while steam explosion is conducted.

5.1 Materials and Methods

5.1.1 Materials

M-Real copier paper, Whatman No. 1 filter paper and dust extracted wheat straw (Dixon Brothers, Norfolk, UK) were used as the substrates for this experiment, the paper substrates were shredded using a PS-67Cs (Fellowes, Doncaster, UK) cross shredder to 3.9 x 50 mm particle size (Din Security Level 3), the straw was supplied shredded into lengths of approximately 40 mm.

5.1.2 Steam explosion

Steam explosion was carried out using pilot scale steam explosion apparatus (Cambi, Asker, Norway), aliquots of 250 g were exploded at a range of different severity factors achieved by altering residence time and temperature. Severity

factors are calculated from Equation 1 (Overend et al., 1987). The steam explosion apparatus was equilibrated to required temperature prior to the addition of material in order to reduce temperature fluctuation during actual explosion. After explosion recovered samples were immediately weighed and then frozen, with aliquots taken for further analyses which were keep under refrigeration. The apparatus was pre-pressurised and exploded several times to ensure the removal of all material before severity factors are altered. Steam explosion apparatus can be seen in Figure 49.
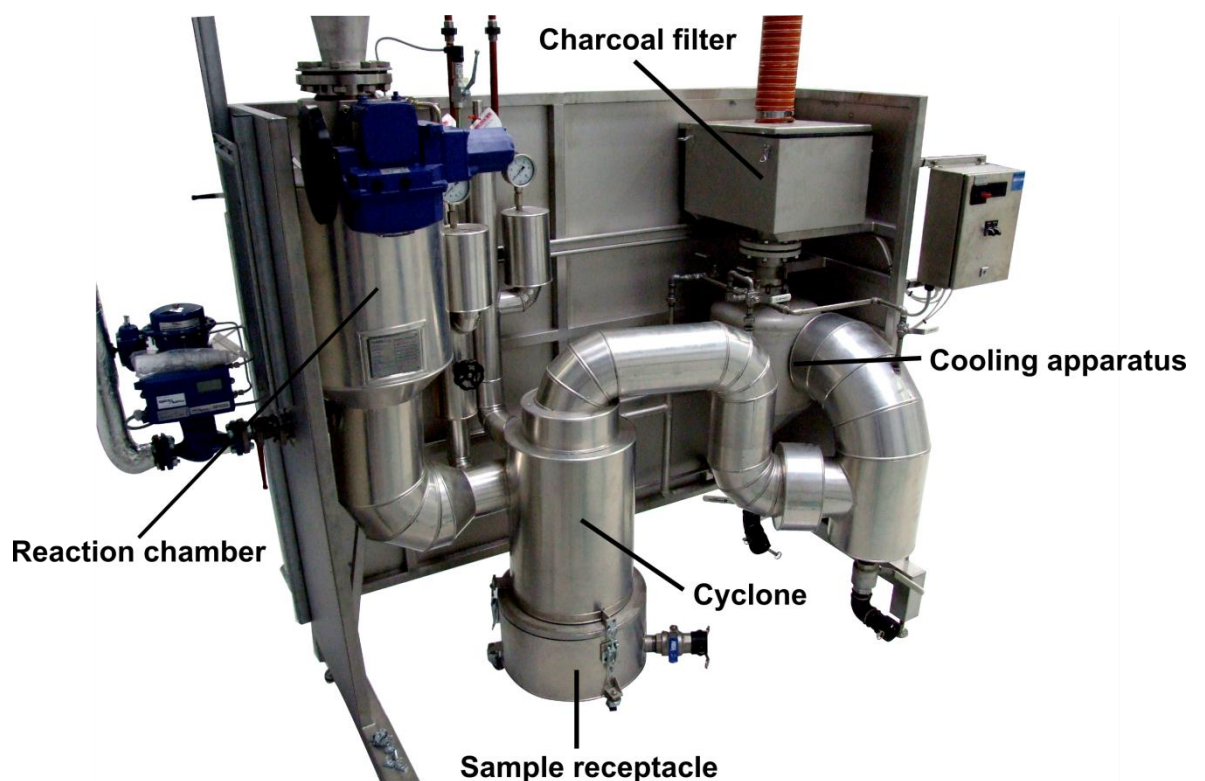
Figure 49. Cambi – pilot scale steam explosion apparatus

5.1.3 Dry weights

Moisture content was calculated for the steam exploded samples by freeze drying (Birchover Instruments Ltd, Hitchin, UK), recording original and final dry weight then calculating moisture by loss in weight. Alternatively the preferred method is to calculate moisture content by use of a Mettler LP-16 Infrared Dryer Balance (Mettler-Toledo Ltd, Leicester, UK).

5.1.4 FT-IR

FT-IR was conducted in triplicate for all samples against an air blank see §2.4.1.

5.1.5 Enzyme digestion

Steam exploded samples were weighed out to give 0.5 g dry weight in 20 g total, therefore 2.5% (w/v) substrate concentration. 200 µL Accellerase® 1500 (16 FPU/g of substrate) and 40 µL βG (20 U/g of substrate). Accellerase® 1500 was chosen on the basis of results from Chapter 4 and with the discontinuation of Accellerase® 1000. Further experimentation using low enzyme additions evolved the reduction of enzyme addition by a factor of ten (1.6 FPU/g of substrate Accellerase® 1500 and 2 U/g of substrate βG)

5.1.6 Simultaneous Saccharification and Fermentation

0.25 g dry weight of each sample was weighed out into 20 mL glass bottles, dry weight was calculated from moisture content derived from Mettler LP-16 Infrared Dryer Balance. Bottles were made up to 8.9 mL with Yeast Nitrogen Base in 0.1 mol/L NaOAc buffer (5.0 pH). NCYC 2826 *Saccharomyces cerevisiae* (NCYC, Norwich, UK) was chosen as the fermenting organism for this experiment due to its high ethanol tolerance (15-20% v/v), see Appendix F for more details on this strain. 1 mL NCYC 2826 in YM media, with a cell count of 6.45×10^7 cells/mL was added along with 75 µL Accellerase® 1500 and 25 µL βG, 20 FPU/g of substrate and 25 U/g of substrate respectively, giving a total volume of 10 mL. A control with no substrate was used to account for any residual fermentable sugars available in the YM inoculum and enzyme addition. Bottles were incubated at 25°C whilst being shaken for 48 hours, then 2 mL samples are taken into gas tight screw cap tubes which were boiled to stop further fermentation/saccharification.

In order to remove unwanted inhibitory compounds samples were also washed with ultra pure water. This was achieved by filtration through GF/C filter paper using a Buchner funnel attached to a vacuum pump. Each sample was subjected to three washes with 150 mL of ultra pure water each time.

5.1.7 HPLC analyses

Soluble/solid sugars, ethanol and organic acid/inhibitors were analysed by HPLC as described in §2.3.5, §2.3.6 and §2.3.7.

5.2 Results and Discussion

5.2.1 Visual degradation

Copier paper was steam exploded for between 10 and 45 minutes over a range of temperatures from 170-230°C spanning a range of severity from SF 3.06 to 5.48. The visual impact of the pre-treatments are shown in Figure 50, and recoveries in Table 13. At high severity (5.48) the moisture content increased considerably (Figure 50) to a point where the sample becomes a slurry. Higher temperatures and residence times require higher pressures and larger quantities of steam therefore imbuing the paper with more moisture (Table 14). It can also be seen that as severity increases that the samples became browner in colour; this is most likely due to the formation of organic acid and furfural products attributed to the caramelisation of the monomeric sugars (Maga Joseph, 1989).
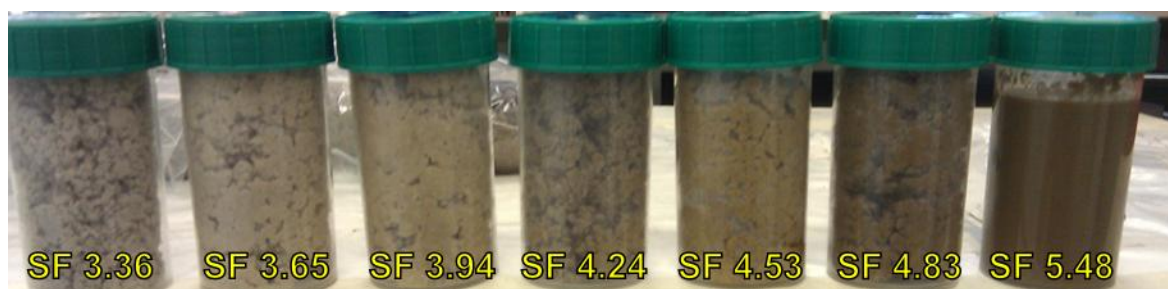
Figure 50. Steam exploded paper – increasing severity from left (SF 3.36) to right (SF 5.48)

Table 14 shows the mass lost during the steam explosion process calculated from mass in and out of the system and the moisture contents of both (5% w/w moisture in paper). The recovered weight accounts for 88-97% (w/w) of the starting material, the loss most likely being contributed to the inability to collect all the post steam exploded material from the reaction chamber. The Cambi™ steam explosion system, at very high intensity, may cause some material to be blown from the vortex into the exhaust port; accounting for the additional loses at high

severity. However, despite the explainable difference in moisture contents and change in colour, inspection of the paper fibre reveals only a marginal difference in consistency.

Temp (°C) / Time (min)	Severity Factor	pH	Moisture Content (% w/w)		Recovered Weight	
			Freeze Dried	IR Dryer	(g)	(% w/w)
170 / 10	3.06	7.8	82.67	82.05	220	92
180 / 10	3.36	7.8	77.92	80.00	214	90
190 / 10	3.65	7.6	85.98	83.12	219	92
200 / 10	3.94	7.8	81.33	80.06	231	97
210 / 10	4.24	7.7	79.28	78.84	222	93
220 / 10	4.53	7.1	86.58	87.85	213	89
230 / 10	4.83	7.0	80.19	80.95	211	89
230 / 45	5.48	6.4	93.32	93.40	209	88

Table 14. Steam explosion moisture contents and pH

5.2.2 Microscopy

Optical microscopy was conducted using an Olympus BX60 brightfield microscope (Olympus, Japan) on the range of steam exploded samples to further investigate the initial visual inspection findings. The results, which can be seen in Figure 51, reconfirm that there has been little visible physical change in the paper fibres with the exception of SF 5.48 where fibre appear to be finer than those in other samples. There is also some change in the general dispersion of the fibres but there appears to be no reduction in fibre length for the samples.

5.2.3 Chemical analysis of steam exploded material

Samples of steam exploded materials were freeze dried and analysed for sugar composition. The results are shown in Figure 52. Two phases of severity can be clearly seen with the transition above SF 4.24. These phases have been detected in studies on the effects of steam explosion on pure microcrystalline cellulose (Jacquet et al., 2011). They showed that low severity causes some thermal degradation of material but at severities above 4 this becomes depolymerisation

(Figure 53). In the case of paper it would appear that this depolymerisation stage is shifted towards higher severity (4.24), this shift is most likely due to the effect of the calcium carbonate in the paper increasing the pH of the system and therefore reducing the effective severity of the steam explosion (Meyer and Pedersen, 2010), this is corroborated by Table 14 where the pH only begins to appreciably reduce above SF 4.24.

Carbohydrate composition (Figure 52) reveals little change during the thermal degradation phase with glucose remaining between 73.9-76.3% (mol/mol) and xylose between 17.9-19.5% (mol/mol). The depolymerisation phase, occurring at severity above 4.24, initiated the steady removal of xylose components from the substrate therefore concentrating the glucose portion. Xylose reducing to 5.6% (mol/mol) while glucose increased to 89.6% (mol/mol). Inhibitory products are formed by the breakdown of carbohydrate cell wall materials (Meyer and Pedersen, 2010), therefore liquid fractions were analysed for these compounds. Figure 54 confirms the formation of inhibitory compounds, minor concentrations being produced during the thermal degradation stage. However the removal of xylose seen in Figure 52 can be seen to correlate with the increase in formation of inhibitory products. The presence of 2-FA at the highest severity (5.48) can again clearly be attributed to the conversion of xylose into this breakdown product (Meyer and Pedersen, 2010).
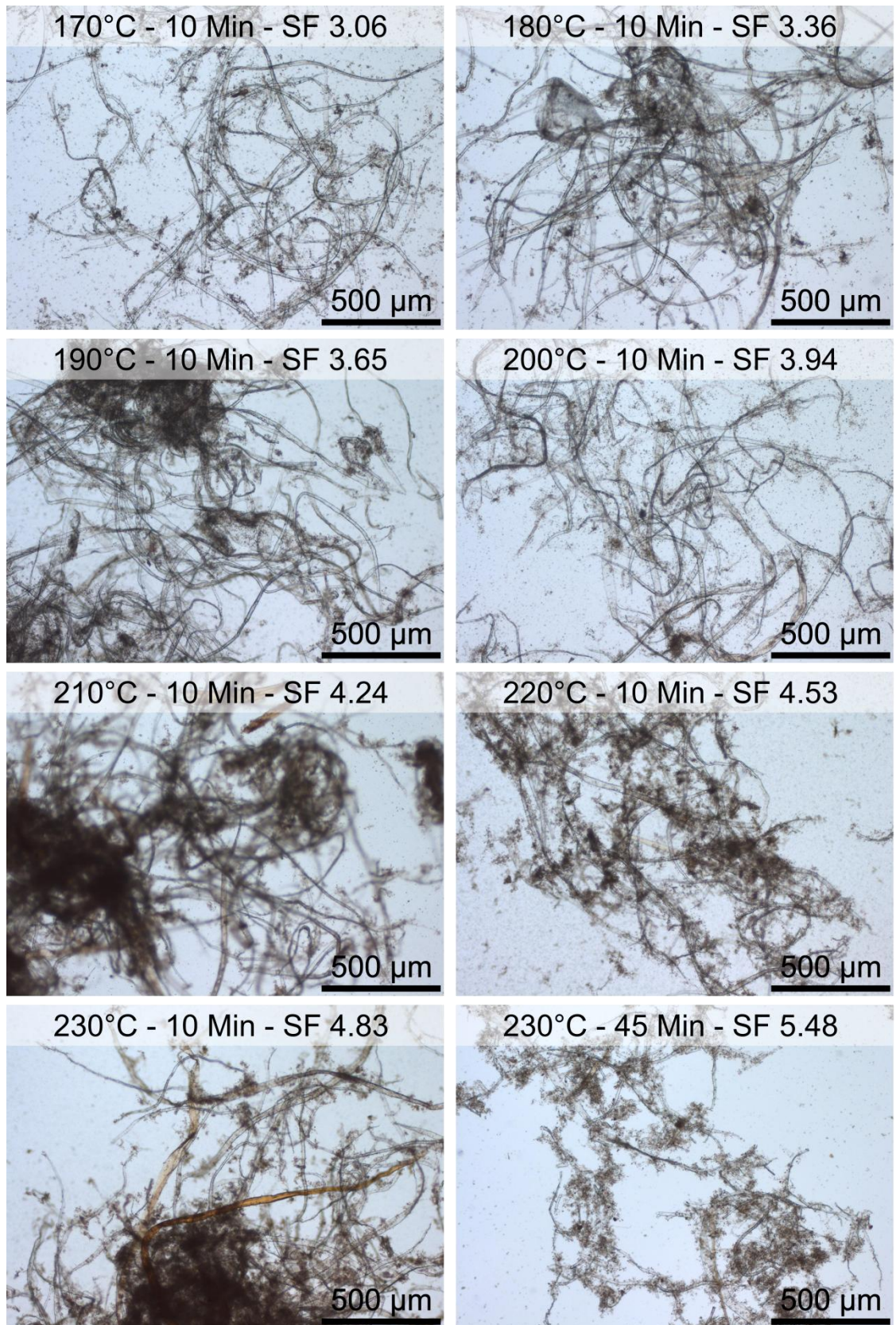
Figure 51. Steam explosion – micrograph using an Olympus BX60 brightfield microscope at 10x magnification
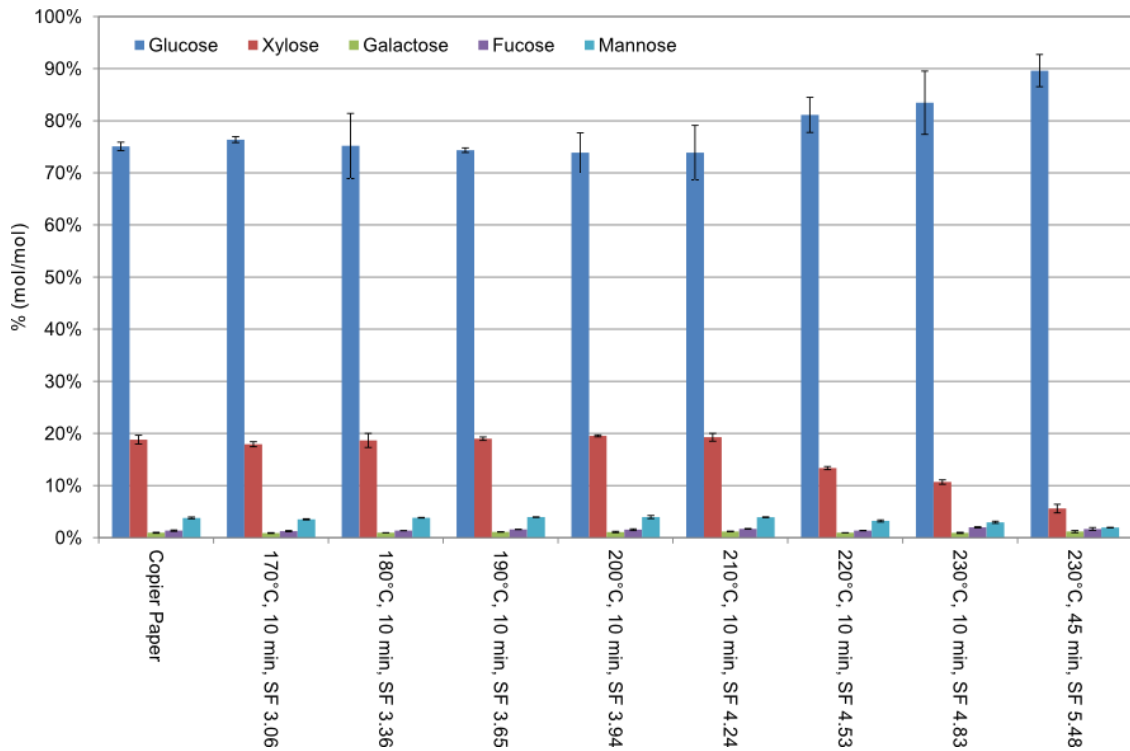
Figure 52. Sugar composition of the steam exploded residues in % (mol/mol)
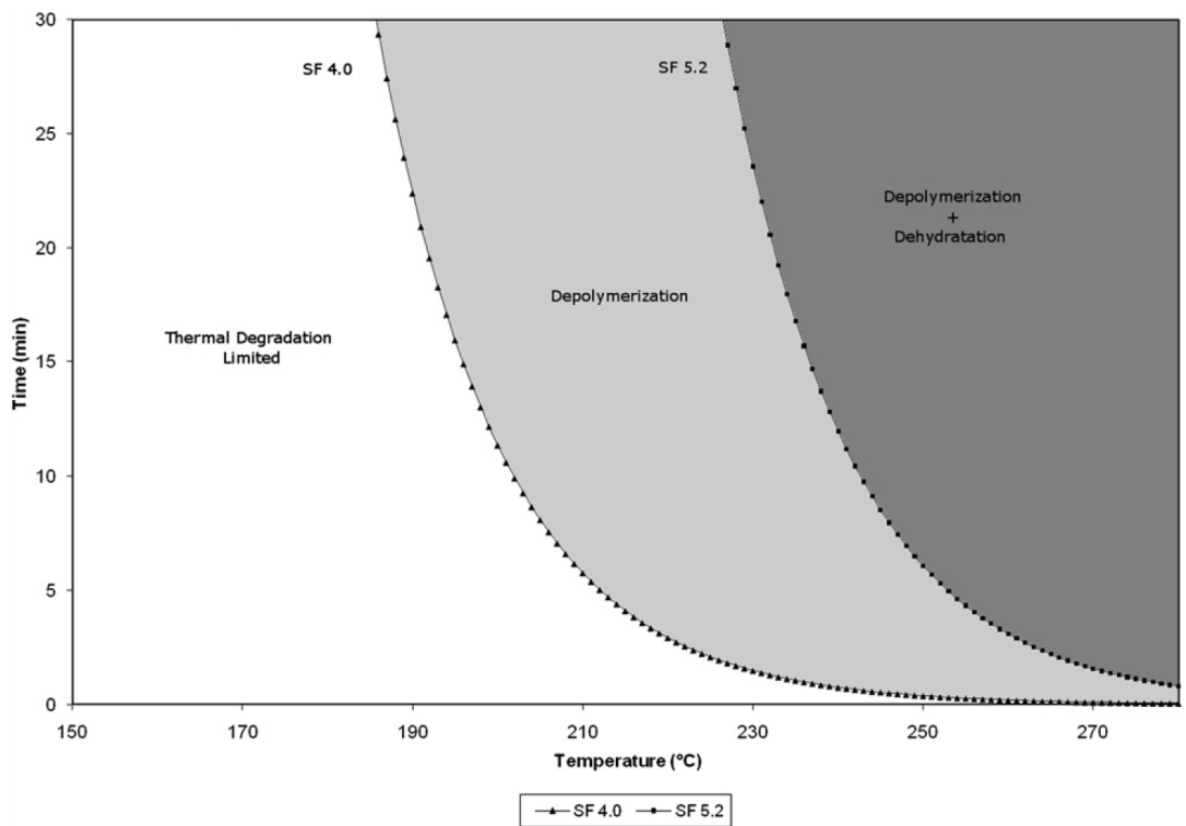
Figure 53. Thermal degradation of cellulose (Jacquet et al., 2011)
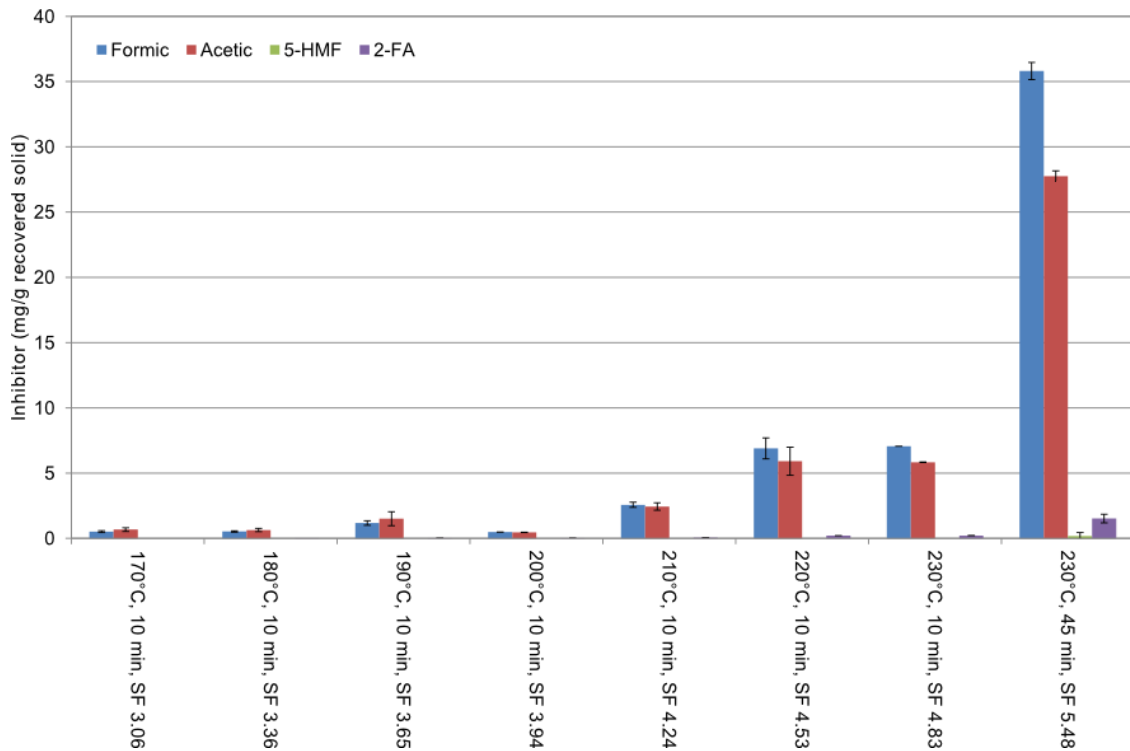
Figure 54. HPLC – Inhibitors from steam exploded residues, mg of inhibitor per g of recovered solids

