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PROCESS IMPROVEMENT FOR THE PRODUCTION OF FERMENTABLE SUGARS USING PAPER PULP DERIVED FROM MUNICIPAL SOLID WASTE

by

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Thesis for the degree of Doctor of Philosophy

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ABSTRACT

Thesis for the degree of Doctor of Philosophy

OPTIMISATION OF THE PRODUCTION OF FERMENTABLE SUGARS USING PAPER PULP DERIVED FROM MUNICIPAL SOLID WASTE (MSW)

Dhivya Jyoti Puri M_{Eng} B_{Sc}

Sugar-lignin bio-refineries using renewable lignocellulosic carbon as an input material could be used in the future to produce a variety of value added products including fuels and specialty chemicals. The bio-refinery aims to replace a proportion of goods currently produced using fossil fuels. Lignocellulosic material has a significant sugar potential in the form of cellulose and hemicellulose and this can be accessed using enzymatic hydrolysis. The lignocellulosic feedstock used in this research was paper pulp derived from municipal solid waste (MSW) and the aim of the work was to maximise the efficiency of producing a concentrated sugar solution from the cellulose (or glucan) component of MSW using commercial enzyme preparations.

Analysis of the pulp by acid hydrolysis showed a ratio of 56: 12: 27: 5 of Glucan: Hemicellulosic sugar chains other than glucan: Lignin & pseudo lignin: Ash on total solids (TS). The hydrolysis behaviour of this pulp was similar to that of other lignocellulosic substrates even though the matrix of this material is perhaps more complex. Glucan conversion could be increased by 6% if the pulp was extracted with acetone to remove solvent soluble compounds. Using the additive PEG 6000 increased conversion by 15 % over 48 hours, and allowed a 40 % reduction in the enzyme requirement. PEG also increased the centrifugal dewaterability of the substrate by up to 13%.

These results were obtained in single stage batch experiments. It was found, however, that both the glucose concentration in solution and the