5.2.4 FT-IR analyses

Figure 56 shows the FT-IR spectra of the usual components of copier paper, filter paper is used to represent the cellulose backbone of the substrate while calcium carbonate and kaolin are the most widely used fillers. Figure 57 summarises the spectra for all steam exploded samples, it can be seen that there is very little difference between the samples with the only visible change being to peak at wave number 1422 cm^{-1} which matches the main peak given in the calcium carbonate spectra. The expression of this peak falls in the depolymerisation phase and is likely due to the removal of carbohydrates, whose spectra have peaks that also fall in this region previously masking the calcium carbonate peak (Pandey and Pitman, 2003).
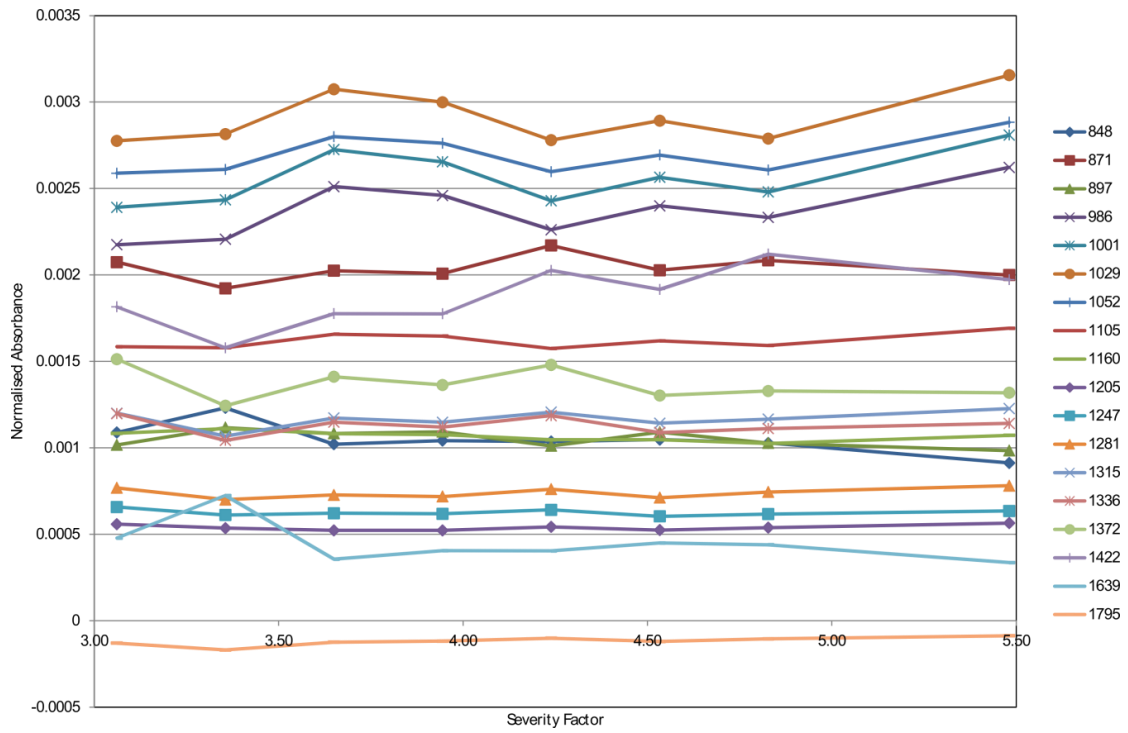
Figure 55. FT-IR Peak heights from normalised spectra 800-1800 cm^{-1}
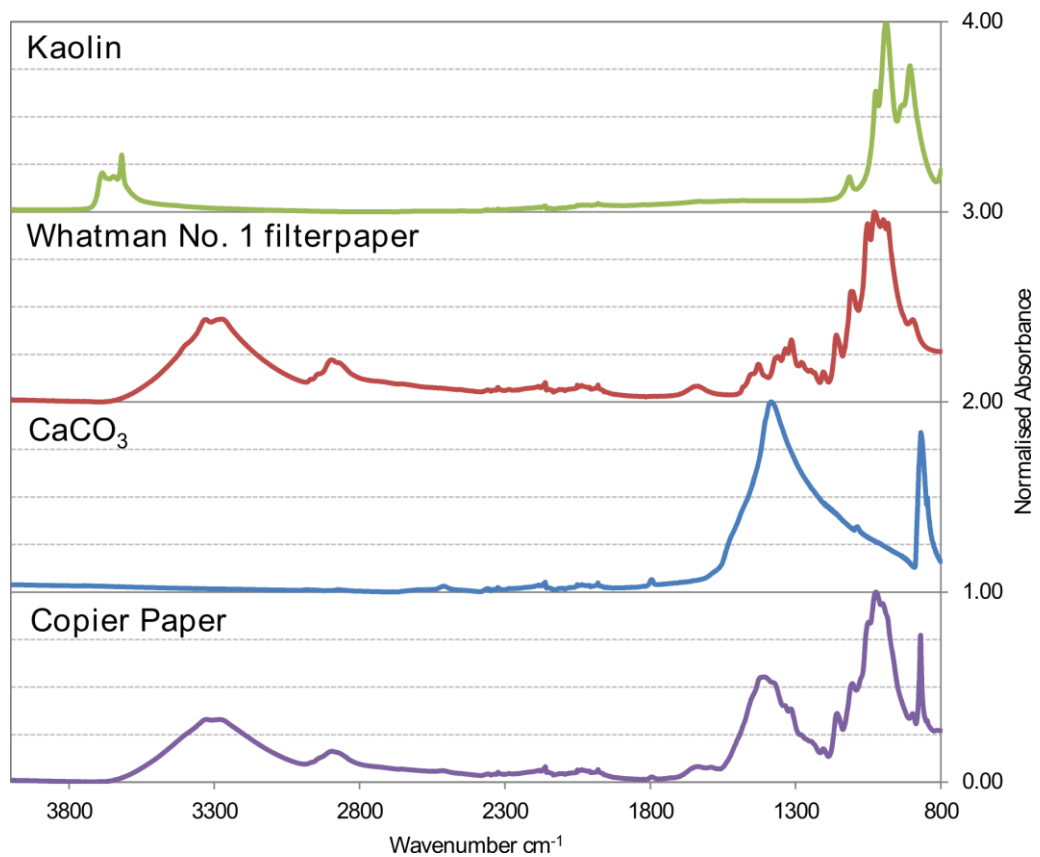
Figure 56. FT-IR Standard copier paper components, spectra are normalised and offset by 1 on the vertical axis
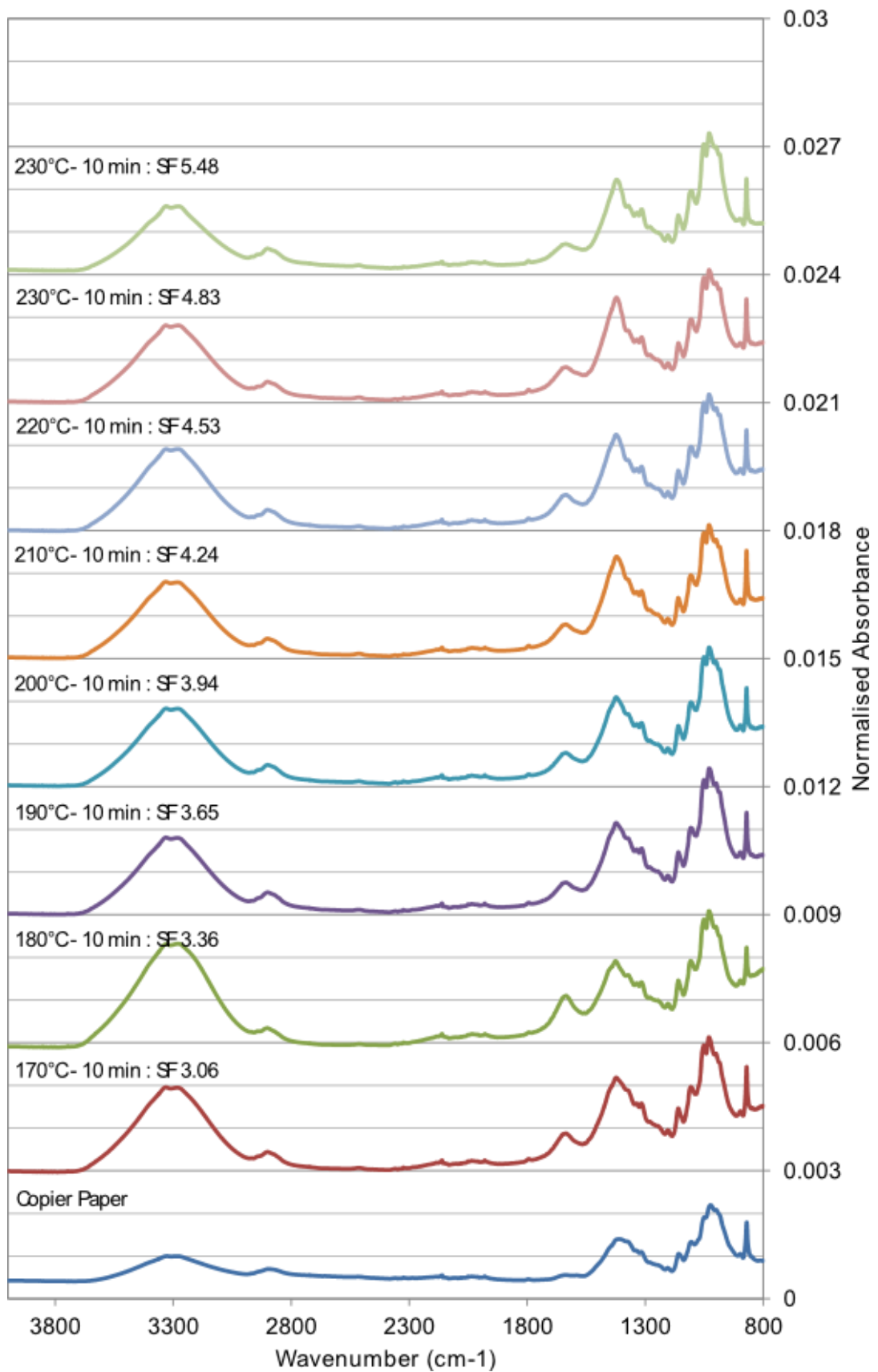
Figure 57. FT-IR – Steam Exploded Paper, spectra are normalised and offset by 0.003 on the vertical axis

Figure 55 portrays the effect of severity on the normalised peaks from the FT-IR spectrum in the region 800 cm^{-1} to 1800 cm^{-1} where the most relevant peaks can be found. Severity can be seen to have only minor effect on the structure with a majority of the peak heights remaining static as severity increases, the peak at 1422 cm^{-1} has already been discussed as becoming more prevalent as hemicellulose is removed. Peaks in the region 986-1052 cm^{-1} show the most turbulence over the change in severity, this is probably due to the formation of degradation products which disrupt the cellulose and hemicellulose peak contours. The peak at 1372 cm^{-1} is also worth highlighting as it is synonymous with crystallinity index of cellulose (Wistara et al., 1999), this also remains relatively constant with severity, suggesting that the cellulose is highly crystalline and only modestly degraded, which would be expected due to the vigorous processing paper is subjected to during manufacture.

5.2.5 Enzyme digestion

With the overall aim of pre-treatment being that of the improvement of enzyme hydrolysis, steam exploded samples were hydrolysed to give a comparison of the effects of severity on digestibility. The maximum yield in all cases was reached within around 48 hours with values reaching a plateau after that point. SF 5.48 gave the greatest yield, 77% (w/w), compared to untreated paper which yielded 54% (w/w) digestion of carbohydrate. It is however dubious that the cost of the energy required for the pre-treatment at this severity can be recuperated by this increase in cellulose digestibility. Lower severities however also gave modest improvement to digestion giving yields between 54-66% (w/w). Further digestions were carried out utilising reduced enzyme concentrations to ascertain if any further improvement could be made to enzyme loading. The results seen in Figure 59 again show a marked improvement over untreated paper, with yields as high as 21% (w/w) in the case of SF 5.48 as compared to 11% (w/w) with untreated paper. This improvement however again comes with the highest severity (5.48) and therefore energy requirement for pre-treatment.
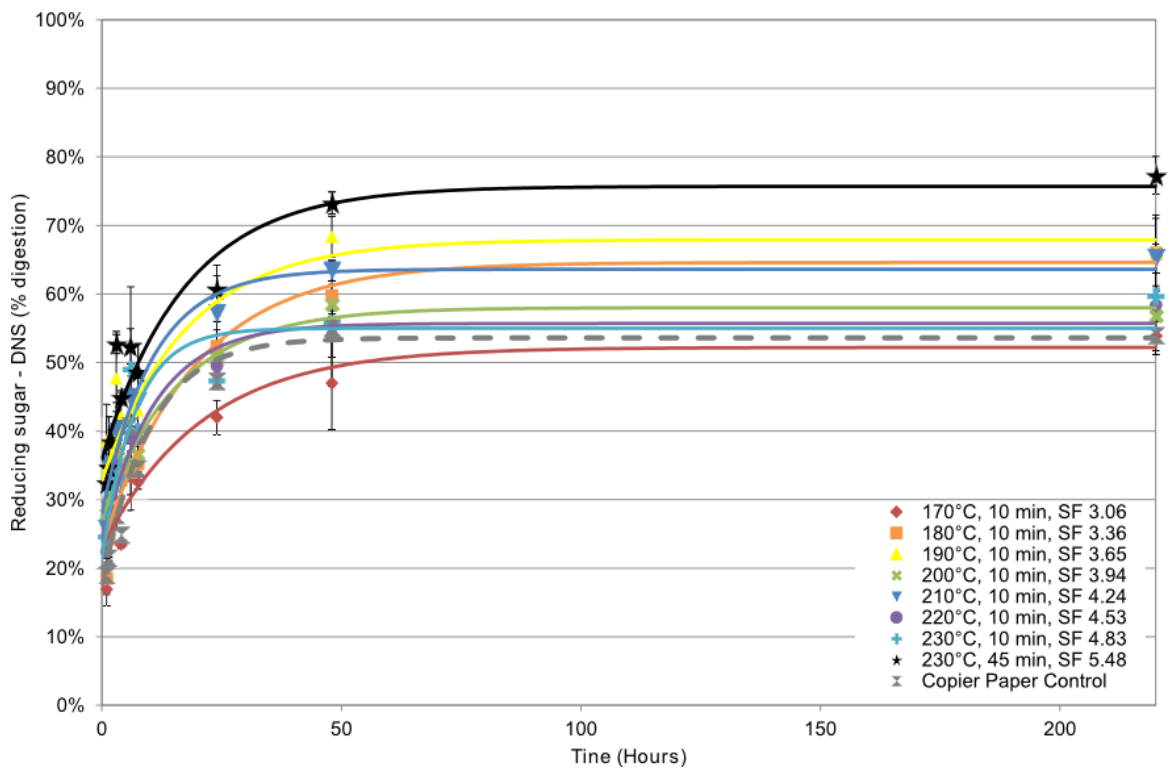
Figure 58. Enzyme digestion; Accellerase 1500 16 FPU/g substrate – % digestion based on total carbohydrate available
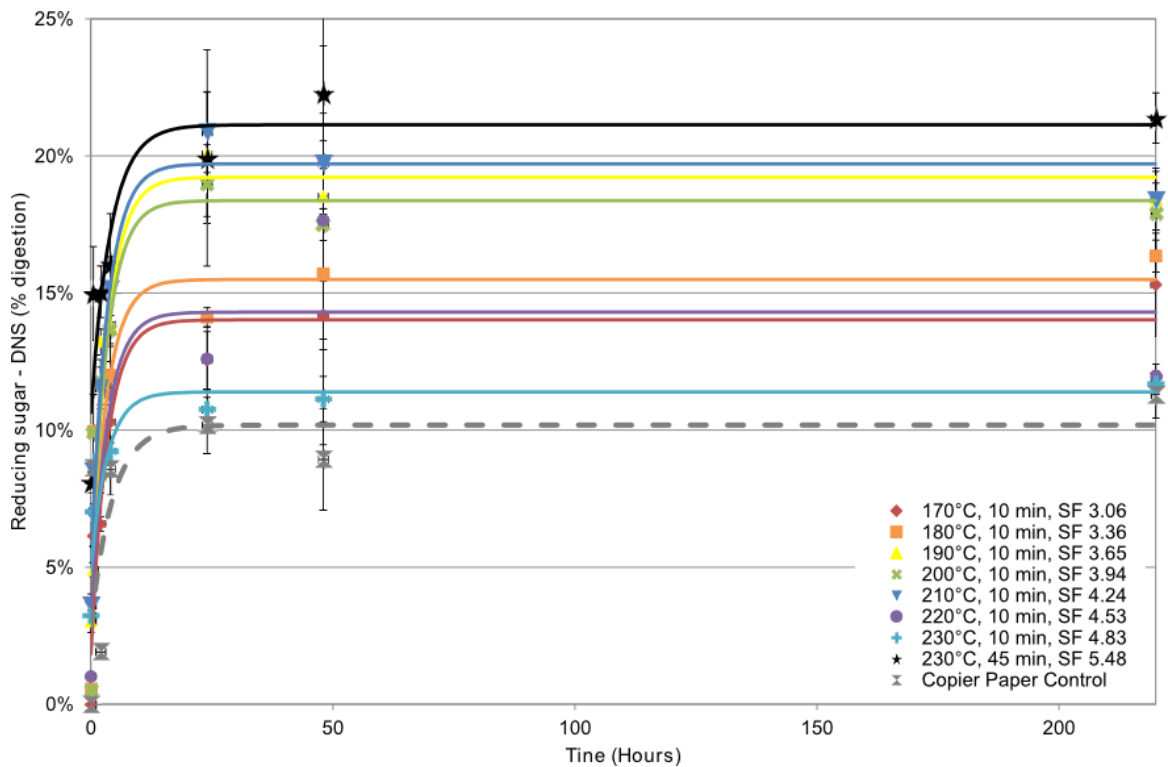
Figure 59. Low enzyme concentration; Accellerase 1500 1.6 FPU/g substrate – % digestion based on total available carbohydrate

5.2.6 Simultaneous Saccharification and Fermentation

Although glucose production is of course important to the bioalcohol process, ethanol is the final product and therefore of the most import. Steam-exploded samples are therefore subjected to SSF over a period of 48 hours to ascertain the effects of severity on this process. Figure 60 shows the percentage yields of ethanol and glucose on the basis of cellulosic material. SF 3.65 starting material, whilst left in for completeness, was later found to have become microbially contaminated and should therefore be ignored from this part of the study. The segregation into two phases can again be clearly seen here, there is a general increasing trend in the thermal degradation phase as the cellulose marginally pre-degraded. The depolymerisation phase shows a sharp drop in yield, this is likely due to the sudden increase in the presence of inhibitors. (Pienkos and Zhang, 2009). It should also be noted however that none of the pre-treated samples achieve a greater yield than that of untreated paper (93% w/w). The use of the SSF methodology can be seen itself to have an improving effect on the process with untreated paper now achieving considerably higher yields than previously seen in the hydrolyses in this chapter. This is likely due to the removal of saccharification products by the yeast and also by the alcohol activation effect highlighted in chapter 4.

As inhibitory compounds are present in potentially inhibitory concentrations in higher severity samples (Table 5), SSFs were repeated on washed samples. This can be seen to give a general improvement to the yields (Figure 61) with SF 3.94 and SF 4.24 now exceeding that of copier paper, suggesting that the presence of inhibitory compounds negatively affected the final yields of the process. However due to copier paper already achieving 93% (w/w) yield would suggest that pre-treatment of this kind is therefore not necessary for paper materials, due to its high crystallinity and base content (calcium carbonate) resisting sufficient degradation whilst still producing significant quantities of inhibitory compounds.
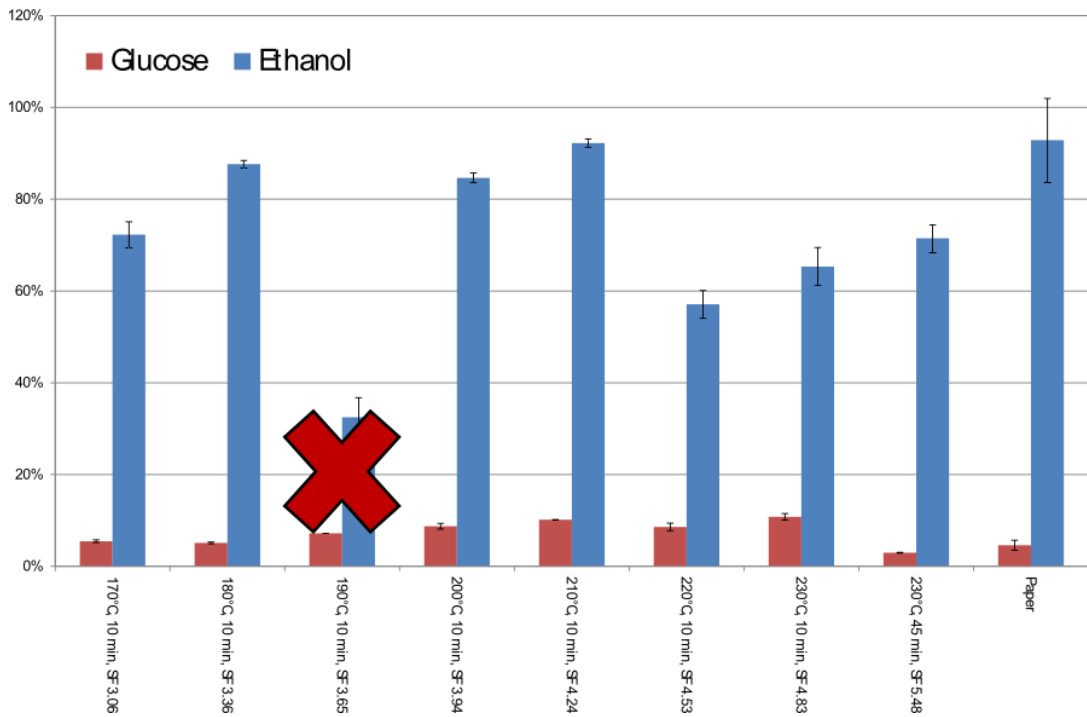
Figure 60. SSF % yields based on cellulose from steam exploded CP at a range of severities; Microbially contaminated sample crossed
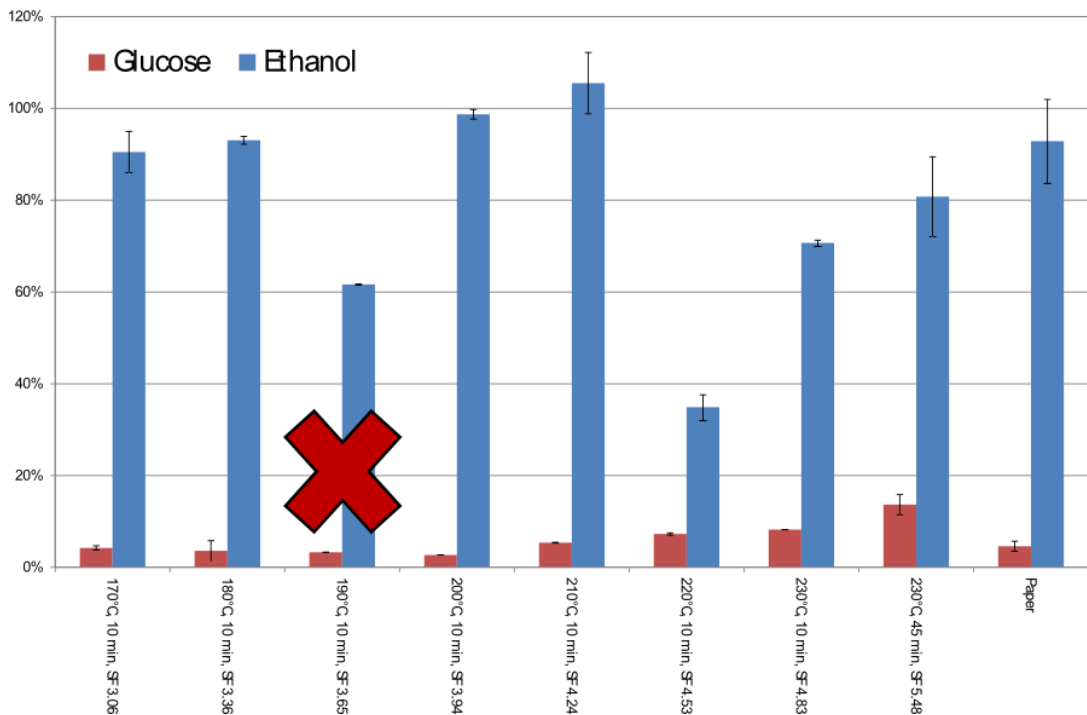
Figure 61. SSF % yields based on cellulose from washed steam exploded CP at a range of severities; Microbially contaminated sample crossed

5.2.7 Paper addition to steam explosion as an inhibitor reducing agent

Wheat straw is one of the main substrates of interest to the second generation bioalcohols industry, but it is well known for its production of inhibitors when subjected to steam explosion pre-treatment (Bellido et al., 2011, Horn et al., 2011, Tomas-Pejo et al., 2008). Detoxification of pre-treated liquors to remove inhibitory compounds has been considered (Cantarella et al., 2004), one such methodology, overliming, uses compounds to neutralise any undesired inhibitory components. As calcium carbonate is present in paper it was hypothesised as to whether the combination of wheat straw and paper would yield less inhibitory breakdown products when compared to wheat straw alone.

Samples of wheat straw were therefore mixed with both calcium carbonate at a ratio of 3:1 and with copier paper in a 1:1 ratio. Steam explosion was conducted at a high severity (4.83) in order to determine the possible positive effects of these additions in conditions that would be most likely to produce inhibitory compounds generally. In addition samples of Whatman No. 1 filter paper were subjected to the same treatment with additions of calcium carbonate and copier paper to establish the effect on a pure cellulose substrate. The results (Figure 62) show that the addition of calcium carbonate in both cases reduces inhibitor concentrations in the final liquor by large factors, the maximum being a 96% (w/w) reduction in the case of the 5-HMF in filter paper. Calcium carbonate appears to offer more protection against the formation of 5-HMF and acetic acid, suggesting that cellulose (whose break down products these constitute) is afforded greater protection than hemicellulose, this is likely due to hemicellulose being degraded more readily by thermal degradation whereas cellulose requires more of a reduction in pH to depolymerise. The addition of paper also has a positive effect on the reduction of 5-HMF and acetic acid, but yields an increase in formic acid and in the case of filter paper an increase in 2-FA. This incidence again can be explained by the inclusion of additional hemicellulose in the paper giving rise to its breakdown products of 2-FA and formic acid (Meyer and Pedersen, 2010).
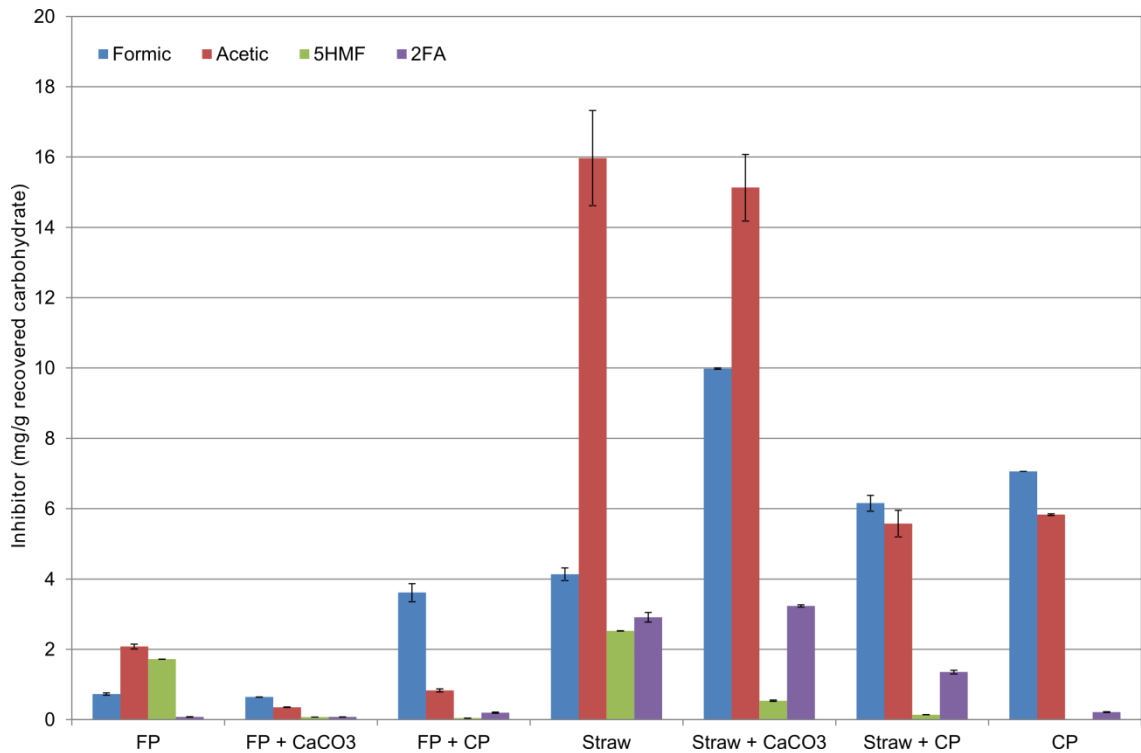
Figure 62. Steam Exploded Filter paper (FP) and straw inhibitors at SF 4.83

5.3 Conclusion

Steam explosion appears to have limited useful effect on the structure of CP, with physical investigation showing only dispersion of fibres and in the case of FT-IR some removal of hemicellulose. There is a marginal increase in the enzyme digestion of the substrate presumably due to the pre-wetting effect of the steam explosion allowing for better transfer of enzyme into the fibres compared to dry paper. However fermentation is hampered by the formation of inhibitory products produced at higher severities, this leads to the conclusion that steam explosion is not productive or economical in the pre-treatment of waste copier paper for bioalcohol production.

It was however noted that the addition of waste paper to other steam exploded substrates, in this case wheat straw, could be beneficial in reducing the inhibitory products produced at high severities. Paper was able to reduce the formation of inhibitory products by up to a 96% (w/w) reduction which has the potential of allowing better fermentation of the steam exploded residues.

6 Semi-Simultaneous Saccharification and Fermentation at a small industrial scale

As previously discussed in §1.13.4, maximising substrate concentration and thence subsequent ethanol concentration is a key objective of this project. This chapter describes studies aimed at increasing the substrate concentrations at higher volumes (1.5 and 5 L). Due to the low moisture content of paper (< 5% w/w) it had been found previously to be difficult to successfully digest with initial substrate concentrations in excess of approximately 10% (w/v) as paper substrate absorbs water so readily making stirring prohibitively difficult. The following experiments explore the possibility of improving substrate concentration considerably by sequential addition of paper.

Initial experimentation focused on saccharification alone in a 2 L vessel and, following on from Chapter 4, evaluated the potential for making multiple sequential additions of substrate and enzyme. Following this, SSSF fermentations were carried out. A tailored pilot-scale vessel (10 L) was then investigated in order to address the problem of mixing suspensions of concentrated solids, and evaluated the potential for increasing both substrate concentration and final ethanol yields.

6.1 Materials and Methods

6.1.1 *Materials*

Commercially available cellulase Accellerase® 1500; *Trichoderma reesei* and accessory enzyme βG, were chosen for use in this study continuing from experimentation in previous chapters. These enzyme preparations were used "as provided" in all experiments without any desalting or other purification steps , thus reflecting practical usage potential in an industrial setting. The substrate used was M-Real Evolve Office 80 g/m^2 paper. 0.1 mmol/L Sodium Acetate Buffer was made using Acetic Acid and Sodium Acetate Trihydrate adjusted to volume with ultra-pure water.

6.1.2 Substrate Preparation

M-Real Evolve paper was shredded using a PS-67Cs cross shredder to 3.9 x 50 mm particle size (Din Security Level 3). These were then portioned into 125 g aliquots and sterilised by autoclaving (121°C for 15 min). No further pre-treatment was utilised following the results of Chapter 5.

6.1.3 2 L Reaction Vessel

A 2 L fermenter (1.5 L working volume) was used for initial experimentation (Figure 63), equipped with an 502D agitator (LH Fermentation, Maidenhead, UK), an LH temperature regulator (LH Fermentation, Maidenhead, UK), a GFM17 mass flow meter (Aalborg®, US) and attached to an MX3 Bio sampler autosampler (New Brunswick Scientific, USA), data was logger using data logged using Orchestrator software (Measurement Systems Ltd (MSL), Newbury, UK). An additional condenser was installed in advance of the mass flow meter in order to prevent the expulsion of water vapour which would both decrease the sample volume and negatively affect the mass flow meter performance. A diagram of this system can be seen in Figure 64.
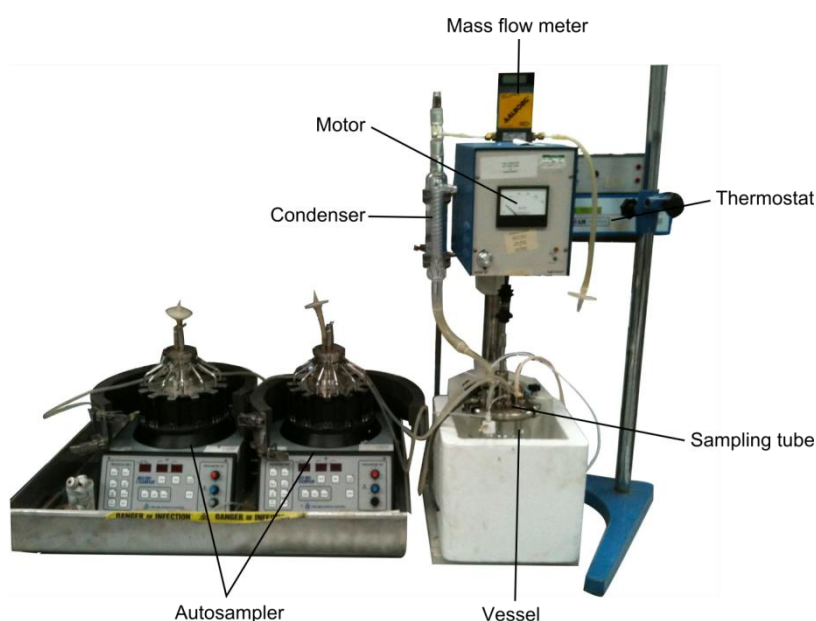
Figure 63. 2 L vessel
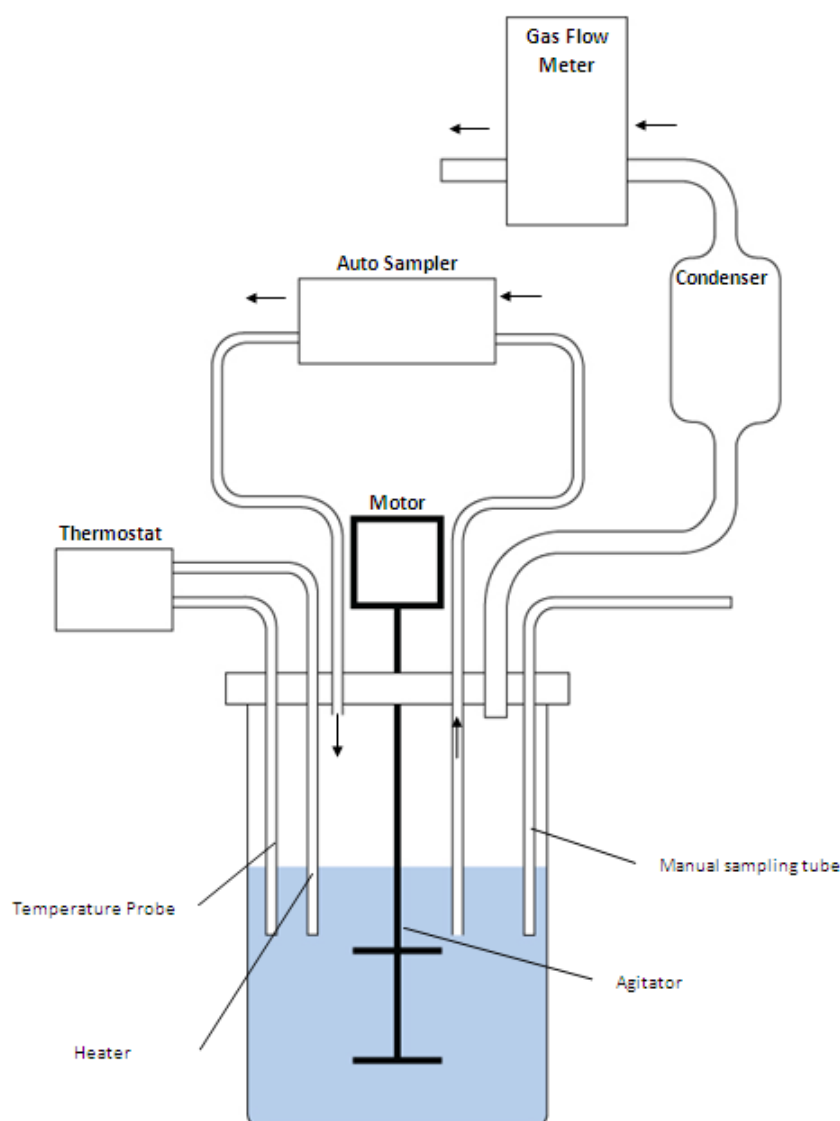
Figure 64. 2 L vessel – schematic diagram

6.1.4   10 L Reaction vessel

A bespoke 10L (5 L working volume) reaction vessel with additional computer control systems installed was used for additional study, Figures 65-67. It was equipped with a high speed mixer and a slow speed agitator (Figure 68) temperature regulated using a Haake C35 (Thermo Scientific, Basingstoke, UK) circulator attached to a water jacket on the vessel. A GFM17 mass flow meter (Aalborg®, US) was attached to the top of the vessel and data logged using Orchestrator software. Samples were taken during incubation from a sampling point at the bottom of the vessel.
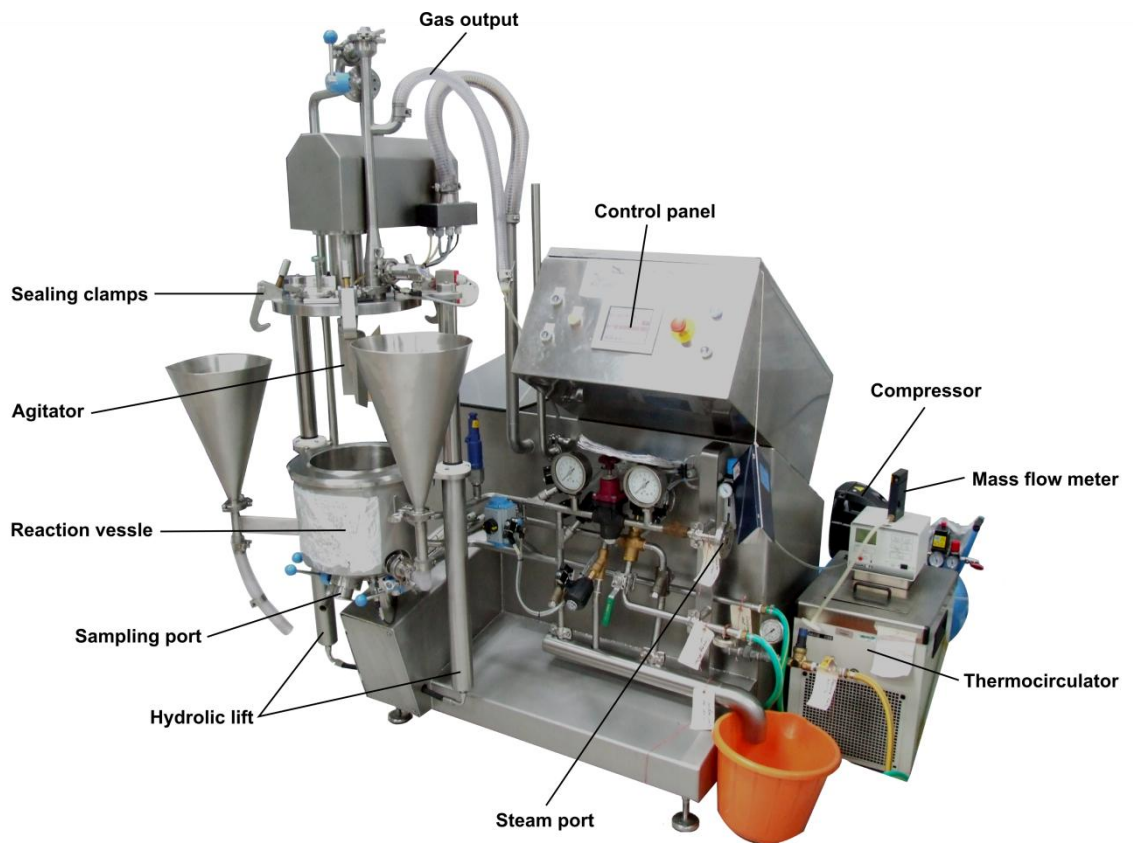
Figure 65. 10 L high torque mixing reactor - front view
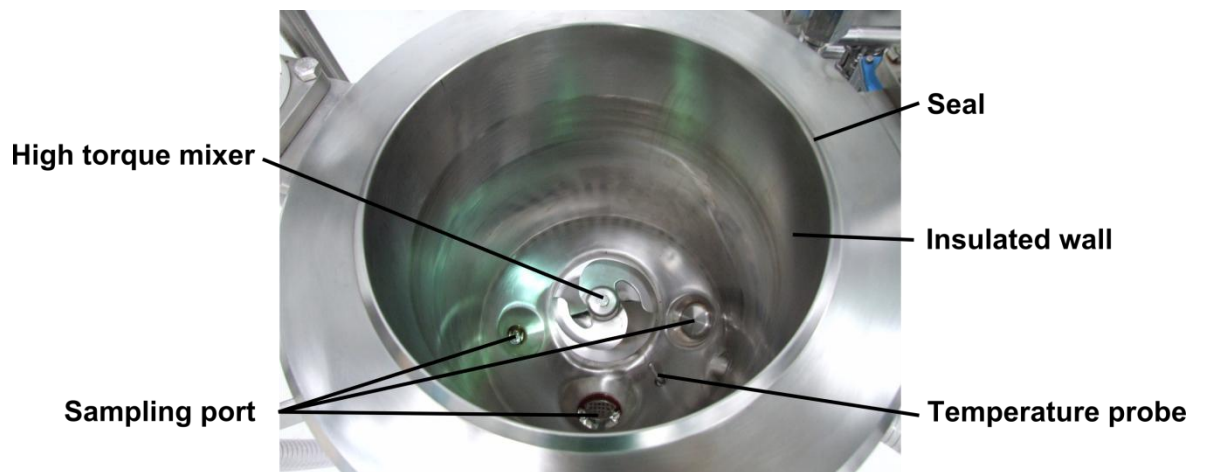
Figure 66. 10 L vessel – top down view

Figure 67. 10 L vessel – agitator side view
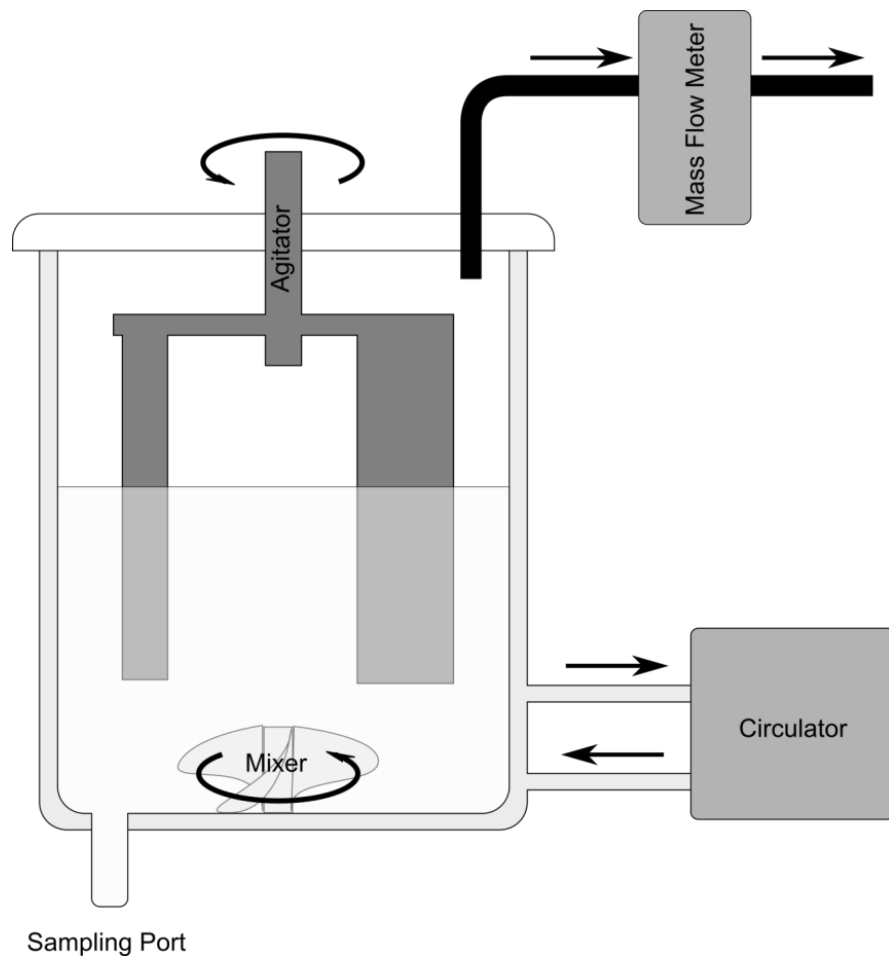
Figure 68. 10 L vessel – schematic diagram

6.1.5 *Initial vessel set-up*

Initial quantities of shredded paper substrate were added to the vessel which was then made up to desired volume (1.5 or 5 L) with 0.1 mol/L NaOAc buffer (pH 5.0). The 2 L vessel was autoclaved, but the 10 L vessel could not be so instead it was heated to 90°C for 10 minutes to sterilise the initial buffer and paper substrate. The vessel was then equilibrated to 50°C the working temperature of Accellerase® 1500. Once cool Accellerase® 1500 (16 FPU/g of substrate) and βG (30 U/g of substrate) were added to the vessel and stirred continuously. Regular samples were taken for later analysis.

6.1.6 *Chromatography*

Samples (2 mL) were put into sealed tubes which were then heated at 100°C for 10 minutes in a water bath so as to denature the enzymes. Residual solids were then removed by centrifugation at 13,000 rpm for 5 minutes. Finally the supernatant was filtered using 0.2 µm syringe filters into 300 µL glass vials. HPLC sugars and alcohol analysis was conducted using method found in §2.3.6.

6.2 Results

6.2.1 *Saccharification studies to increase substrate concentrations*

Initial saccharification studies (summarised in Table 15) were carried out using the 2 L vessel without autosampler or mass flow meter. Substrate concentrations were limited to 5% (w/v) as the mixer on this vessel was unable to stir any greater quantities in a reliable fashion (Figure 69A). Repeat experiments, H1 and H2, were conducted in this way giving sugar concentrations of 7.5 mg/mL and 14.4 mg/mL respectively, therefore yields equating to 30% and 57% (w/w) compared to a theoretical glucose maximum of 25.2 mg/mL. Ineffective stirring due to the substrate loading appeared to be responsible for both low and variable yields from these experiments. Experiment H3 was therefore carried out with a reduced substrate loading of 2.5% (w/v) to enable more vigorous stirring. The reduced substrate concentration resulted in a visually more degraded sample so after 12 hours digestion the reaction was stopped and solid material was removed by filtration through GF/C glass fibre filter paper. The supernatant was returned to the

vessel along with an additional 2.5% (w/v) substrate, adjusted to 1.5 L volume with buffer and autoclaved. Once equilibrated to 50°C further enzyme was added (Accellerase® 1500 16 FPU/g of substrate and βG 30 U/g of substrate) as before. This process was repeated to give a total of four additions resulting in a final glucose concentration of 30.8 mg/mL, equating to a yield of 61% (w/w). This multiple addition method therefore increased both yield and effective substrate concentration. Subsequently the final filtered supernatant was returned to the vessel and SHF performed by the addition of 200 mL of yeast inoculum (1.4 x 10^8 cells/mL NCYC 2826 in nitrogen base).This resulted in an ethanol concentration of 1.2% (v/v) equating to 63% (v/v) yield from released glucose, 37% (v/v) yield from total glucose in the original substrate.

5% substrate concentration
1 Addition
0 Hours digestion

10% substrate concentration
4 Additions - 2 Hours apart
96 Hours digestion

10% substrate concentration
4 Additions - 24 Hours apart
96 Hours digestion
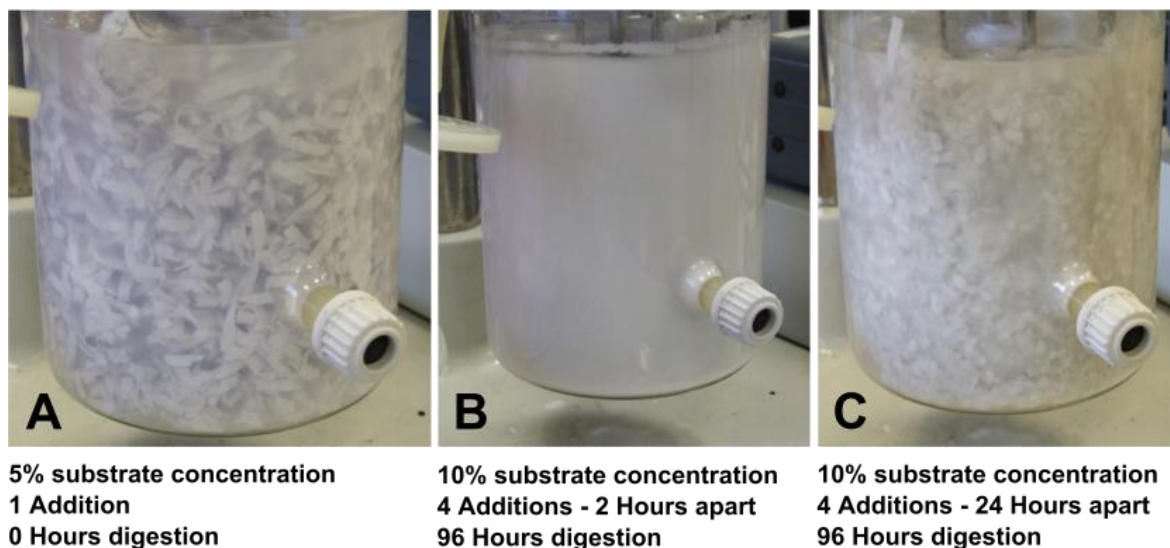
Figure 69. Visualisation of substrate concentrations in 2 L vessel A) 5% (w/v) substrate concentration B) 10% (w/v) substrate digested for 96 hours with a 2 hour addition regime C) 10% (w/v) substrate digested for 96 hours with a 24 hour addition regime

Separation of saccharified solutions from insoluble solids was considered to be time consuming and inefficient. As a result, trials of stepwise addition without the removal of previously undigested materials were made using additions of increases of 2.5% (w/v) substrate with the addition 16 FPU/g of substrate Accellerase® 1500 and 30 U/g of substrate βG. In trial one, three further 2.5% (w/v) additions were made on a 2 hourly basis, in trial two these additions were made every 24 hours in order to maximise digestion between additions, no further

enzyme as added in either case. Figure 69B shows the level of digestion with 2 hours between additions regime as opposed to Figure 69C which shows the same experiment with 24 hours between additions. It can be seen that there is considerably less digestion when additions are made with 24 hours between them.

6.2.2   SSSF1 – high substrate concentration

Building on progress made in experiments H1-3 the methodology was extended with the use of SSSF. Experiment SSSF1 again employed the 2 L vessel and exploited the autosampler and mass flow meter. An initial hydrolysis phase was undertaken with additions of 2.5% (w/v) substrate, Accellerase® 1500 (16 FPU/g of substrate) and βG (30 U/g of substrate) made every two hours to prevent any inactivation of enzyme between additions. After 12 hours hydrolysis 200 mL (see §2.7 for method) of yeast inoculum was added to the system (1.02 x 10^8 cells/mL NCYC 2826 in nitrogen base). Further substrate additions were made after this addition without additional enzyme. These along with ethanol and CO_2 production can be seen in Figure 70.
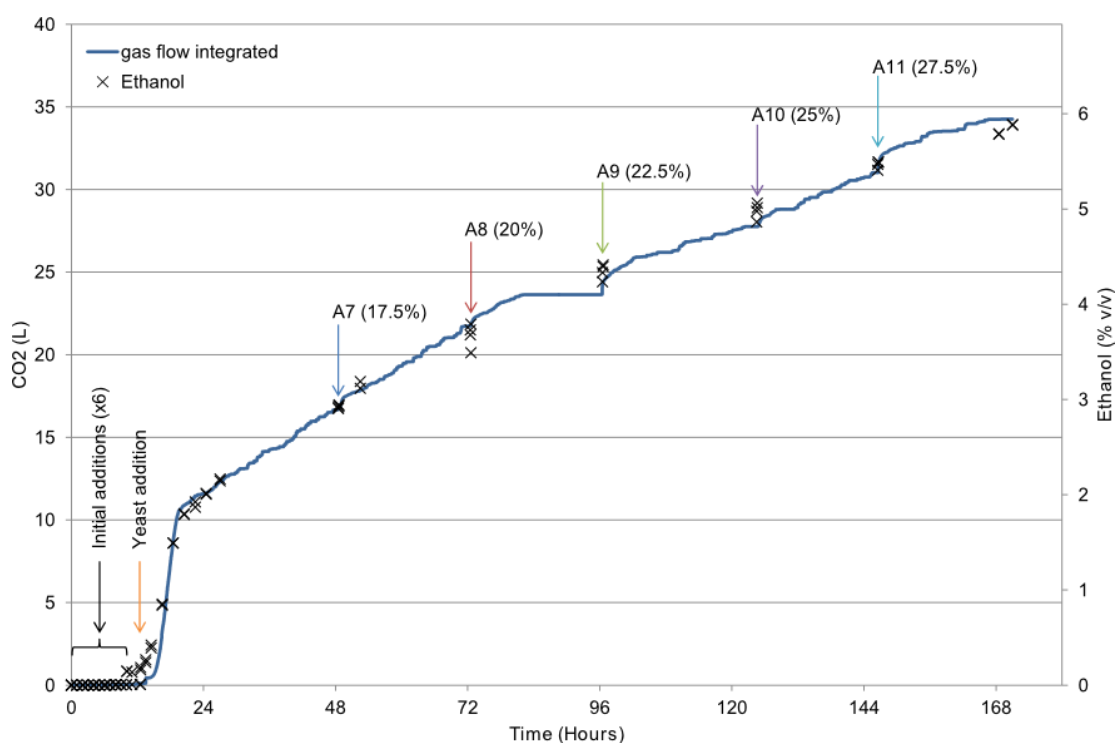
Figure 70. SSSF 1 – 2 L vessel, integrated gas and HPLC-determined ethanol plots. Substrate addition points are indicated with equivalent substrate concentration reached in brackets

Ref	Vessel (Volume)	Enzyme concentration				Substrate			Glucose		Ethanol	
		Accellerase® (FPU/g)		βG (U/g)		Initial (% w/v)	Additions	Final (% w/v)	mg/mL	Yield (% w/w)	% (v/v)	Yield (% v/v)
		Initial	Final	Initial	Final							
H1	2 L (1.5 L)	16.0	16.0	30.0	30.0	5.0	1	5.0	7.5	30.0	-	-
H2	2 L (1.5 L)	16.0	16.0	30.0	30.0	5.0	1	5.0	14.4	57.0	-	-
H3	2 L (1.5 L)	16.0	16.0	30.0	30.0	2.5	4	10.0	30.8	61.0	1.2	37
SSSF1	2 L (1.5 L)	16.0	8.7	30.0	16.4	2.5	11	27.5	-	-	5.9	65
SSSF2	10 L (5 L)	16.0	3.7	30.0	6.9	2.5	26	65.0	23.1	27.5	6.9	29
SSSF3	10 L (5 L)	16.0	3.7	30.0	6.9	2.5	26	65.0	30.5	36.3	11.6	54

Table 15. Hydrolysis experiments summary

The paper additions can be seen to be closely followed by increases in ethanol production, and a final concentration of 5.9% (v/v) ethanol was achieved in this experiment equating to a 65% (v/v) yield (maximum theoretical 8.96% v/v). The eleventh addition was the final made due to stirring again becoming impaired by the high substrate concentration (equivalent to a final total of 27.5% w/v) however it can be seen that the ethanol production is still increasing suggesting that if there was improved stirring additional substrate could be added in order to increase the ethanol still further.

6.2.3 SSSF2 – scale up to higher shear 10 L vessel in order to increase workable substrate concentration

Due to the necessity of an increased mixing capability in order to handle even higher substrate concentrations a specialised bioreactor with 10 L capacity (5 L working volume) was employed (Figure 65). This vessel with its combined 550 W agitator and 4 kW mixer was developed to enable the necessary mixing to be achieved.

Similar to SSSF1, an Initial hydrolysis stage was carried out to build up the glucose levels to start fermentation. This stage consisted of six aliquots of 125 g (2.5% w/v) copier paper each dosed with 16 FPU/g of substrate Accellerase® 1500 and 30 U/g of substrate βG. These were added to the vessel, containing 5 L 0.1 mol/L sodium acetate buffer, at two hour intervals. This enabled a total accumulation of 750 g copier paper during the initial hydrolysis which consisted of 50.4% (w/w) cellulosic material according to GC analysis (Table 2). Here the total available for initial hydrolysis was 382.5 g. Taking into account the hydration of the cellulose during hydrolysis a theoretical complete hydrolysis would be expected to yield 420.75 g glucose in 5 L total volume or 84.15 mg/mL. A glucose concentration of 23.12 mg/mL was achieved (Figure 71, red line) equating to an initial yield of 27.5% (w/w).
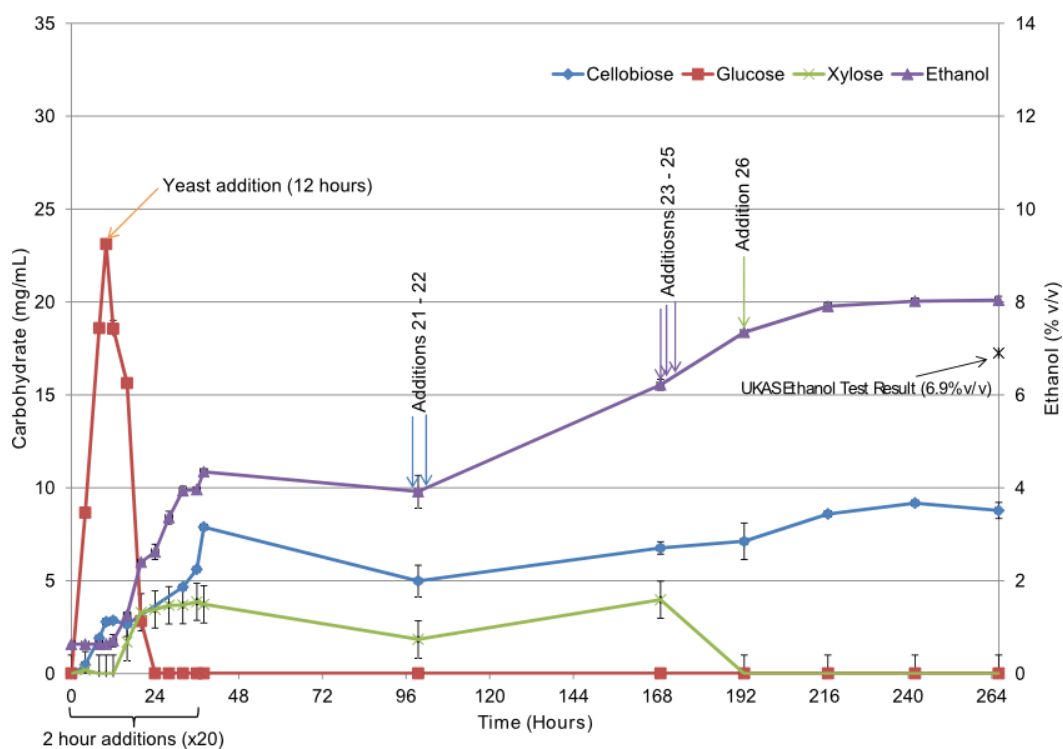
Figure 71. Carbohydrate and ethanol production from SSSF2

The vessel temperature was reduced to 30°C and 500 mL yeast inoculum (§2.7) added (2 x 10^8 viable cells/mL) was added to the vessel after the initial 12 hour hydrolysis. The available glucose from the hydrolysis was quickly metabolised by the yeast as seen in Figure 71. Subsequently, the glucose concentration in the liquor remained low (less than 2.1 mg/mL), while ethanol concentration steadily increased. As the glucose production was completely repressed by the fermentation to ethanol this suggests that the enzymatic hydrolysis of the substrate was the rate determining factor at this point. Substrate additions (125 g) continued to be made in 2 hours periods through-out this section, again without any appreciable increase in glucose concentration, but increasing the concentration and, for short periods, the rate of ethanol production. In total 26 additions of 125 g paper were made (20 in the fermentation stage and 6 during hydrolysis only) while no additional enzyme was added after the initial hydrolysis stage was finished. A final substrate concentration of ~65% (w/v) was achieved in this experiment with additions totalling 3.25 kg.

Estimated ethanol production from carbon dioxide evolution (§2.8) showed an approximate ethanol concentration of 9.5% (v/v) (Figure 72) compared to 8.0%

(v/v) by HPLC (Figure 71). This difference was most likely due to the combination of both, the marginal increase in volume due to addition of yeast, and the requirement of the vessel to be opened in order to add additional substrate, affecting the pressure of the system and also introducing some small quantities of oxygen to the system. The oxygen therefore allowing for standard respiration via the Krebs cycle, which although utilises less glucose, as explained by the Pasteur effect (Strathern et al., 1981), is likely to have also reduced the production of ethanol and thus the final concentration achieved. External verification of this ethanol concentration was also sought. This verification was conducted by Campden Technology Ltd (Chipping Campden, UK) using their UKAS accredited TES-AC-567 method. This gave a concentration of 6.9% (v/v), result certificate can be seen in Appendix G.
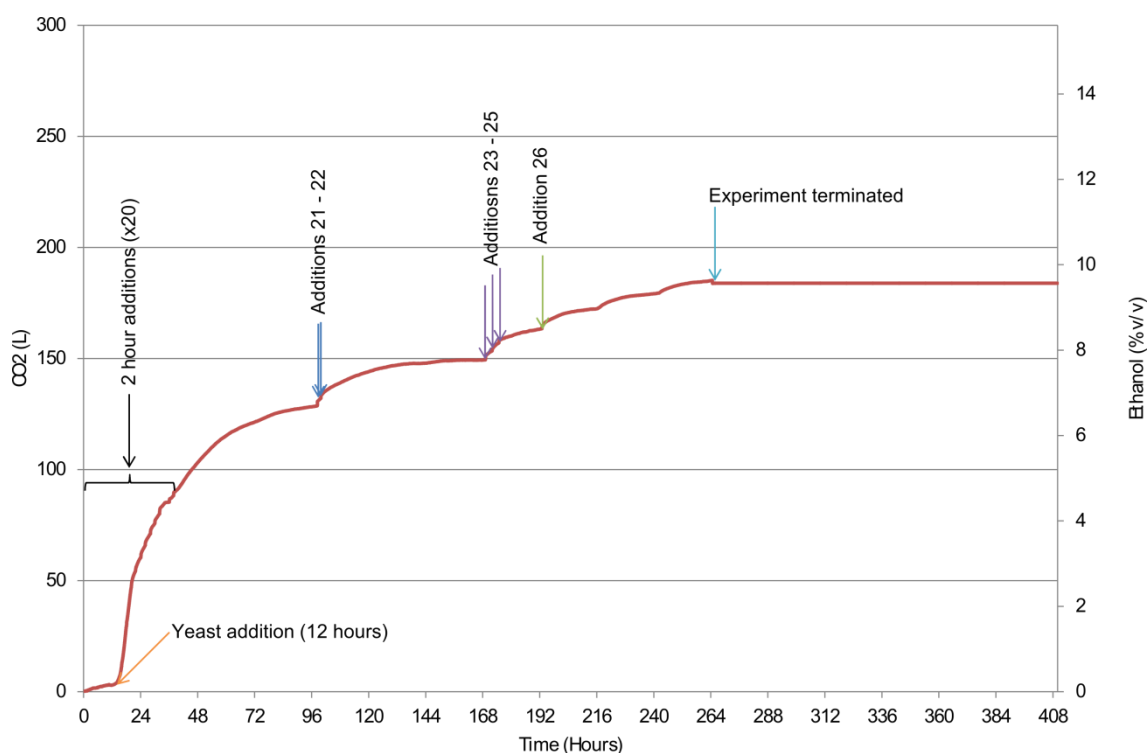
Figure 72. Integrated gas output with theoretical ethanol yield – SSSF2

The theoretical concentration of ethanol achievable with 100% (w/w) conversion to glucose and then on to ethanol can be calculated as in Equation 10, where CP_s is the quantity of copier paper added to the system, in this case 3250 g, 50.4% (w/w) of which is cellulose 51.11% (w/w) of which can be converted into ethanol, 1.111 factor takes into account the water of hydrolysis (glucose, 180 g/mol /

anhdryoglucose, 162 g/mol = 1.111), 930 g ethanol therefore being the theoretical maximum.

$$Ethanol \; g \; = 0.504 \times CP_S \times 0.511 \; \times 1.111 \tag{10}$$

Equation 10. Maximum Theoretical Ethanol

The final volume for SSSF2 was measured at 6700 mL, 28.53% of which was found to be dry matter, therefore a liquid content of 5053 g with a volume of 4955 mL from density measurements (Density meter, Anton Paar DMA 5000, Anton Paar GmbH, Graz, Austria). This equates to a volume of ethanol of 342 mL or a mass of 270 g (based on 6.9% ethanol v/v), giving a final yield of 29%. It was also noted in this experiment that the constant addition of paper every two hours led to a highly viscous substrate after 20 additions, not unlike bread dough in consistency, this is likely to have retarded the enzyme digestion by reducing free movement and the availability of water and possibly mopping up the free enzyme through binding.

6.2.4   SSSF3 – ad hoc paper addition regime

As SSSF2 showed that the addition of paper in a regimented two hour period eventually caused the substrate to become highly viscous, a further experiment was conducted where after the initial hydrolysis; phased additions were made in an ad hoc manner at points where the material was deemed to have digested sufficiently.

An initial glucose concentration of 30.54 mg/mL was achieved in SSSF3 equating to an initial yield of 36.3%, a similar amount to that achieved in SSSF2 and in the same residence time as expected. The glucose concentration dropped sharply and remained low after the addition of yeast. However, addition of further substrate on a reasonably regular basis, as digestion permitted, resulted in continual hydrolysis, fermentation, and production of ethanol (Figure 73). However, after 315 hours, the glucose level started to rise, reaching 12.1 mg/mL. This is most likely due to ethanol inhibition of the fermentation process above 10% v/v (Figure 73) or simply

the yeast coming to the end of its life cycle. This does however suggest that there was still enzymatic activity present within the reaction liquor at this point.
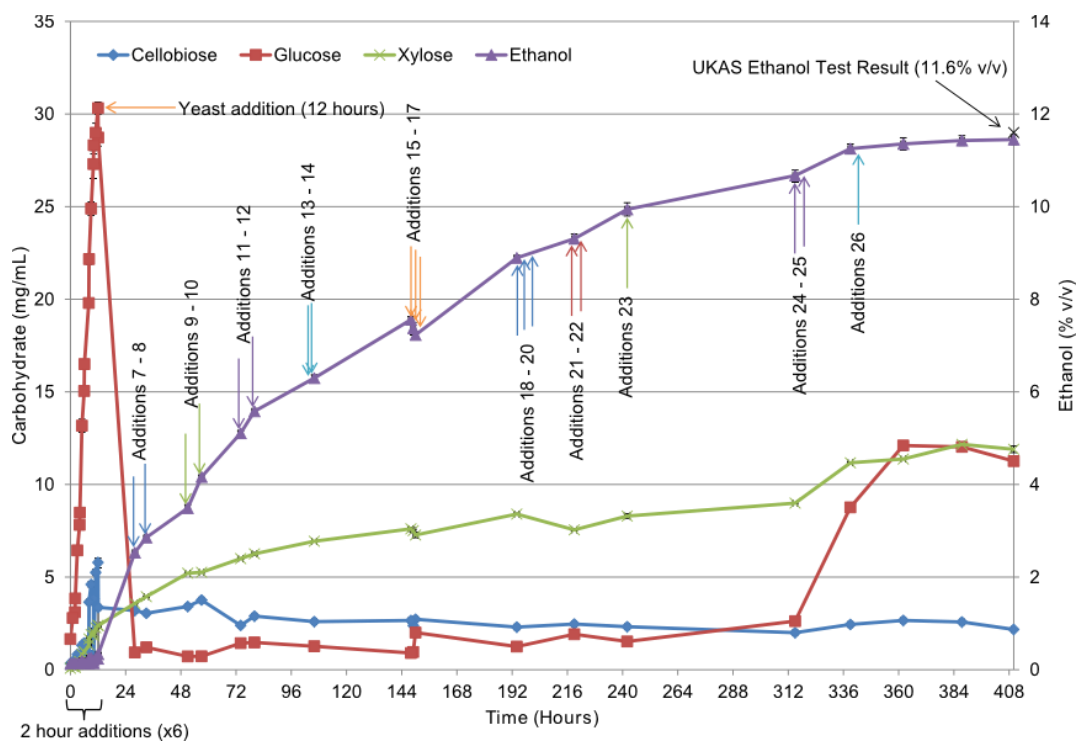
Figure 73. Carbohydrate and ethanol production from SSSF3

Ethanol estimation from carbon dioxide production (§2.8) showed an approximate ethanol concentration of 14% (v/v) (Figure 74) compared to 11.6% (v/v) by HPLC as confirmed by Campden Technology Limited for SSSF3 (Figure 73).

The final ethanol yield of SSSF3 was calculated using the same method as for SSSF2 giving 54% (v/v of theoretical maximum). This final yield is likely to have been retarded as the yeast appears to have been limited towards end of the experiment as highlighted by the resurgence of the glucose concentration, suggesting that the yeast was no longer fermenting. The highest yield however was achieved after 148 hours and 14 additions, being 65.5% (v/v of theoretical maximum - based on a liquid content of 5.5 L).
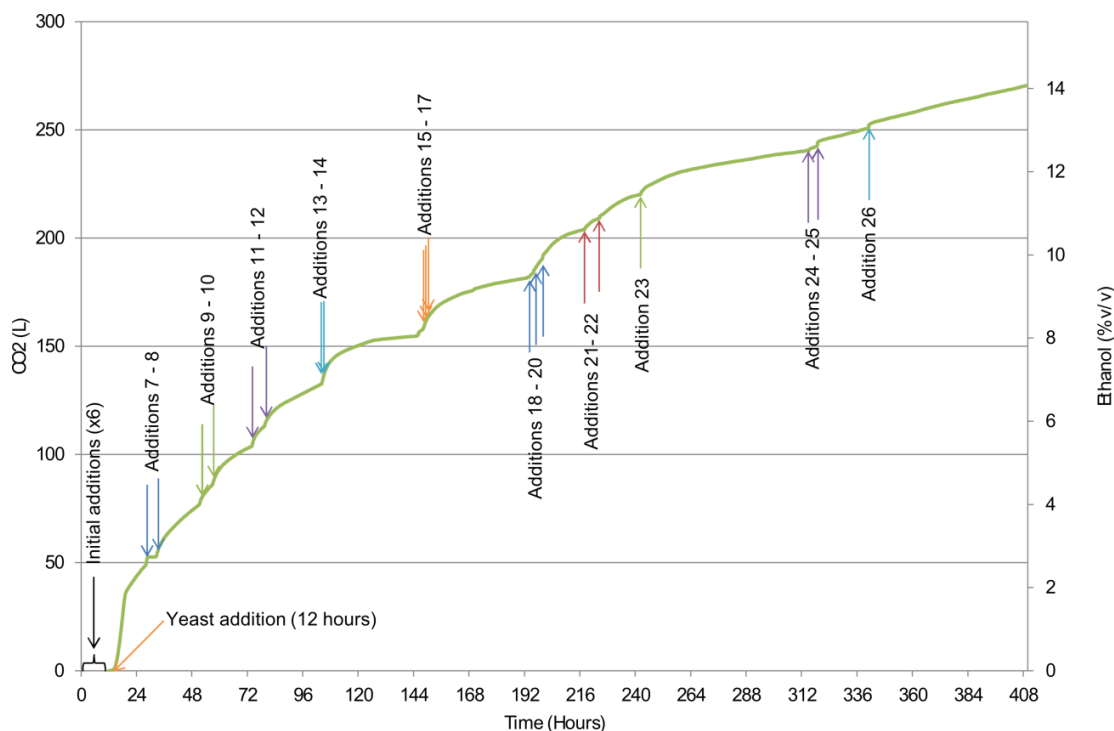
Figure 74. Integrated Gas Output – SSSF3, arrows represent addition points

Sugars analyses of the insoluble residue at key time points in SSSF3 were conducted and the results are shown in Table 16. A general trend of declining sugar concentrations can be seen. With final dry weight, liquid volume and mass of total paper addition known, the expected ethanol concentration can be calculated from sugars results for the final time point 410 hours. This calculation yields an expected 9% (v/v) ethanol, marginally lower than the actual.

Time	Carbohydrate (% w/w)							
(Hours)	Glu		Xyl		Gal		Man	
1	66.60	[0.11]	12.78	[0.16]	2.97	[0.31]	6.33	[0.58]
12	56.51	[0.24]	9.80	[0.15]	1.28	[0.61]	4.65	[0.52]
28	49.80	[0.17]	8.82	[0.10]	1.64	[0.81]	5.38	[0.61]
194	43.06	[0.21]	7.27	[0.17]	0.04	[1.07]	4.55	[0.42]
315	46.86	[0.13]	6.87	[0.05]	< 0.01	[1.01]	4.02	[0.26]
410	48.01	[0.22]	7.30	[0.09]	< 0.01	[0.66]	4.43	[0.37]

Table 16. SSSF 3 Sugar analysis of insoluble solids (HPLC), standard deviation in square brackets

6.3 Discussion

Initial hydrolyses H1-2 whilst having low glucose concentration and yields, provided the impetus to move from using prohibitively high substrate concentrations that cause reduction in stirring efficiency, to utilising lower substrate concentrations that allow ease of stirring and iteratively building concentration by step-wise addition as in H3. This methodology coupled with findings in §4.2.4 lead to the design of SSSF1 and the facilitation of substrate to a concentration equivalent to 27.5% (w/v) becoming achievable where previously 5% (w/v) was the maximum. This is also an improvement on recent literature as summarised in Table 17. SSSF1 had to be terminated solely for the reason that the substrate concentration had become too thick and prohibitive to stirring; ethanol production could be seen to still be increasing suggesting that further additions, if possible, would have facilitated further ethanol production.

SSSF2 and 3 therefore employed a bespoke 10 L vessel with enhanced stirring capabilities. Initial glucose yields of SSSF2 (23.12 mg/mL) and SSSF3 (30.54 mg/mL), whilst appearing low, were achieved rapidly, with the most recent addition of paper only having two hours residence time at this point. Recent work in the area, summarised in Table 17, can be seen to give greater conversion rates, up to 76.1% (w/w) but require a considerably longer residence time, in the order of 72 hours or more. The quantity of paper to volume of liquid added at this point is 15% (w/v) which is considered a high substrate loading (Modenbach and Nokes, 2012, Wang et al., 2012)

Table 17 also highlights the fact that literature results characteristically have substrate concentrations in the rage of 6-17% (w/v). Here the low moisture content of the (waste) paper is what enables achievement of such a high substrate concentrations, since with other cellulosic substrates such as paper sludge large quantities of water are included which effectively dilute the reaction mixture and keeping the solids concentrations low. The dry paper added in batches, in conjunction with the enhanced stirring facility of the 10 L vessel allowed the exploitation of substrate which would have been equivalent to a single additional to

a concentration of 65% (w/v), exceeding anything found in recent literature, Table 17.

With a total of 26 substrate additions coupled with only six enzyme doses, in total 12,000 FPU cellulase (50 mL x 40 FPU/mL x 6 additions) and 22,500 U βG (15 mL x 250 U/mL x 6 additions) were added to the reaction. For the first six additions this equates to the previously stated, 16 FPU/g of substrate cellulase and 30 U/g of substrate βG, but after all additions of substrate were made the concentration decreases to 3.7 FPU/g of substrate Cellulase and 6.92 U/g of substrate βG significantly lower than found in similar studies in the literature (Table 17).

The ethanol concentration achievable from this system is clearly an important factor, SSSF2 gave a concentration of 6.9% (v/v) and a yield of 29% (v/v) whilst SSSF3 gave a concentration of 11.6% (v/v) and a yield of 54% (v/v). These levels are both higher than those achieved in recent literature with SSSF3 being almost double the highest found (Kang et al., 2011). Distillation is known to be the most heavily energy intensive part of the bioethanol production process and it has been long known that the higher the ethanol concentration is before this part begins the more energy efficient it becomes (Hengstebeck, 1961). Thus the increase in ethanol concentration by stepwise introduction of substrate could be an important step therefore in increasing the overall efficiency of the system. Recent yields reported in the literature, Table 17, are greater than those achieved in SSSF2-3 but are at considerably lower substrate concentrations (15% w/v or less). Although the final yield of SSSF3 was 54% (v/v) higher yields were attained during the time course prior to completion, the highest being 65.5% (v/v) suggests that the reduction in the number of additions may have had the effect of increasing yield whilst still achieving a similar ethanol concentration due to the latter additions being poorly fermented as seen in Figure 73.

6.3.1 Substrate Addition Strategies

SSSF2 and SSSF3 only differed in their substrate addition strategies, with additions made on an ad hoc basis with SSSF3 when sufficient hydrolysis had occurred to liquefy the previous substrate addition. Additions in SSSF2 on the

other hand were made at regular two hour intervals irrespective of degradation of the previous addition until twenty additions had been made at which time the viscosity was judged to be too great for the adequate stirring and ad hoc additions commenced for final six additions. The regime of paper additions every two hours was used due to finding shown in Figure 69B and C, where if additions are made over a protracted length of time the enzyme digestion appears to be adversely effected.

Initially SSSF2 produced ethanol at a higher rate than SSSF3 (first 120 hours) but as more additions were made SSSF3 is seen to be more effective. This was probably owing to the reduction in free water during SSSF2 by absorption into the copier paper, therefore leaving both less water available for hydrolysis and fermentation to take place and also reducing the ability of the cellulase and yeast to circulate adequately. Furthermore the reduction in free water could effectively concentrate the solubilised glucose and ethanol content potentially causing product inhibition of both cellulase and yeast. This coupled with the observation of enzyme inactivation if addition timings are too protracted, Figure 69, means that there is an observable necessity to achieve an addition regime that is neither too fast and regimented nor too slow and ad hoc to allow for optimal ethanol production and also prevent non-productive enzyme binding.

The addition regime utilised above appears to diminish the problems associated with enzyme blocking (Yu et al., 2012), where predominantly CBHs become non-productively bound to the substrate and therefore block attempts by other CBHs to productively bind to the substrate (Ma et al., 2008) The addition of new substrate increases the number of active sites in the mixture therefore allowing hydrolysis to continue despite blocked sites on the original substrate. The reduction of competition for relatively few active sites, by addition of new ones may also enable previously blocked enzyme to recommence hydrolysis and eventually detach from the substrate.

Reference	Substrate (% w/v) (max)	Cellulase FPU/g (min)	Glucose yield (% w/w) (max)	Ethanol (% v/v)	Yield (% v/v)
(Sangkharak, 2011)		20	43.7	2.1	43.7
(Prasetyo et al., 2010)		15		4.0	66.3
(Kang et al., 2011)	13.5	5		6.0	70.0
(Shen and Agblevor, 2011)	6	9.7		0.7	78.5
(Zhang and Lynd, 2010)	17	10		4.0	
(Kang et al., 2010)				4.5	70.0
(Ballesteros et al., 2010)	10	15	47.9		79.7
(Wang et al., 2012)	15 (High)	7.5	76.1		n/a
(Kuhad et al., 2010)	6	5	59.8	1.5	
Elliston, 2012	**65**	**3.7**	**36.3**	**11.6**	**54.0**

Table 17. Summary of literature results, author's results in bold for comparison

6.3.2   Possible use of solid by-product as a paint additive

It was observed after experimentation that the recalcitrant material from SSSF was bright white in colour, suggesting that it was made up predominantly of calcium carbonate, as would be expected. This observation leads to the possible re-use of this by-product as a paint additive (Carr and Frederick, 2000) in addition to being re-used in the paper making process. Figure 75 shows an example of post-fermentation residue painted onto a dark coloured surface.

Figure 75. Fermentation residue painted onto a dark surface to highlight its possible use as paint additive

6.4 Conclusion

The use of stepwise addition in synchronisation with SSSF allows for many potential improvements to the current bioethanol production regime. Initial addition of enzyme followed by only addition of substrate to the system allows for overall low enzyme concentrations (3.7 FPU/g of substrate) to be achieved due to enzymes being kept in productive activity throughout the process. Stepwise substrate addition also allows for substantial final substrate concentration (equivalent to 65% w/v) by liquefying small quantities at each stage. This therefore enables the production of high levels of ethanol (11.6% v/v) due to the extent of substrate available for degradation, this high ethanol concentration will itself lead to improved distillation efficiencies through energy conservation. This chapter has highlighted that additional research is needed into the timings and number of the paper additions as these can affect the overall yields of the system to a large extent. The final process flow used in this chapter can be summarised by process flows given in Figure 76 and Figure 77.
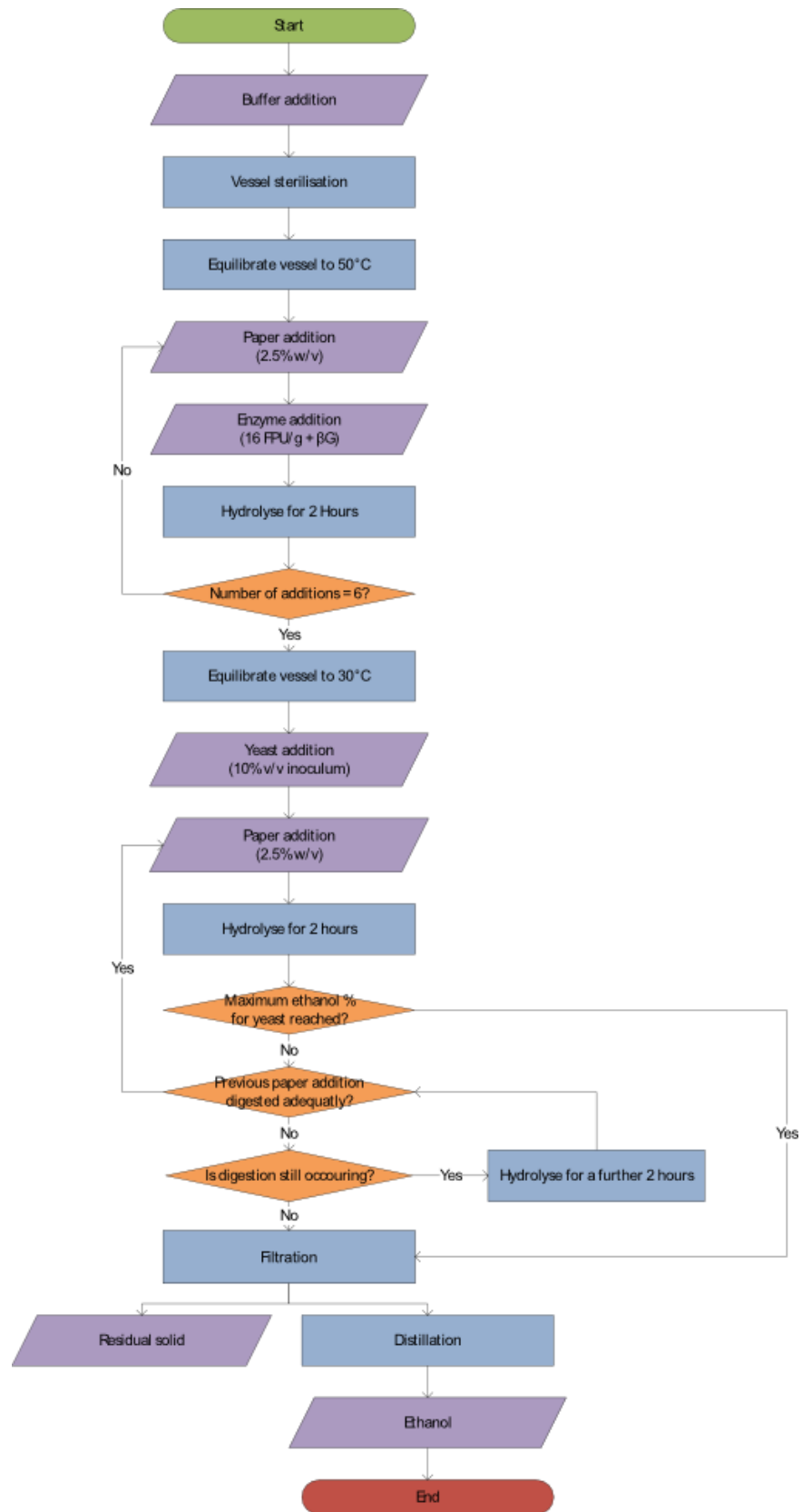
Figure 76. SSSF process flow chart
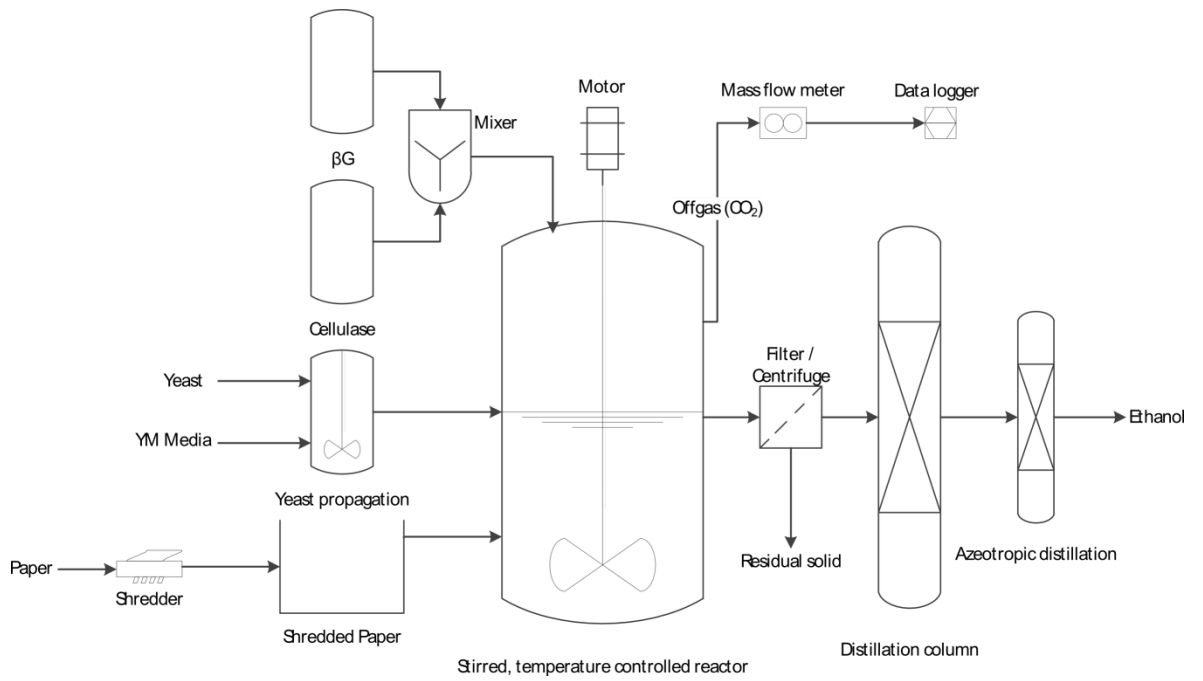
Figure 77. SSSF process flow diagram

7   Temperature Tolerance of Yeast

One of the discontinuities evident in SSF is the fact that enzymatic saccharification has a higher temperature optimum (50°C for Accellerase®) compared to that of the yeast fermentation (typically 25-35°C). With this in mind it was therefore valuable to find yeasts with temperature tolerances approaching the 50°C enzyme optimum. This would enable the enzyme to work as efficiently as possible therefore both increasing yield and potentially reducing residence time.

Saccharomyces cerevisiae is the most widely used yeast species for ethanol production so this was targeted as the candidate for temperature tolerance experimentation. As part of the *Saccharomyces* Genome Resequencing Project (SGRP), a collaborative project between The Sanger Institute and The University of Nottingham, the NCYC produced a strain set including a number of *S.cerevisiae* and *S.paradoxus* strains. *S.paradoxus* was also included, as it is the closet known relative of *S.cerevisiae* (Johnson et al., 2004) and therefore a logical species to also include in the screen.

The strain set were screened under a number of different temperatures ranging from 25 to 45°C. Turbidity and therefore cell growth was measured.

7.1   Materials and Methods

7.1.1   Master strain plate

The SGPR strain set, a 96-well plate of *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* yeast strains in glycerol (stored at -80°C), was used for this set of experiments (NCYC, Norwich, UK). This contained a selection of both strains, a complete list with supporting information for the strains can be found in Appendix H.

7.1.2   YM Media plate preparation

1 mL of sterile YM media was transferred by liquid handling robot (Tecan, Switzerland) to a 96 deep well plate. The master strain plate was thawed at room temperature, then mixed by liquid handling robot and 100 µL added to the YM in

the deep-well plate. The YM plate was incubated at 25°C for three days mixing occasionally, and the master plate returned to the -80°C freezer.

7.1.3   Temperature Screening

180 µL YM Media was transferred to a 96 well micro-titre plate, 20 µL of yeast culture from YM plate was then added (10% v/v inoculum). This plate was then covered with a lid and incubated at test temperature in a FL-600 micro-plate reader (Bio-tek® Instruments Inc, Winooski, USA). Plate was read at 590 nm every 30 minutes for 48 hours, mixed from 1 minute prior to each reading to prevent the yeast settling. This process was repeated for each chosen temperature; 25, 30, 35, 37, 40 and 45°C.

7.1.4   Yeast adaptation to thiomersal

A solution of YM Media with 0.01% (w/v) thiomersal addition was prepared, and a dilution series from 2 to 18 times was then made. 180 µL of thiomersal dilutions and a YM control were added to a 96 well micro-titre plate in triplicate, a 10% (v/v) inoculum of NCYC 2826 (20 µL) was then added to all wells. The plate was covered with a lid and incubated at 30°C in a FL-600 micro-plate reader. The plate was incubated for 48 hours and read every 30 minutes at 590 nm, the plate is mixed for 1 minute prior to each reading. After 48 hours yeast from the minimum dilution factor which showed signs of growth is selected for further analysis, this descendent is inoculated into pure YM Media and allowed to incubate for a further 48 hours at 30°C. The experiment is then repeated with the yeast descendent as many times as necessary in order to achieve adaptation.

7.2   Results and Discussion

A total of 71 yeasts were screened (36 *S.cerevisiae*; 35 *S.paradoxus*) giving growth curves at all six temperatures. The growth curve was integrated over a time frame of 24 hours to give an indication of how well the strain grew at a given temperature (Figure 78). These figures were divided into percentile groups for *S.cerevisiae* and *S.paradoxus* separately, over 75th percentile being deemed as

good growth, over 50th percentile moderate growth, over 25th percentile low growth and less than 25th percentile negligible growth.
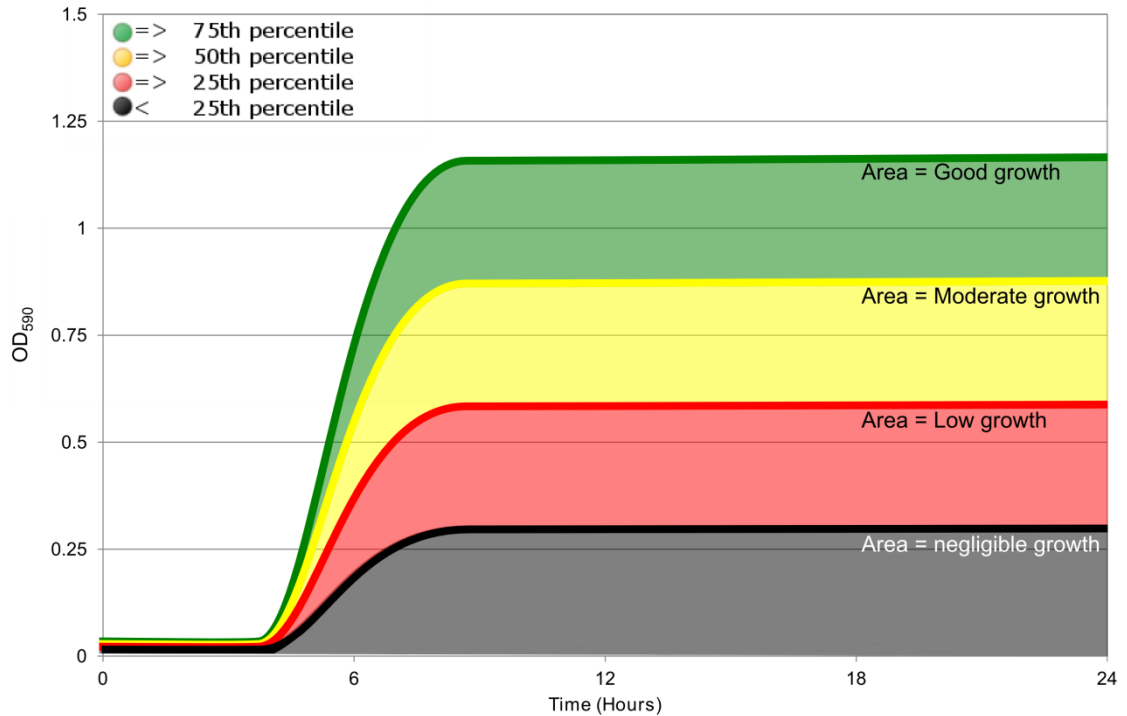
Figure 78. Visualisation of calculation of yeast growth factor

7.2.1   Saccharomyces cerevisiae temperature tolerance

Figure 79 shows the growth of *S.cerevisiae*, it can be seen that all except NCYC 3264, whose geographic isolation is unknown, sustain at least moderate growth up to 37°C. Only six strains produce good growth at 40°C and they also have low growth at 45°C, these strains are summarised in Table 18.
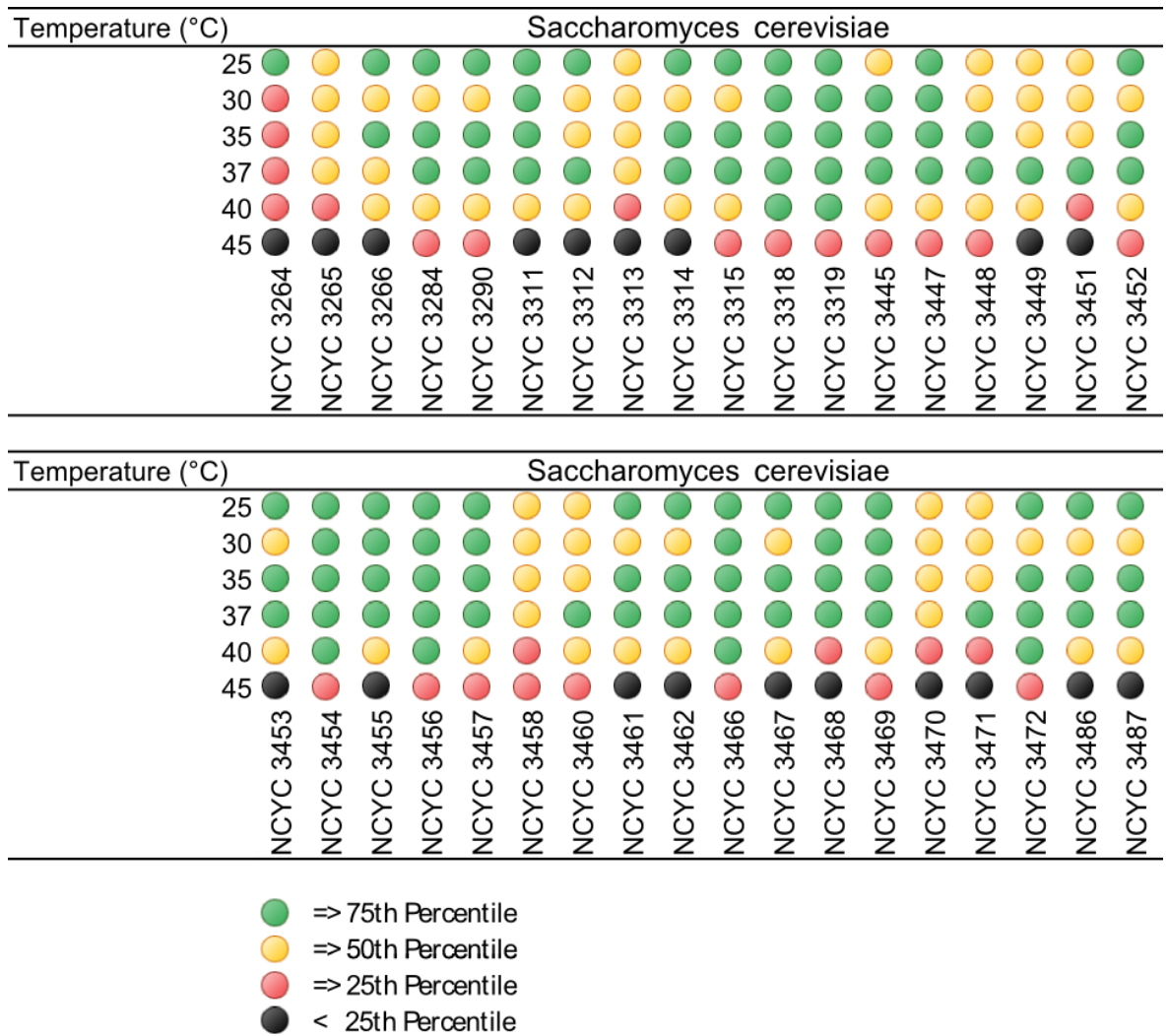
Figure 79. *S.cerevisiae* **temperature tolerance**

Of the highlighted six strains two are clinical in nature which is unsurprising due to their preference for marginally higher temperature (37°C body temperature). The remaining four strains are all from moderate or tropical climates (Koppen-Geiger climate classification (Koppen, 1936) therefore more likely to have an innately higher temperature tolerance. Of the six shortlisted for temperature tolerance NCYC 3318 and NCYC 3319 were focussed on due to being strains already used for ethanol production therefore more likely to have higher ethanol tolerance.

Saccharomyces cerevisiae			
Ref	Strain	Geographic / Isolated by / Year	Source / Reference
NCYC 3318	L-1374	Cauquenes, Chile Ganga A 1999	Fermentation from must País
NCYC 3319	L-1528	Cauquenes, Chile Ganga A 1999	Fermentation from must Cabernet
NCYC 3454	YS9	Singapore - -	Baker strain (Bell et al., 2001)
NCYC 3456	378604X	Royal Victoria Infirmary, Newcastle UK Galloway A -	Clinical isolate (Sputum)
NCYC 3466*	S288c	Merced, California, USA Mrak E 1938	Rotting fig (Mortimer and Johnston, 1986)
NCYC 3472	YJM975	Ospedali Riuniti di Bergamo, Italy - 1994-6	Isolated from vaginitis patient (McCullough et al., 1998)

Table 18. Shortlist of temperature tolerant *S.cerevisiae* (Liti et al., 2009)

7.2.2 Saccharomyces paradoxus temperature tolerance

The growth of *S.paradoxus* strains can be seen in Figure 80; again as with *S.cerevisiae* other than one strain NCYC 3480, which was isolated in Russia, all are able to sustain at least moderate growth up to 37°C. However in this species only one strain was able to maintain good growth at 40°C, NCYC 3335, but this strain had negligible growth at 45°C. Only 20% of *S.paradoxus* strains had greater than negligible growth compared to 47% of *S.cerevisiae*, suggesting that at least within this strain set *S.cerevisiae* is the most thermophilic species. This occurrence has been noted before by Salvadó et al (2011), however it is important to take into account that in this case there may be some skewing of data due to the location of strain collection. *S.cerevisiae* strains were collected in more moderate, dry or tropical climates (e.g. Australia, Singapore and Malaysia) whereas *S.paradoxus* strains were obtained from moderate or continental climates (e.g. UK, Russia and Canada).
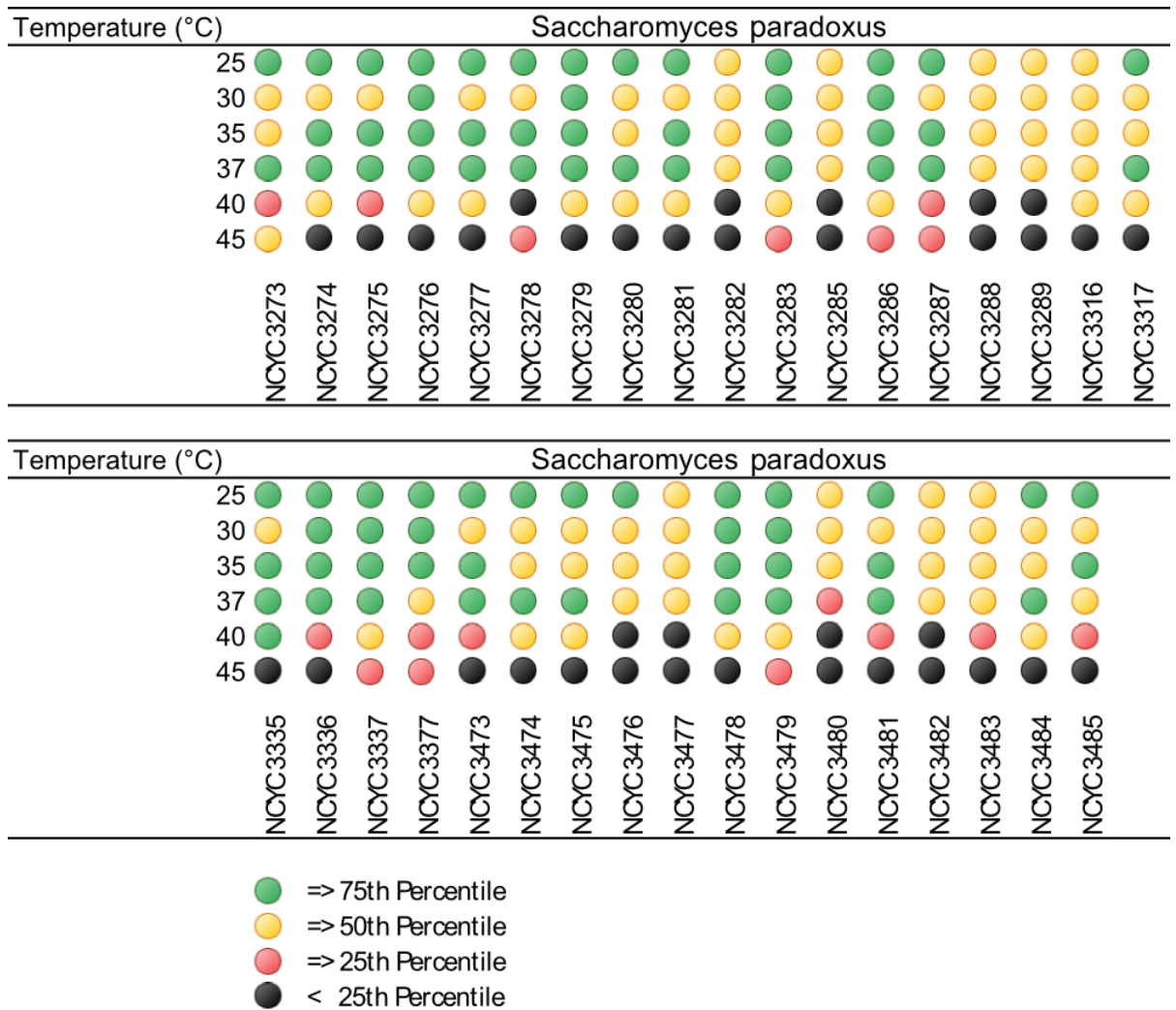
Figure 80. *S.paradoxus* **temperature tolerance**

Of the 35 strains of *S.paradoxus* five were marked as possible SSF use, these are summarised in Table 19. It can be seen that all five strains have been isolated from *Quercus spp* (Oak tree) but this is unsurprising as the studies that produced the samples were focussed on Oak.

		Saccharomyces paradoxus	
Ref	Strain	Geographic / Isolated by / Year	Source / Reference
NCYC 3273	N-45	Ternei, Russia Naumov G 1987	Exudate of *Q.mongolica* (Naumov et al., 1997)
NCYC 3283	Z1.1	Silwood Park, UK Koufopanou V 2003	Bark of *Quercus spp* (Koufopanou et al., 2006)
NCYC 3286	Q95.3	Windsor Great Park, UK Koufopanou V 1998	Bark of *Quercus spp* (Johnson et al., 2004)
NCYC 3337	Q89.8	Windsor Great Park, UK Koufopanou V 1998	Bark of *Quercus spp* (Johnson et al., 2004)
NCYC 3479	Z1	Silwood Park, UK Koufopanou V 2003	Bark of *Quercus spp* (Koufopanou et al., 2006)

Table 19. Shortlist of temperature tolerant *S.paradoxus* (Liti et al., 2009)

7.2.3 Temperature optimum for selected strains

Figures 81 and 82 show the normalised growth versus temperature of the selected strains of each species, more detailed temperature points would be necessary to pinpoint temperature peaks for the individual strains but it can be seen that in all cases except NCYC 3273 this would lie around the 37°C point. The difference in species can again be seen here with *S.paradoxus'* growth declining more steeply following the 37°C point that that of *S.cerevisiae.*
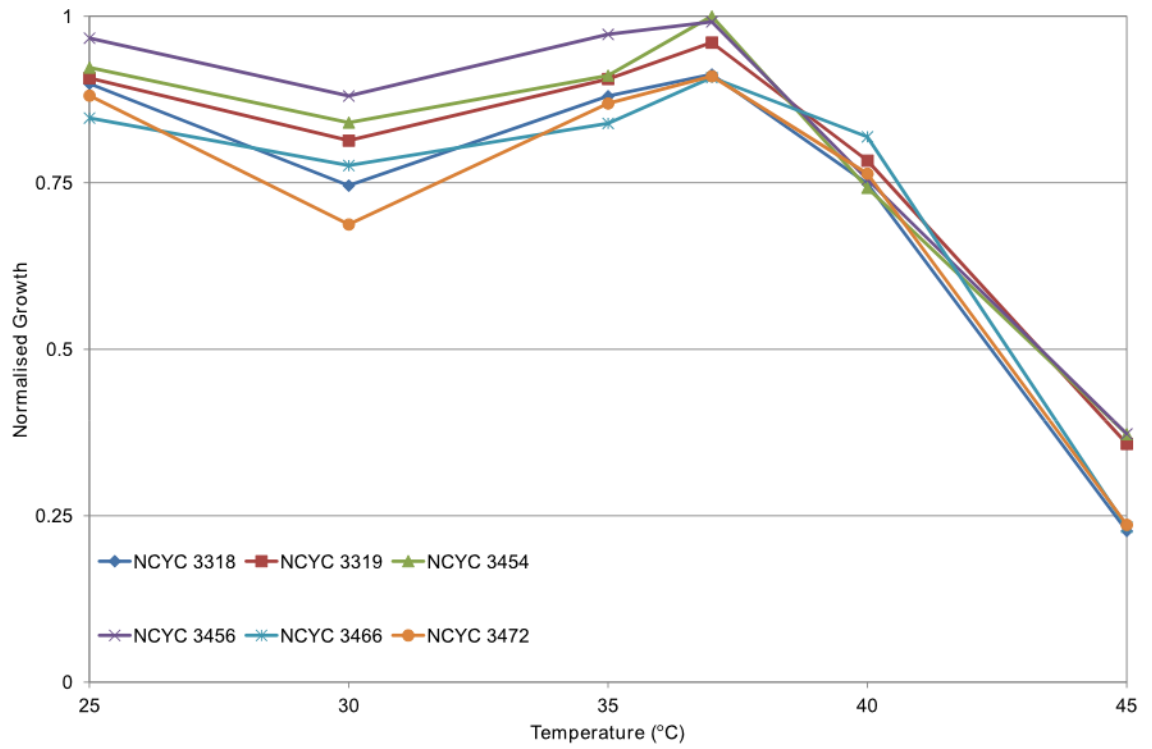
Figure 81. Normalised growth against temperature for selected *S.cerevisiae* strains
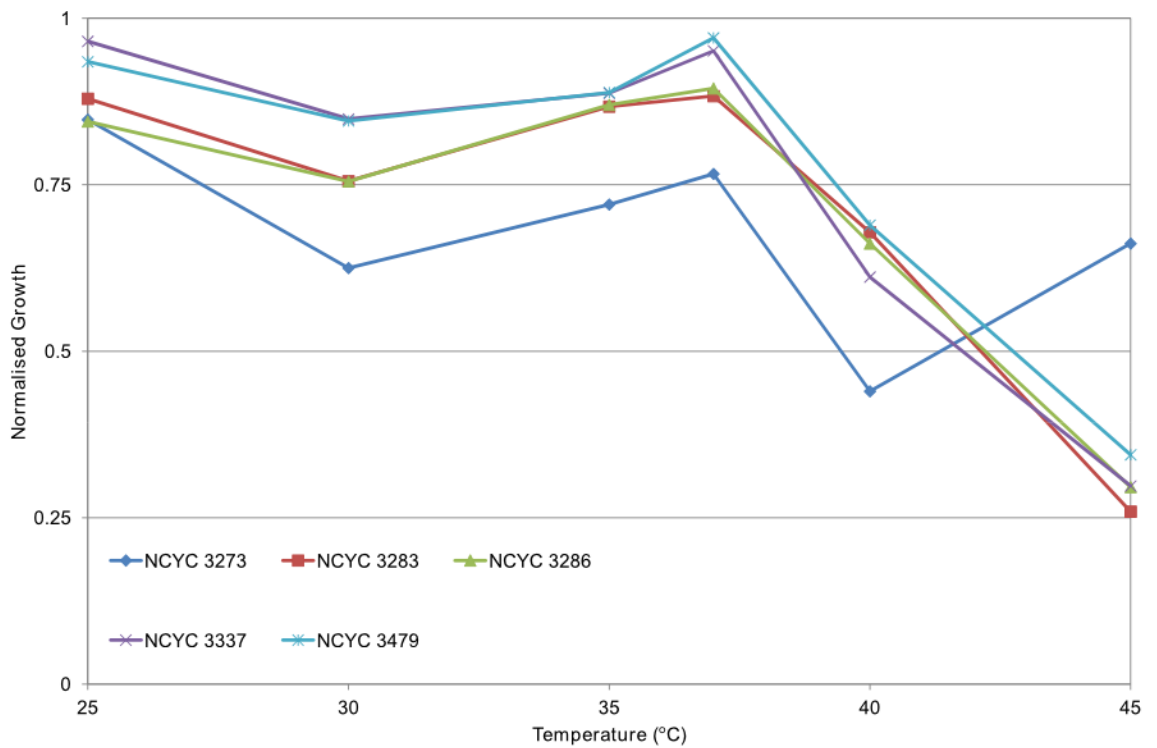
Figure 82. Normalised growth against temperature for selected *S.paradoxus* strains

7.2.4 Comparison with currently utilised strains

Temperature tolerance experiments were also conducted on previously utilised strain NCYC 2826 which is not included in the SGRP strain set and industrial strain Ethanol Red® (Fermentis, France), both of which are *S.cerevisiae* strains. Ethanol Red® is a commonly used dried yeast strain (Kawa-Rygielska and Petrzak, 2011, Mukhtar et al., 2010, Pelaez et al., 2011). Figure 83 shows the temperature tolerances (data is calculated as part of the *S.cerevisiae* population data, Figure 79) it can be seen that the strains grow moderately well up to 40°C then there is a sharp decline in cell growth, by previous selection criteria neither of these strains would have been selected for further study. However this study is focussed on yeast biomass and not ethanol production, an additional screen of ethanol production may highlight different yeast strains including these industrial ones.

Temperature (°C)	NCYC 2826	Ethanol Red®
25	🟡	🟡
30	🟡	🟡
35	🟡	🟡
37	🟡	🟡
40	🟡	🟡
45	⚫	🔴

🟢 => 75th Percentile
🟡 => 50th Percentile
🔴 => 25th Percentile
⚫ < 25th Percentile
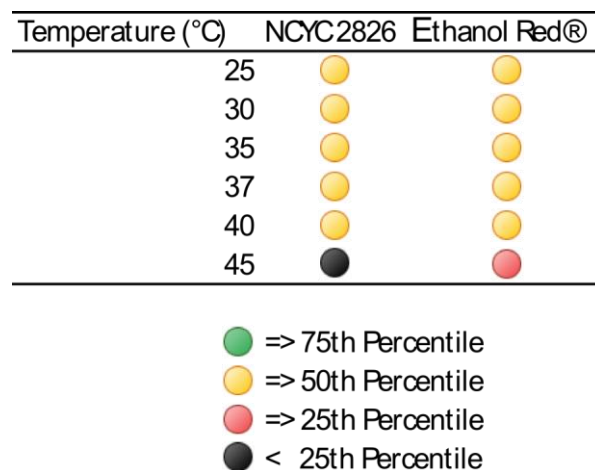
Figure 83. Utilised strains temperature tolerance

The optimum temperature can be seen to be approximately 37°C in Figure 84, NCYC 2826 providing better growth but falling more sharply when temperature exceeded this point. This data highlights the possibility of increasing the temperature used in SSSF experimentation, such as in chapter 6, to 40°C in order to increase the enzyme efficiency. However, alcohol production by NCYC 2826 at this elevated temperature would be necessary to confirm this potential improvement.
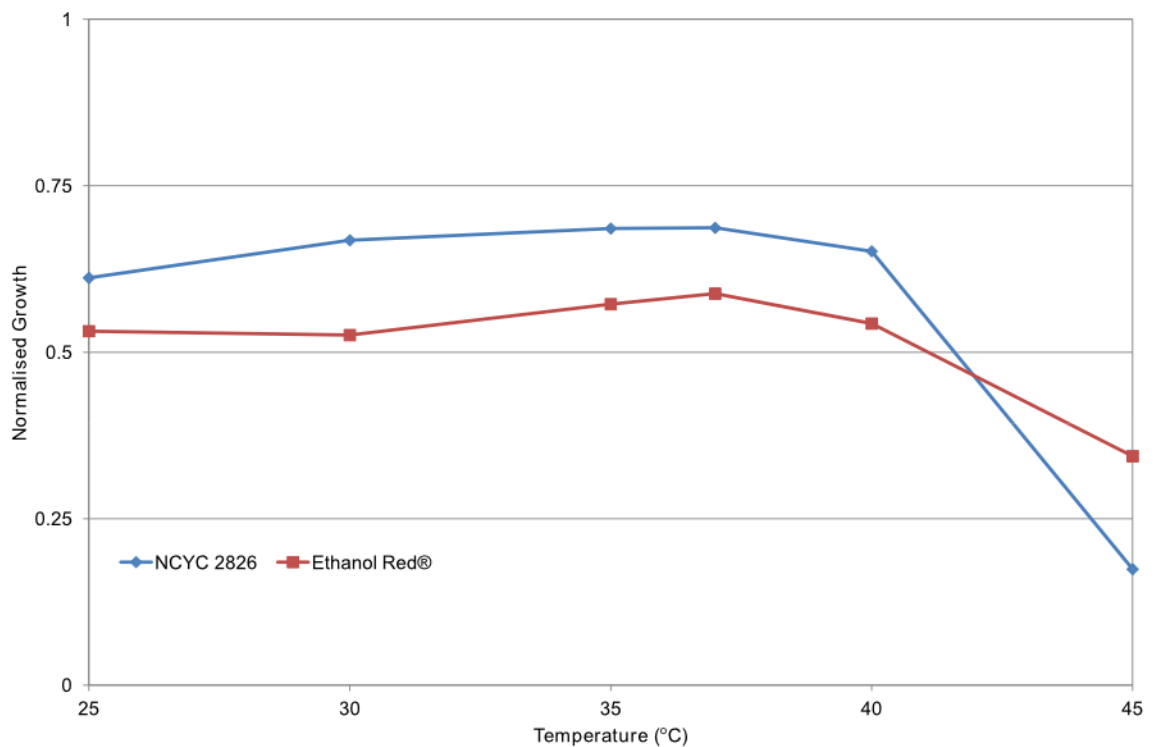
Figure 84. Normalised growth against temperature for utilised strains

7.2.5 Methodology benefits

Using this method allows for a large number of strains to be screened at one time, with the addition of the liquid handling robot this number can be multiplexed permitting even bigger strain sets to be screened or multiple factors evaluated quickly. In contrast a similar study, Edgardo et al (2008), had a strain set of only eleven and utilised larger incubations (250 mL).

7.2.6 Yeast Adaptation

There have been several papers on the adaptation of micro-organisms by evolutionary engineering (Kuyper et al., 2005, Long-McGie et al., 2000). The aim of the following study was to adapt the yeast strain NCYC 2826 to an antimicrobial (thiomersal) to help prevent the unwanted contamination of fermentation by other micro-organisms. The study intended to produce a methodology that would speed up the adaptation of yeast to any given condition using a 96 well system, this method would therefore be the apparent next step in improving the thermotolerance of the selected yeast strains above. Yeast strains were subjected

to a challenging condition (in this case thiomersal) at a range of severities and the sample that had the greatest growth against the harshest severity was selected for further study. The experiment was then repeated with the selected more tolerant descendent until such time as an adaptation occurred. Figures 85-89 show the adaptation to thiomersal over five generations, Figures 85 and 86 only growth on YM can be seen. Descendent 2 (Figure 87) and Descendent 3 (Figure 88) have begun to show some adaptation in that they are beginning to grow on low levels (14-18 times dilutions) of thiomersal addition. Descendent 4 (Figure 89) has begun to show moderate growth on low levels of thiomersal (14-18 times dilution) and therefore signs of adaptation compared to the original strain NCYC 2826.
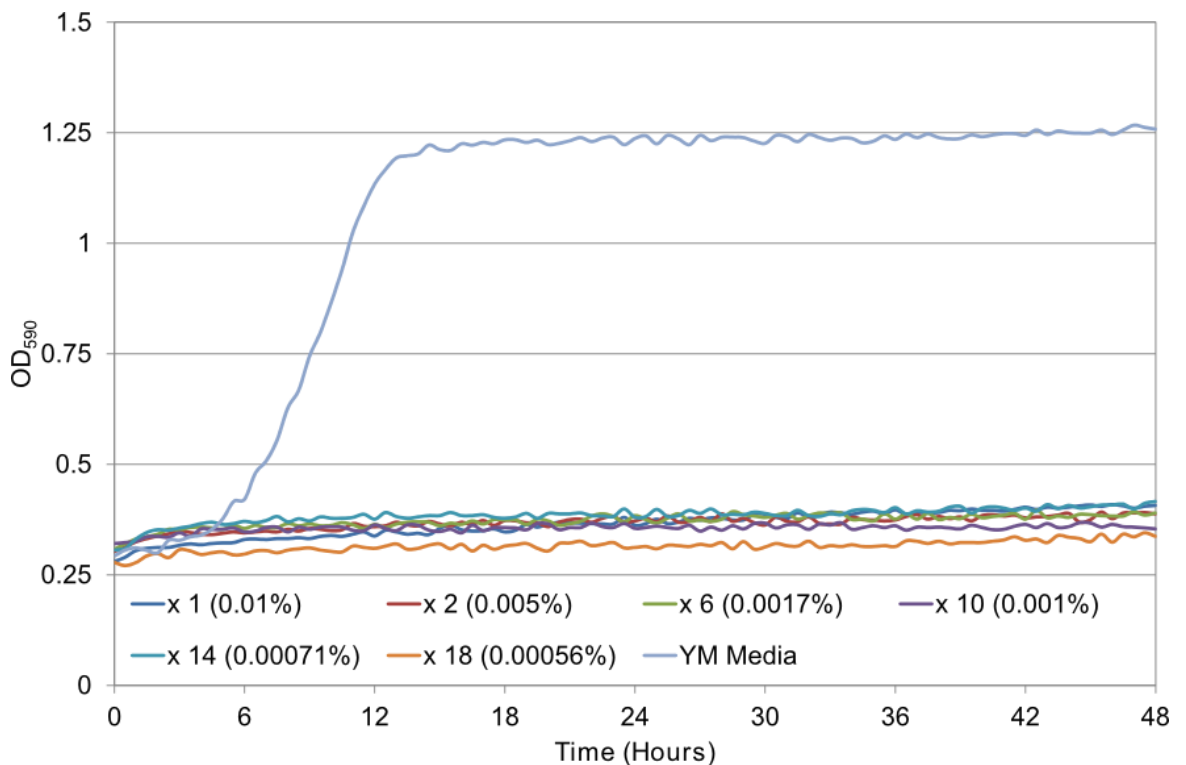
Figure 85. Thiomersal adaptation – NCYC 2826 – growth measured by turbidity at 590 nm
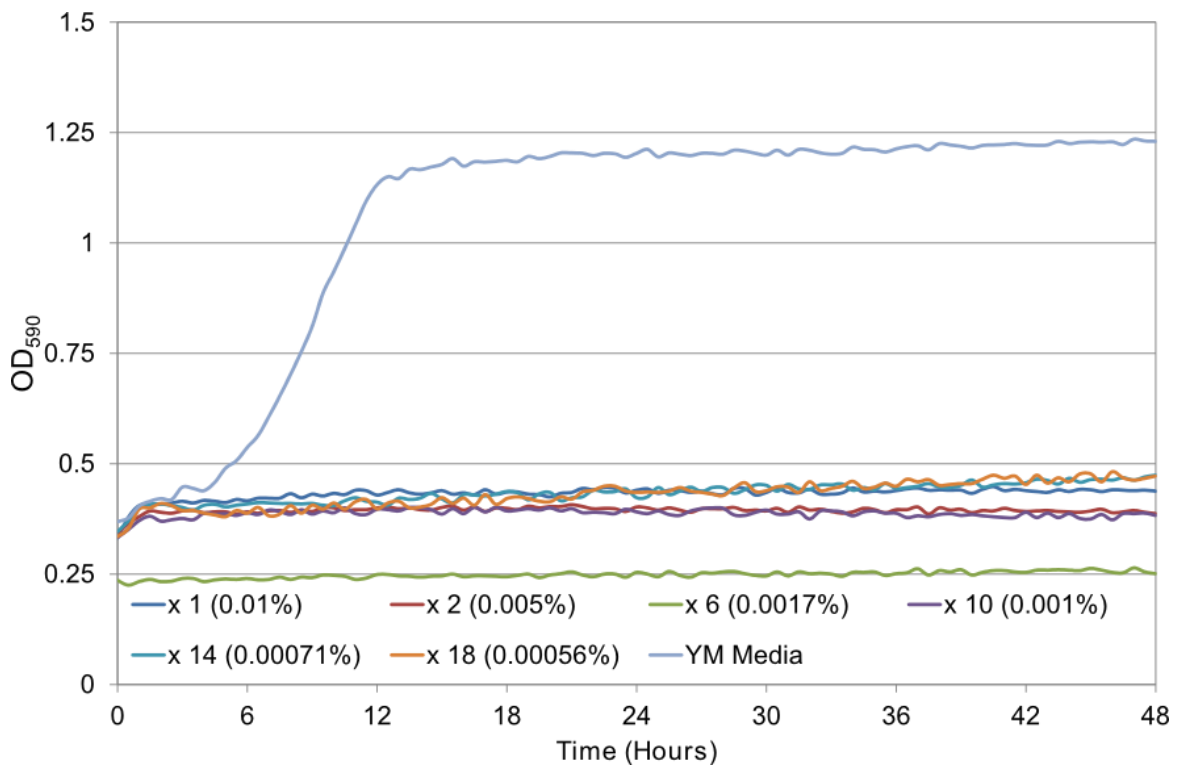
Figure 86. Thiomersal adaptation – NCYC 2826 Descendent 1 – growth measured by turbidity at 590 nm
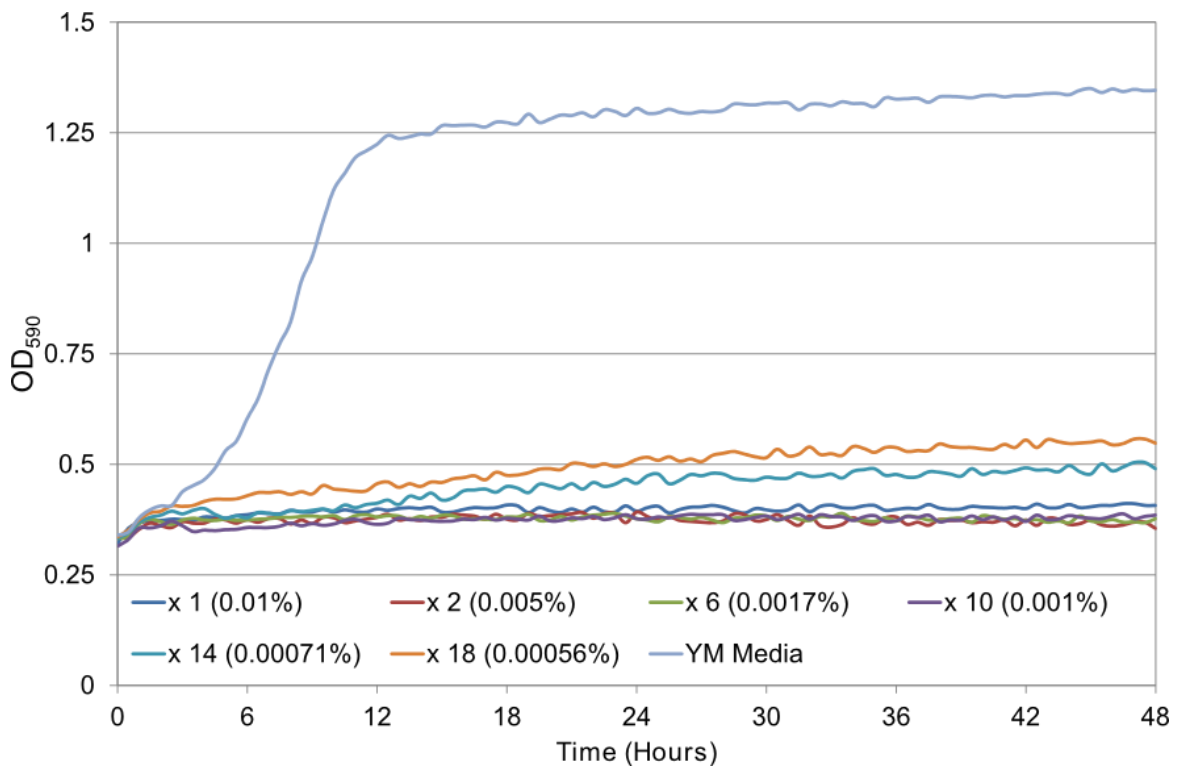
Figure 87. Thiomersal adaptation – NCYC 2826 Descendent 2 – growth measured by turbidity at 590 nm
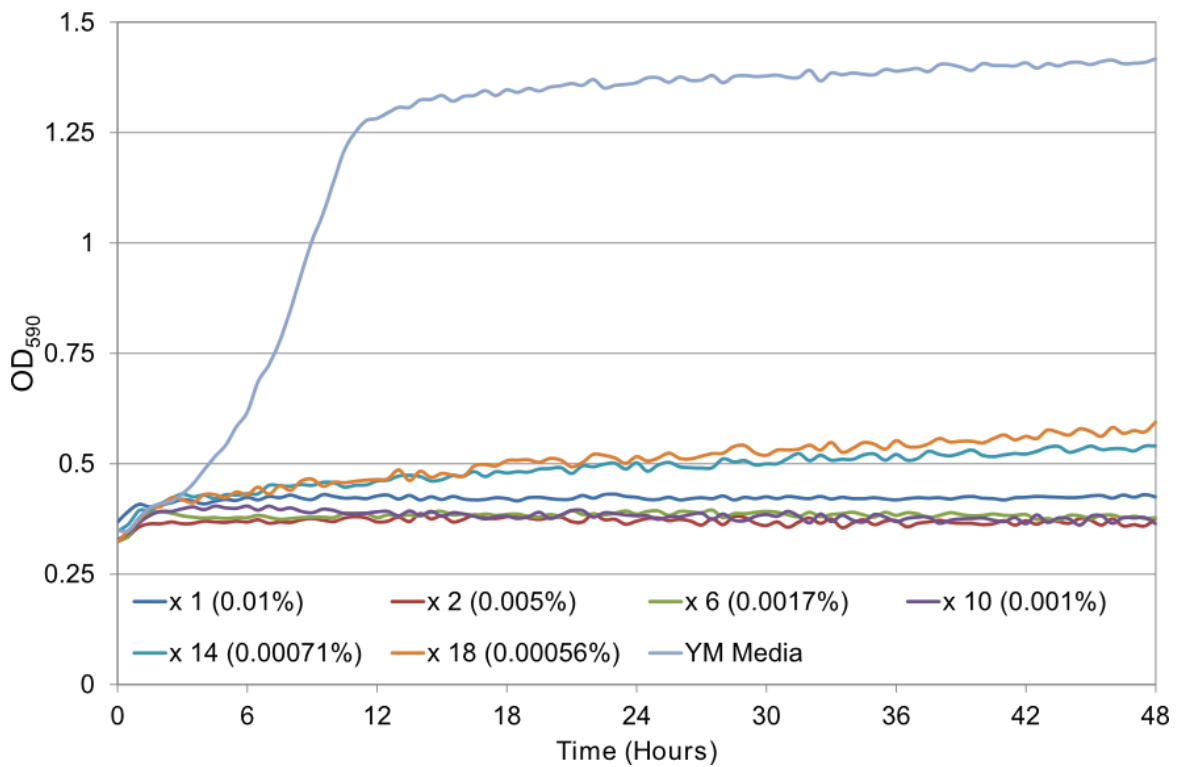
Figure 88. Thiomersal adaptation – NCYC 2826 Descendent 3 – growth measured by turbidity at 590 nm
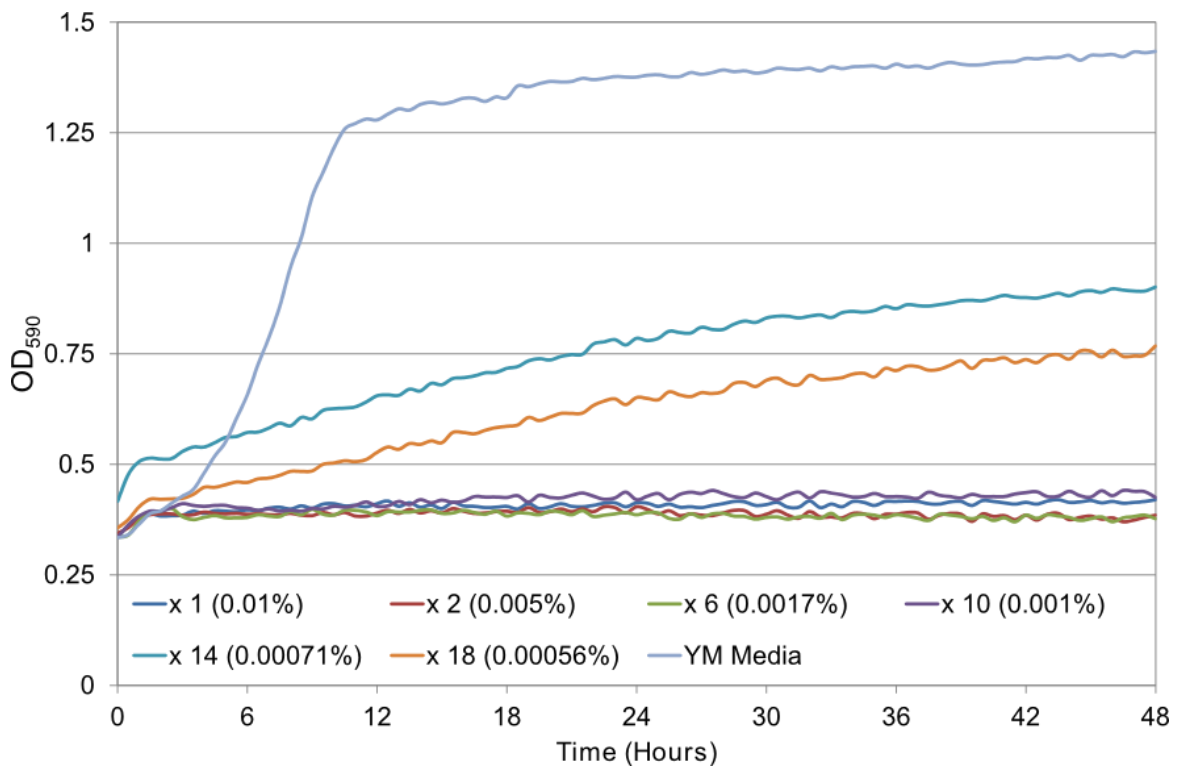
Figure 89. Thiomersal adaptation – NCYC 2826 Descendent 4 – growth measured by turbidity at 590 nm

7.3   Conclusion

This chapter has highlighted a number *S.cerevisiae* and *S.paradoxus* strains that show potential tolerance approaching cellulase optimum temperatures (~50°C), therefore they would be valuable in an SSF or SSSF environment where temperatures usually have to be reduced in order to accommodate the yeast, therefore reducing the efficiency of the enzyme. Currently used strain NCYC 2826 was found to have a possible temperature optimum of 40°C but further study would be required to ascertain the ethanol output at this temperature.

The methodology in this chapter allows for an accelerated screening of multiple strains or conditions for a number of purposes including those outside the scope of this project. Fast adaptation to adverse conditions is also possible using a 96 well plate system over a short period of time.

8 General Discussion and Conclusion

The overall aim of the investigation was to improve the production of cellulosic ethanol from MSW with a focus on paper waste streams, with special reference to:

- Characterisation of waste to ascertain the feasibility of the use of such waste streams in the production of bioalcohol;
- Optimisation of enzyme digestion in order to allow for greatest potential saccharification with the least possible enzyme usage;
- Increasing the concentration/availability of fermentable sugars by increasing the levels of substrate digested in a given volume;
- Optimisation of Fermentation methodology via SSF/SSSF and strain evaluation, to produce as high an ethanol concentration and yield as possible in order to reduce distillation cost;
- Finally judging the feasibility of the overall process in an integrated manner by integrating the above factors.

The conclusions for these are given below:

8.1 Characterisation of waste

In Chapter 3, MSW FW and CP were found to contain significant cellulosic and sugar rich components, 38.9% (w/w) glucose and 50.4% (w/w) glucose respectively, and therefore would both make useful source materials for the waste to biofuel process. With the likelihood of a presence of a number of undesirable chemicals such as, inks, glues and microbial breakdown products, within the MSW which are not determined by the analyses carried out, and due to the results of the microscopy highlighting the similarity in plant fibre content within FW and copier paper samples shredded copier paper was chosen as the most appropriate substrate for further experimentation.

8.2 Enzyme optimisation

Chapter 4's results show that for the effective enzymatic hydrolysis of copier paper, single-step quantitative hydrolysis was achieved with Accellerase®

concentration between 10 and 20 FPU/g of substrate in the presence of additional βG and an anti-microbial agent. βG not only increased the extent of digestion, but also the initial rate of digestion. Sufficient levels of enzyme were also able to overcome the solids effect (Kristensen et al., 2009). Enzyme loading could be effectively reduced whilst facilitating high glucose concentrations when using step-wise substrate addition (discussed further in §8.4).

Ethanol was also found to have potential when used as an additive, stimulating the hydrolysis action of cellulases at low concentrations, and enabling increased glucose yield. This stimulation was also repeated with the addition of Tween[®] 20 detergent.

Steam explosion was investigated in chapter 5 as a pre-treatment process potentially capable of giving an improvement in cellulose accessibility to the enzymatic hydrolysis. However it appeared to have limited useful effect on the digestibility of CP. This may have been due to the presence of calcium carbonate which was included during the paper making process. The latter would have the effect of preventing a reduction in pH within the steam explosion environment and therefore its effectiveness. Physical investigation showed only dispersion of fibres and in the case of FT-IR some removal of hemicellulose; this gave some increase enzymatic digestion possibly due to the pre-wetting effect of the steam explosion allowing for better transfer of enzyme, but there was no clear impact on fermentation which was quite variable, possibly due to variances in the levels of inhibitory products. For these reasons steam explosion is not recommended as a pre-treatment to improve the accessibility of waste paper substrates.

It was however noted that the addition of waste paper to other waste sources, in this case wheat straw, might be beneficial in reducing the inhibitory products produced at high severities of steam explosion. However, the overall impact on saccharification requires further investigation.

The use of stepwise addition of substrate in conjunction with SSSF in chapter 6 allowed for initial addition of enzyme followed by only addition of substrate to the system as first postulated in chapter 4. This process allowed for the reduction of

enzyme concentration from an initial 16 FPU/g of substrate to 3.7 FPU/g of substrate considerably improving the efficiency of enzyme use and therefore the cost implications thereof. However the run times were still long.

8.3 Fermentation optimisation

The benefits of stepwise addition shown in chapter 6 enabled the production of high ethanol concentrations (up to 11.6% v/v) with yeast. This is a considerable improvement, approximately double, over that found in the literature (Table 17). The timings of the paper additions were found to be important to achieve this effect. Yeast fermentation is clearly dependent on the strain used, therefore methodology was developed to screen a number of yeast strains against temperature tolerance. Chapter 7 highlighted a number *S.cerevisiae* and *S.paradoxus* strains that show potential tolerance to higher temperatures necessary for cellulase optimisation (~50°C). These would therefore be valuable in an SSF environment where temperatures usually have to be reduced in order to accommodate the yeast, therefore reducing the efficiency of the enzyme. A rapid screening approach was also developing with liquid-handling robotics allow for yeasts to be quickly evaluated for growth at a variety of conditions.

8.4 Substrate concentration

Chapters 4 and 6 both looked at the possibility of the reduction of enzyme and in the case of chapter 6 the increase in ethanol concentration by the increase of substrate digested. Batch additions enabled outputs comparable to employing substrate concentrations of up to an equivalent of 65% (w/v), high concentration is normally considered as anything above 15% (w/v) (Modenbach and Nokes, 2012). Hence, this result is therefore a significant finding which in turn enables the production of higher concentrations of ethanol (11.6% v/v).

8.5 Feasibility

A number of factors are brought together in Chapter 6 with the use of shredded paper as a substrate, the optimised enzyme cocktail, and the use of stepwise addition. This allowed for the reduction of the effective enzyme concentration to

3.7 FPU/g of substrate and the production of high ethanol concentration at 11.6% (v/v). These improvements help alleviate some of the typical problems associated with second generation bioalcohol production, with enzyme addition being considered the biggest cost factor and therefore a drawback (Black and Veatch Limited, 2008). The low ethanol concentrations typically achieved (Table 17) are generally considered uneconomic to purify. The additional possibility of utilisation of calcium carbonate by-products (Figure 75) increases the feasibility of the overall system. Therefore an important step towards economic viability has been made for these levels of ethanol to have been reached from high substrate concentrations.

8.6 Limitations

A limitation of the work conducted was the use of virgin shredded copier paper rather than an actual waste product complete with inks, glues and other contaminants. This may be relevant to the exploitation of the results, due to the fact that the experimentation was carried out on an uncontaminated substrate with no knowledge of the effects that probable contaminants would cause. Nevertheless, microscopic analysis confirmed the utility of the system developed.

The high level of heterogeneity of MSW leads to the question as to whether a further sample would be as rich in degradable compounds. To ascertain an average carbohydrate content a number of samples would need to be taken over a period of time. This would also provide an estimate of substrate variability. However, on the plus side, MSW evaluated in Chapter 3 was a fraction averaged from many thousands of tonnes of waste, and is therefore probably reliable and representative.

8.7 Future work

8.7.1 Inks and glues

As mentioned in §8.6, studies in Chapter 3 would be enhanced by the additional characterisation of inks and glues. Similar carbohydrate and inhibitor analyses should be carried out along with additional examination warranted by further study into the generic make up of the substrates. The effects of known standard additions would then be tested on a bench scale (~20 mL) to investigate the

effects on enzymatic saccharification, as in §4.1.7 on alcohol inhibition, and also the effects on fermentation as in §5.1.6.

8.7.2 Enzymatic binding

Additional study into enzymatic binding to cellulose and the effects alcohol addition has on this would be beneficial; it was hypothesised as the probable cause of the alcohol activity effect in chapter 4 but further study would help to confirm the cause of this phenomenon. It was shown in chapter 6 that if additions were made over a protracted time period digestion was adversely affected. From the work of Yu et al (2012) it was postulated that non-productive binding followed by denaturation could be the cause, more regular additions alleviating this problem with the introduction of fresh active sites to the system. Therefore, binding information, such as quantitation of both bound and free protein would be advantageous in calculating enzyme loadings at high substrate concentrations and indeed the timings of these additions to best optimise the process outputs.

8.7.3 Yeast Selection and adaptation

Chapter 7 highlighted a number of yeast strains that showed potential of high temperature tolerance. Further work in this area would involve evaluating the effect of temperature on ethanol production by these selected strains allowing the number of potential strains to be reduced. Ethanol production data for these elevated temperatures would immediately be useful for strain NCYC 2826 in increasing SSSF experimentation optimisation.

Furthermore, the condensed subset of strains could then be subjected to temperature stress and resistant cultures progressively produced through selection experiments as described in §7.2.6. Additionally this methodology could be used to look at the possibility of xylose exploitation due to the quantities available in the supernatant liquor, following work such as Kuyper et al. (2005) that showed the capability of evolutionary engineering in this area.

8.7.4 Combined system methodology

SSSF was trialled in Chapter 6 and this brought together a number of factors from throughout this body of work such as; optimised enzyme mixtures and high substrate concentrations. However further experimentation would allow for supplementary factors to be trialled on this scale. These would include the addition of ethanol/Tween® 20 as an activation component (§4.1.7), the use of an optimised yeast strain and therefore higher process temperatures (Chapter 7) and the reduction of the number of enzyme additions. Optimal substrate addition timings and number of additions would also be the aim of any further experimentation. Furthermore the use of SSEF methodology would allow the removal of ethanol during the fermentation therefore overcoming the problems associated with enzyme inhibition (§4.2.5) and also yeast death due to high concentrations as in §6.2.4. The use of a filtration system would allow for the removal of recalcitrant material while the process is still operating, this would also potentially allow for a continuous process to be achieved, a process flow for this can be seen in Figures 90 and 91. These adaptations to the overall process would have the potential of increasing both efficiency and reducing time therefore increasing the overall feasibility of the process.

Finally experimentation with both ink contaminated paper and the original MSW fraction would be carried out, this coupled with the exploitation of processing by-products such as calcium carbonate for paint (Figure 75) would significantly improve the overall feasibly of the process.
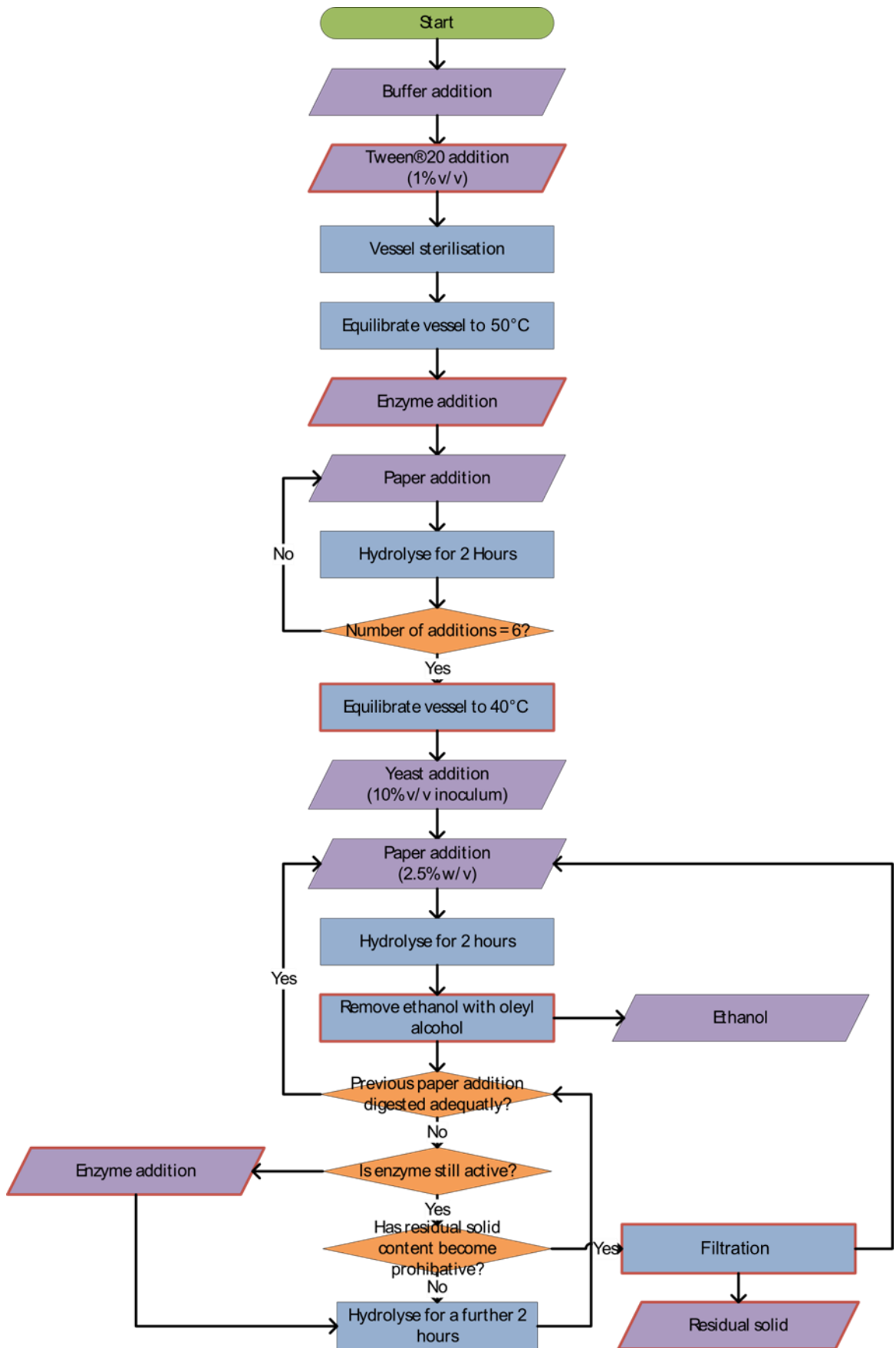
Figure 90. SSEF process flow, including potential improvements to methodology
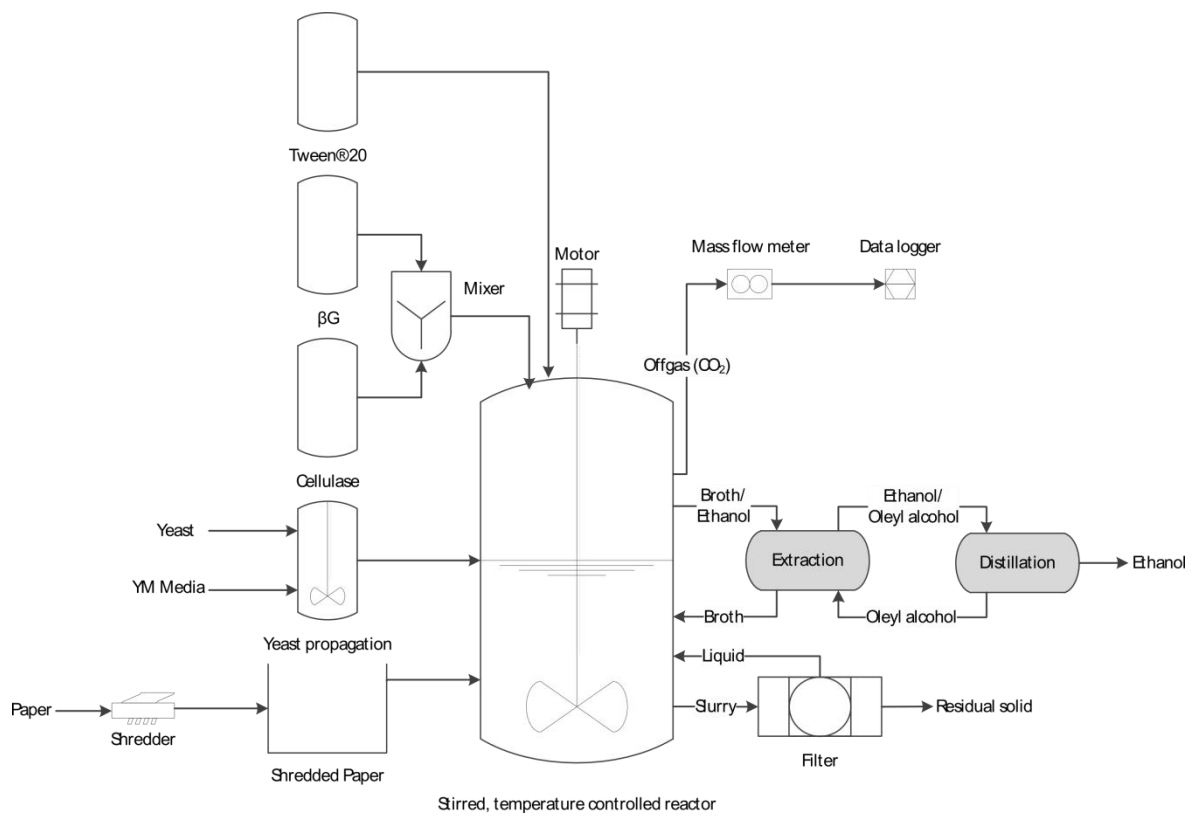
Figure 91. SSEF vessel flow diagram, including potential improvements to methodology

9 Appendices

9.1 Appendix A – GC Method: Sugars Analysis

Instrument Control Method

Instrument Type: Perkin Elmer AutosystemXL

Channel Parameters

Data will be collected from channel A

Delay Time: 0.00 min	Run Time: 50.00 min
Sampling Rate: 12.5000 pts/s	Analogue Output: NT
Attenuation: 5.0 mV	

Autosampler Method

Syringe Capacity: 5.0 µL	Injection Speed: Normal
Viscosity Delay: 0	Pre-injection Solvent Washes: 0
Post-injection Solvent Washes (A): 8	Injection Volume: 2.0 µL
Sample Pumps: 6	Wash/Waste Vial Set: 1
Pre-injection Sample Washes: 2	

Carriers Parameters

Carrier A control: PFlow – He	Column A length: 15.00 m
Vacuum Compensation: OFF	Split Flow: 0.0 mL/min
Initial Setpoint: 2.0 PSI	Diameter: 320 µm
Initial Hold: 999.00 min	

Valve configuration and settings

Valve 1: SPLIT On	Valve 2-6: NONE

Detector Parameters

Detector A: FID	Detector B: NONE
Range: 1	Time Constant: 200
Autozero: ON	

Heated Zones

Injector A: PSSI	Initial Setpoint: 250°C

Initial Hold: 999.00 min   Injector B: NONE

Setpoint: OFF   Detector A: 250°C

Detector B: 0°C   Auxiliary (NONE): 0°C

Oven Program

Cryogenics: Off   Initial Temp: 140°C

Initial Hold: 5.00 min   Total Run Time: 50.00 min

Maximum Temp: 240°C   Equilibration Time: 2.0 min

Ramp 1: 2.5 0/min to 210,
hold for 17.00 min

Timed Events

SPL1 set to 60 at 4.00 min   SPL1 set to 10 at 10.00 min

Real Time Plot Parameters

	Pages	Offset (mV)	Scale (mV)
Channel A	1	-2.000	32.000

Processing Parameters

Bunch Factor: 12 points   Noise Threshold: 20 µV

Area Threshold: 100.00 µV

Peak Separation Criteria

Width Ratio: 0.200   Valley-to-Peak Ratio: 0.010

Exponential Skim Criteria

Peak Height Ratio: 5.000

Adjusted Height Ratio: 4.000

Valley Height Ratio: 3.000

Component Information

Component	Retention Time	Search Window
Rha	19.380 min	0.00 s, 0.50%
Fuc	19.740 min	0.00 s, 1.00%
Ara	22.160 min	0.00 s, 1.00%
Xyl	24.580 min	0.00 s, 1.00%
2-DOG	26.240 min	0.00 s, 1.00%
Man	30.110 min	0.00 s, 1.00%
Gal	30.920 min	0.00 s, 1.00%
Glc	31.880 min	0.00 s, 1.00%

9.2 Appendix B – HPLC Method: Carbohydrate

Instrument Control Method

Device Name: FX10ASCO-3

Model: Flexar FX-10 UHPLC Autosampler Cool Only

Pump Section

Device Name: FX10Pump-2

Transition type: Isocratic Pressure units: psi

Standby time (min): 30.000	Standby flow (mL/min): 0.2
Stop time after equil (min): 120.000	Lower pressure limit: 0
Initial equil time (min): 0.100	Upper pressure limit: 10000

Model: Flexar FX-10 UHPLC Pump

Program Solvent Reservoir

1 Run   42.000   0.6   100 0 0

0 Equil   0.000   0.6   100 0 0

Channel Name: RI

Device Name: FXRIDet-4

End Time (min): 42.000   Sampling rate (pts/s): 20 Range: High

Channel name: RI Temperature (°C): 35 Time Adjustment (min): 0.000

Unretained peak time (min): 0.000

Channel Name: Photo Diode Array Detector

Time Adjustment (min): 0.000   Unretained peak time (min): 0.000

Oven Section

Oven Temperature: 65°C   Flexar Peltier Column Oven

Component information

Component	Retention time (min)	Peak search start (min)	Peak search end (min)	Matching
Glucose	13.032	12.558	13.506	Use closest
Xylose	14.262	13.750	14.773	Use closest
Mannose	18.066	17.192	18.427	Use closest
Fucose	16.446	15.716	16.860	Use tallest
Galactose	15.196	14.270	15.324	Use closest
Myo-Inositol	36.357	34.500	39.700	Use tallest

9.3 Appendix C – HPLC Method: Organic Acids/Inhibitors

Instrument Control Method

Device Name: FX10ASCO-3
Model: Flexar FX-10 UHPLC Autosampler Cool Only

AutoSampler Section

Flush speed: Fast Tray Temperature (°C): 20

Loop size (µL): 200	Needle level (mm): 4.0
Tolerance (± °C): 2	Mode: Partial loop (45 µL)
Injection Delay Time (min): 0.000	Injection Volume (µL): 20
Syringe size (µL): 250	Flushes: 2
Flush volume (µL): 500	Sample speed: Medium
Pre-inject flush cycles: 0	Air cushion (µL): 5
Post-inject flush cycles: 1	

Pump Section

Device Name: FX10Pump-2
Model: Flexar FX-10 UHPLC Pump

Transition type: Isocratic	Pressure units: psi
Standby time (min): 90.000	Standby flow (mL/min): 0.2
Stop time after equil (min): 120.000	Lower pressure limit: 0
Initial equil time (min): 0.100	Upper pressure limit: 10000

Program Solvent Reservoir

1 Run	60.000	0.6	100 0 0
0 Equil	0.000	0.6	100 0 0

Oven Section

Device Name: FXPOven-5

Model: Flexar Peltier Column Oven

Oven temperature: 65 °C

Channel name: RI

Device Name: FXRIDet-4 1

Model: Refractive Index Detector

End Time (min): 60.000	Sampling rate (pts/s): 10
Range: Low	Temperature (°C): 35

Channel name: 210nm

Device Name: PDADet-1

Model: Photo Diode Array Detector

Time (min)	Analytical Wavelength (nm)	Analytical Bandwidth (nm)	Reference Wavelength (nm)	Reference Bandwidth (nm)
0	210	10	400	10

Channel name: 280nm

Device name: PDADet-1

Model: Photo Diode Array Detector

Time (min)	Analytical Wavelength (nm)	Analytical Bandwidth (nm)	Reference Wavelength (nm)	Reference Bandwidth (nm)
0	280	10	400	10

Channel name: 325nm

Device name: PDADet-1

Model: Photo Diode Array Detector

Time (min)	Analytical Wavelength (nm)	Analytical Bandwidth (nm)	Reference Wavelength (nm)	Reference Bandwidth (nm)
0	325	10	400	10

Component information

Component / Detector	Retention time (min)	Peak search start (min)	Peak search end (min)	Matching
Citric / RI	7.643	7.330	7.955	Use closest
Malic / RI	9.067	8.712	9.422	Use closest
Succinic / RI	10.945	10.534	11.357	Use closest
Formic / RI	13.278	12.797	13.760	Use closest
Acetic / RI	14.443	14.000	14.600	Use closest
2FA / RI	40.322	39.029	41.615	Use closest
TFA / RI	6.360	6.086	6.635	Use closest
5HMF / RI	27.123	26.226	28.020	Use closest
5HMF / 210 nm	26.953	26.061	27.844	Use closest
2FA / 210 nm	40.130	38.842	41.417	Use closest
Citric / 210 nm	7.460	7.153	7.767	Use closest
Succinic / 210 nm	10.756	10.350	11.162	Use closest
Malic / 210 nm	8.879	8.529	9.229	Use closest
Acetic / 210 nm	14.268	14.000	14.400	Use closest
Formic / 210 nm	13.090	12.614	13.566	Use closest
TFA / 210 nm	6.162	5.894	6.431	Us closest
5HMF / 280 nm	26.931	26.040	27.822	Use closest
Levulinic / 280 nm	14.591	14.400	14.800	Use tallest
2FA / 280 nm	40.034	38.750	41.319	Use closest
2FA / 325 nm	40.131	38.843	41.418	Use closest
5HMF / 325 nm	26.931	26.040	27.822	Use closest

9.4 Appendix D – HPLC Method: Size Exclusion Chromatograph

Instrument Control Method

Instrument Type : Quaternary LC Pump Model 200Q/410 with Series 200 Autosampler

Channel Parameters

Data will be collected from channel A   Delay Time: 0.00 min

Run Time: 90.00 min   Sampling Rate: 0.5682 pts/s

Signal Source: LCD200

Autosampler Method

Injection Source: Autosampler   Injection volume: 50.0 µL

Loop size: 200 µL   Flush volume: 1000 µL

Fixed mode: Off   Flush speed: Fast

Excess volume: 10 µL   Flush cycles: 2

Air cushion: 10 µL   Pre-injection flush cycles: 1

Sample syringe size: 250 µL   Post-injection flush cycles: 2

Sample speed: Medium   Post-method flush cycles: 0

Needle level: 10%   Inject delay time: 0.00 min

Peltier tray temperature: OFF   Peltier tolerance (+/-): 1°C

Detector Parameters

A (nm): 254 nm   B (nm): 280 nm

BWA (nm): 20 nm   BWB (nm): 20 nm

RWA (nm): 360 nm   RWB (nm): 360 nm

Spectral Aquisition Mode: Time   Sampling Period: 3.52 s

Lamp off at end of run: No

Pump Parameters

Step	Time Flow	Flow	NaNO3	B	C	D
0	0.5	0.50	100.0	0	0	0
1	90.0	0.50	100.0	0	0	0

Ready Time: 999.0 min   Standby Time: 15.0 min

Standby Flow: 0.10 mL/min   Solvent Saver: No

Saver Equ Time: 0.0 min   Shutdown: No

Min Pressure: 0 PSI   Max Pressure: 1000 PSI

Timed Events

There are no timed events in the method

Real Time Plot Parameters

	Pages	Offset (mV)	Scale (mV)
Channel A	1	-30.000	1000.000

Processing Parameters

Bunch Factor: 1 points   Noise Threshold: 1 µV

Area Threshold: 100.00 µV

Peak Separation Criteria

Width Ratio: 0.200   Valley-to-Peak Ratio: 0.010

Exponential Skim Criteria

Peak Height Ratio: 5.000   Adjusted Height Ratio: 4.000

Valley Height Ratio: 3.000

Baseline Timed Events

Event #1 - Set Bunching Factor 5.000 at 1.000

Optional Reports

No report format files given

Component Information

Component	Retention Time	Search Window
mw2M_Dextran	35.000 min	2.00 s, 3.00%
mw1660000	37.600 min	2.00 s, 3.00%
mw380000	41.630 min	2.00 s, 3.00%
mw212000	43.960 min	2.00 s, 3.00%
mw100000	47.140 min	2.00 s, 3.00%
mw48000	50.230 min	2.00 s, 3.00%
mw23700	53.480 min	2.00 s, 3.00%
mw12200	55.860 min	2.00 s, 3.00%
mw5800	58.130 min	2.00 s, 3.00%
mw738_Stachyose tetrahrdrate	62.350 min	2.00 s, 3.00%
Cellobiose	63.500 min	2.00 s, 3.00%
mw180_Glucose	64.660 min	2.00 s, 3.00%

SEC   **Standard**   **Curve**
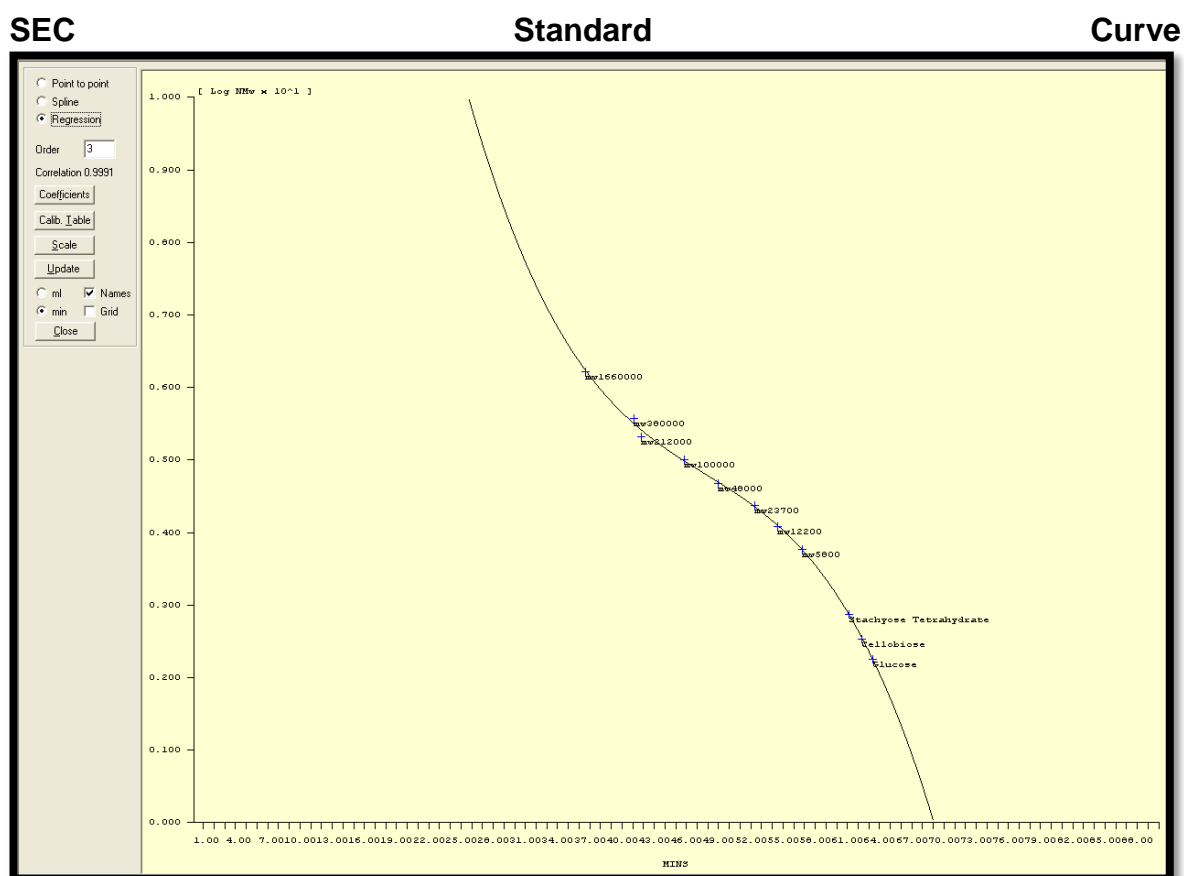
9.5   Appendix E – Paper profile: M-Real Evolve Office 80 g/m^2

paper profile

m·real

EMAS
Verified
environmental
management
REG.NO. UK-000138

Product	**EVOLVE Office 80 g/m^2**
Company	**M-real**
Mill	**SRP New Thames, UK**

Information gathered from   01.01.2007   to   31.12.2007

Date of issue   12.05.2008

E n v i r o n m e n t a l   p r o d u c t   d e c l a r a t i o n   f o r   p a p e r

Environmental Management

Certified environmental management system (at the mill since)   **ISO 14001 (1996) & EMAS (2004)**

Company systems ensure traceability of the of the origin of wood __ yes __ no __X 100% recovered paper

Certified FSC and PEFC chain of custody at the mill since 2005.
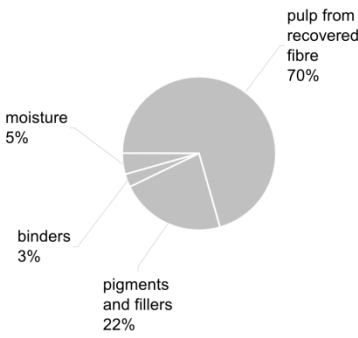
Environmental parameters

The figures are based on methods and procedures
of measurement approved by the local (or national)
environmental regulators at the production site.
The figures include both paper and pulp production.

Water	COD	4.4	kg/t
	AOX	0.0	kg/t
	N$_{Tot}$	0.149	kg/t
	P$_{Tot}$	0.011	kg/t
Air	SO$_2$	0.04	kg/t
	NO$_x$	0.38	kg/t
	CO$_2$ (fossil)	728	kg/t

Solid waste landfilled	42	BDkg/t

Purchased electricity consumption		
/ tonne of final product	**245**	kWh

Product composition

pulp from
recovered
fibre
70%

moisture
5%

binders
3%

pigments
and fillers
22%

More information

Contact person   **Kane Wootton**

Address   M-real UK services limited

Sittingbourne, Kent, ME10 2SG

Phone   +44 1795 564425

E-mail   kane.wootton@m-real.com

More information about Paper Profile can be found on www.paperprofile.com

Supplementary information I

m·real

Product   **EVOLVE Office 80 g/m^2**

White uncoated 100% recycled woodfree office paper
Main end uses: Daily office use for high volume copying, printing of e-mails, draft
documents, faxes, internal reports and other general documents for internal use and
circulation. More information on: www.evolve-papers.com

Mill info

Located in the village of Kemsley, just outside Sittingbourne, Kent, M-real New Thames
comprises a Recycled Fibre Plant and a Paper Mill. The Recycled Fibre Plant processes
approximately 180,000 t/a of recovered paper to make pulp which is used in the adjacent
paper mill. The Paper Mill produces approximately 220,000 t/a of uncoated business and
offset papers. Waste water is treated at the on site effluent treatment plant and the cleaned
water discharged to the Swale.

Transport

RAW MATERIAL - FIBRES

Transport modes   71%

27%

2%
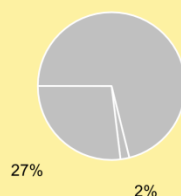
Average transport distance

Wood	**0** km
Pulp	**170** km
Recovered paper	**92** km

(Info. gathered from 01.01.2007 to 31.12.2007)

PRODUCTS

New Thames produces paper in both cut size, folio and reel form. The UK is our main market
and our products are delivered to our domestic customers by truck. If our products are
exported, they are sent by ship and then by train or truck depending on distance and customer
requirements.

Handling after use

PRODUCT

EVOLVE Office is fully recycled and fully
recyclable. Help to close the loop by
recycling the used product.

PACKAGING

EVOLVE Office is packaged in cardboard,
polyethylene (PE) coated paper and stretch
wrap. The packaging should be recycled
where possible or incinerated to produce
energy.

Date of issue   *12.05.08*

EMAS
Verified
environmental
management

Supplementary information II

m·real

Product   EVOLVE Office 80 g/m^2

Origin of wood

Not applicable

Management systems

ISO 14001 Environmental management – LRQ 0770198/A
ISO 9001 Quality standard – LRQ 0770198/A
PEFC Chain of custody – BMT-PEFC-0206
FSC Chain of custody – TT-COC-2108
EMAS Environmental management – UK-000130

Chemicals used as raw materials

No substances classified as carcinogenic, mutagenic or reprotoxic (CMR) are used as raw materials.

Date of issue   12.05.08

9.6   Appendix F – NCYC 2826: Strain information

NCYC Number: 2826

Name: *Saccharomyces cerevisiae*

Depositor: CECT   Deposit Name: *Saccharomyces cerevisiae*

Deposit Month: Nov   Deposit Year: 1998

Equivalent Strain Designations: CECT 1438, IFI 649

Habitat: Grape Must

References: Identification confirmed by 26s rDNA Sequence analysis. Listed in CECT catalogue as producing 15-20% Alcohol.

Physical Characteristics

Optimum Temperature: °   Minimum Temperature: °

Maximum Temperature: °

Cells

Shape: Short-Oval to Long-Oval   Min Broth Breadth: 4

Max Broth Breadth: 7   Min Broth Length: 6

Max Broth Length: 10   Min Agar Breadth: 4

Max Agar Breadth: 7   Min Agar Length: 5

Max Agar Length: 7   Arrangement: Single

Colour on Agar: Cream   Surface on Agar: Slightly shiny

Texture on Agar: Slightly Rough   Deposit in Broth: Non-Flocculent

Ring in Broth: Absent   Ring Colour: N/A

Pellicle in Broth: Absent   Pellicle Appearance: N/A

Pellicle Habitat: N/A

Cell Division

Budding: Multipolar   Fission: Absent

Filamentous Growth

Pseudomycelium: Absent   Pseudomycelium Branch: N/A

Pseudomycelium Form: N/A   Blastospores: N/A

Blastospore Shape: N/A   Blastospore Location: N/A

Blastospore Habit: N/A   True Mycelium: Absent

Clamp Connections: Absent

Asexual Spores

Ballistospores: Absent   Arthrospores: Absent

Endospores: Absent   Chlamydospores: Absent

Sexual Spores

Ascospores: Present   Ascospore Shape: Round

Ascospore Wall: Smooth   Ascospore No Per Ascus: 2-4

Ascus Shape: Oval   Conjugation: Absent

Teliospores: Absent   Teliospore Shape: N/A

Miscellaneous

Assay: Unknown   Salt Tolerant: 10% Weak

Killer: Unknown   Plasmid: Unknown

Semi-Anaerobic Fermentation

Glucose: +   Galactose: +

Sucrose: +   Maltose: -

Cellobiose: -   Trehalose: -

Lactose: -   Melibiose: -

Raffinose: +   Melizitose: -

Inulin: -   Soluble Starch: -

Xylose: Unknown   A M D Glucoside: -

Aerobic Utilistaion and Growth

Glucose: +   Galactose: +

Sorbose: -   Sucrose: +

Maltose: -   Cellobiose: -

Trehalose: +   Lactose: -

Melibiose: -   Raffinose: +

Melizitose: -   Inulin: -

Soluble Starch: -   Xylose: -

L Arabinose: -   D Arabinose: -

Ribose: -   Rhamnose: -

Ethanol: +   Glycerol: -

Erythritol: -   Ribitol: -

Galactitol: -   Mannitol: -

Sorbitol: -   A M D Glucoside: -

Salicin: -   Lactic Acid: +

Succinic Acid: -   Citric Acid: -

Inositol: -   Gluconolactone: -

Glucosamine: -   Methanol: -

Xylitol: -

Aerobic Utilistaion and Growth - Sole Sources of Nitrogen

NH4 2SO4: +   KNO3: -

Ethylamine: -   Cadaverine: -

Lysine: -

Other

Vitamin Free Growth: Unknown   Cyclohex 100ppm: -

Cyclohex 1000ppm: -   50% Glucose Growth: +

60% Glucose Growth: Weak/Latent

Lipolytic: -

Acid Production: -   37c Growth: +

40c Growth: +   Arbutin Hydrolysis: -

Urease Activity: -   Starch Production: -

Acid Tolerant: -

Sequence Data:

Sequence Data: Data not available.

9.7 Appendix G – Ethanol testing by Campden BRI

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GL55 6LD, United Kingdom

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Colney
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NR4 7UA

Report No: IS/REP/126835/30 - 31

Report Date: 04/05/2012

CONFIDENTIAL
CERTIFICATE OF ANALYSIS
Chemistry and Biochemistry
Food Composition Section

Notes

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Results quoted are not corrected for recovery where applicable.
No comments on compliance against any legislative or commercial values are given in this Certificate of Analysis

Prepared/Checked By:

Authorised By:

Authorised Signatories:	R.Butler (Laboratory Manager)	
	K.Grey (Laboratory Manager)	
	J.Cox (Senior Analyst)	
	P. Marriott (Senior Analyst)	

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Report No: IS/REP/126835/30 - 31   **Page 1 of 2**

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provided without liability in its application and use.

ISO 9001 CERTIFIED

UKAS TESTING 1079

Sample Code:	IS/126835/00030		Date of Receipt:	03/05/2012
Description:	Liquid		Condition on Receipt:	Satisfactory for Analysis
Client Code:	H9-11		Testing Performed:	04/05/12
Comments:	P.O. Number: IFR0019847			
Test		**Campden BRI Test Reference**		**Result**
Alcohol (Density)		TES-AC-567 UKAS		11.6 % v/v

Sample Code:	IS/126835/00031		Date of Receipt:	03/05/2012
Description:	Liquid		Condition on Receipt:	Satisfactory for Analysis
Client Code:	H10-12		Testing Performed:	04/05/12
Comments:	P.O. Number: IFR0019847			
Test		**Campden BRI Test Reference**		**Result**
Alcohol (Density)		TES-AC-567 UKAS		6.9 % v/v

9.8 Appendix H – Yeast Strain Information

		Saccharomyces cerevisiae	
Ref	Strain	Geographic / Isolated by / Year	Source / Reference
NCYC 3264	DBVPG6765	Unknown - -	Unknown [55,56]
NCYC 3265	SK1	USA Kane S pre-1974	Soil [49]
NCYC 3266	Y55	France Winge Ö 1930-60	Grape [52]
NCYC 3284	YPS128	Pennsylvania, USA Sniegowski P 1999	Soil beneath Q. alba [62]
NCYC 3290	DBVPG6044	West Africa Guilliermond A 1925	Bili wine, from Osbeckia grandiflora [55,56]
NCYC 3311	DBVPG1788	Turku, Finland Capriotti A 1957	Soil [55,56]
NCYC 3312	DBVPG1373	Netherlands Capriotti A 1952	Soil [55,56]
NCYC 3313	DBVPG1853	Ethiopia Rossi J 1959	White Teff [55,56]
NCYC 3314†	BC187	Napa Valley, USA Bisson L -	Barrel fermentation [54]
NCYC 3315†	YPS606	Pennsylvania, USA Sniegowski P 1999	Bark of Q. rubra [62]
NCYC 3318	L-1374	Cauquenes, Chile Ganga A 1999	Fermentation from must País
NCYC 3319	L-1528	Cauquenes, Chile Ganga A 1999	Fermentation from must Cabernet

NCYC 3445	Y12	Ivory Coast - pre-1981	Palm wine strain [61]
NCYC 3447	DBVPG1106	Australia Fornachon J 1947	Grapes
NCYC 3448	UWOPS83-787.3	Great Inagua Island, Bahamas Lachance M 1983	Fruit, *Opuntia stricta*
NCYC 3449	UWOPS87-2421	Puhelu Road, Maui, Hawaii Lachance M 1987	Cladode, *Opuntia megacantha*
NCYC 3451	NCYC361	Ireland Gilliland R 1952	Beer spoilage strain from wort [58]
NCYC 3452	K11	Japan - 1981	Shochu sake strain [61]
NCYC 3453	YS4	Netherlands Barnett J 1975	Baker strain [53]
NCYC 3454	YS9	Singapore - -	Baker strain [53]
NCYC 3455	322134S	Royal Victoria Infirmary, Newcastle UK Galloway A -	Clinical isolate (Throat sputum)
NCYC 3456	378604X	Royal Victoria Infirmary, Newcastle UK Galloway A -	Clinical isolate (Sputum)
NCYC 3457	273614N	Royal Victoria Infirmary, Newcastle UK Galloway A -	Clinical isolate (Fecal)
NCYC 3458	YJM978	Ospedali Riuniti di Bergamo, Italy - 1994-6	Isolated from vaginitis patient [59]

NCYC 3460	Y9	Indonesia - pre-1962	Ragi (similar to sake wine) [61]
NCYC 3461	UWOPS03-461.4	Telok Senangin, Malaysia Wiens F 2003	Nectar, Bertram palm [60]
NCYC 3462	UWOPS05-217.3	Telok Senangin, Malaysia Lachance M 2005	Nectar, Bertram palm
NCYC 3466*	S288c	Merced, California, USA Mrak E 1938	Rotting fig [48]
NCYC 3467*	W303	Created by Rothstein R by multiple crossing	NA [50,51]
NCYC 3468	UWOPS05-227.2	Telok Senangin, Malaysia Lachance M 2005	Trigona spp (Stingless bee)
NCYC 3469	DBVPG6040	Netherlands - 1947	Fermenting fruit juice [57]
NCYC 3470†	YIIc17_E5	Sauternes, France - -	Wine
NCYC 3471	YJM981	Ospedali Riuniti di Bergamo, Italy - 1994-6	Isolated from vaginitis patient [59]
NCYC 3472	YJM975	Ospedali Riuniti di Bergamo, Italy - 1994-6	Isolated from vaginitis patient [59]
NCYC 3486	NCYC110	West Africa Guilliermond A pre-1914	Ginger beer from *Z.officinale* [63]
NCYC 3487	YS2	Australia - -	Baker strain [53]

Adapted from (Liti et al., 2009)

		Saccharomyces paradoxus	
Ref	Strain	Geographic / Isolated by / Year	Source / Reference
NCYC 3273	N-45	Ternei, Russia Naumov G 1987	Exudate of Q. mongolica [68]
NCYC 3274	UFRJ50816	Tijuca Forest, Rio de Janeiro, Brazil pre-1992	Drosophila spp [70]
NCYC 3275	N-44	Ternei, Russia Naumov G 1987	Exudate of Q. mongolica [68]
NCYC 3276	N-17	Tartastan, Russia - -	Exudate of Q. robur [66]
NCYC 3277	T21.4	Silwood Park, UK Koufopanou V 1998	Bark of Quercus spp [64]
NCYC 3278	Q59.1	Windsor Great Park, UK Koufopanou V 1998	Bark of Quercus spp [64]
NCYC 3279	YPS138	Pennsylvania, USA Sniegowski P 1999	Soil beneath Q. velutina [62]
NCYC 3280	S36.7	Silwood Park, UK Koufopanou V 1997	Bark of Quercus spp [64]
NCYC 3281	Y7	Silwood Park, UK Koufopanou V 2003	Bark of Quercus spp [65]
NCYC 3282	Q32.3	Windsor Great Park, UK Koufopanou V 1998	Bark of Quercus spp [64]
NCYC 3283	Z1.1	Silwood Park, UK Koufopanou V 2003	Bark of Quercus spp [65]
NCYC 3285	DBVPG4650	Marche, Italy Bartolini pre-1992	Fossilized guano in a cavern [55,56]
NCYC 3286	Q95.3	Windsor Great Park, UK Koufopanou V 1998	Bark of Quercus spp [64]

NCYC 3287	N-43	Vladivostok, Russia Naumov G 1987	Exudate of Q. mongolica [68]
NCYC 3288	CBS5829	Denmark Jensen V pre-1967	Mor soil, pH3.6 [66]
NCYC 3289	DBVPG6304	Yosemite, California, USA Phaff H 1951	Drosophila pseudoobscura [69]
NCYC 3316	A4	Mont St-Hilaire, Quebec, Canada Bell G and Replansky T 2003	Bark of Quercus rubra [65]
NCYC 3317	A12	Mont St-Hilaire, Quebec, Canada Bell G and Replansky T 2003	Soil beneath Q. rubra [65]
NCYC 3335	Y6.5	Silwood Park, UK Koufopanou V 2003	Bark of Quercus spp [65]
NCYC 3336	Q62.5	Windsor Great Park, UK Koufopanou V 1998	Bark of Quercus spp [64]
NCYC 3337	Q89.8	Windsor Great Park, UK Koufopanou V 1998	Bark of Quercus spp [64]
NCYC 3377	KPN3828	Novosibirsk, Siberia, Russia Yurkow A 2003	Bark of Q. robur [67]
NCYC 3473	Y9.6	Silwood Park, UK Koufopanou V 2003	Bark of Quercus spp [65]
NCYC 3474	Q74.4	Windsor Great Park, UK Koufopanou V 1998	Bark of Quercus spp [64]
NCYC 3475	Q69.8	Windsor Great Park, UK Koufopanou V 1998	Bark of Quercus spp [64]
NCYC 3476	W7	Silwood Park, UK Koufopanou V 1996	Bark of Quercus spp [64]

NCYC 3477	Q31.4	Windsor Great Park, UK Koufopanou V 1998	Bark of Quercus spp [64]
NCYC 3478	Y8.5	Silwood Park, UK Koufopanou V 2003	Bark of Quercus spp [65]
NCYC 3479	Z1	Silwood Park, UK Koufopanou V 2003	Bark of Quercus spp [65]
NCYC 3480†	CBS432	Moscow area, Russia - pre- 1931	Bark of Quercus spp [66]
NCYC 3481	Y8.1	Silwood Park, UK Koufopanou V 2003	Bark of Quercus spp [65]
NCYC 3482	KPN3829	Novosibirsk, Siberia, Russia Yurkow A 2003	Bark of Q. robur [67]
NCYC 3483	UFRJ50791	Catalao point, Rio de Janeiro, Brazil pre-1992	Drosophila spp [70]
NCYC 3484	IFO1804	Japan - -	Bark of Quercus spp [68]
NCYC 3485	UWOPS91-917.1	Saddle Road, Island of Hawaii Lachance M 1991	Flux of Myoporum sandwichense

Adapted from (Liti et al., 2009)

10 Glossary

2-FA	2-Furaldehyde
2-DOG	2-Deoxyglucose
5-HMF	5-Hydroxymethylfurfural
ABE	Acetone, Butanol, Ethanol process
ADP	Adenosine diphosphate
AD	Anaerobic digestion
AFEX	Ammonia fiber expansion
AIR	Alcohol Insoluble Residue
AKI	Anti-knock index
Ara	Arabinose
ATP	Adenosine triphosphate
βG	Beta-glucosidase
BBSRC	Biotechnology and Biological Sciences Research Council
CBH	Cellobiohydrolase
CBM	Carbohydrate Binding Domain
CBP	Consolidated Bioprocessing
CP	Copier paper
DAD	Diode Array Dectector
DCM	Dichloromethane
DNS	Dinitrosalicylic acid
DP	Degree of Polymerisation
Defra	Department for Environment, Food and Rural Affairs
EG	Endo-glucanase
FFV	Flex Fuel Vehicle
FID	Flame ionisation detector
FPA	Filter Paper Assay
FPU	Filter Paper Unit
FT-IR	Fourier transform Infrared Spectroscopy
Fuc	Fucose
FW	Fibrous Waste
Gal	Galactose

GC	Gas Chromatography
Glu	Glucose
GOPOD	Glucose Oxidase Peroxidase
HPLC	High performance Liquid Chromatography
IR	Infrared
IU	International Enzyme Unit
IUBMB	International Union of Biochemistry and Molecular Biology
Man	Mannose
MON	Motor octane number
MS	Mass Spectrometry
MSW	Municipal Solid Waste
NAD	Nicotinamide adenine dinucleotide
NCYC	National collection of Yeast Cultures
NREL	Nation Renewable Energy Laboratory
OD	Optical density
PCR	Polymerase chain reaction
PDA	Photo Diode Array
PEM	Protein Exchange Membrane
PON	Pump octane number
PW	Particulate Waste
Rha	Rhamnose
RI	Refractive Index
RON	Research octane number
SEC	Size Exclusion Chromatography
SF	Severity Factor
SGRP	Saccharomyces Genome Resequencing Project
SHF	Separate Hydrolysis and Fermentation
SI	Système international d'unités
SSCF	Simultaneous Saccharification and Co-fermentation
SSEF	Simultaneous Saccharification and Extractive Fermentation
SSF	Simultaneous Saccharification and Fermentation
SSSF	Semi-Simultaneous Saccharification and Fermentation
U	Unit (enzyme)

UV	Ultraviolet
UV-VIS	Ultraviolet and visible light
Xyl	Xylose
YM	Yeast and Mould

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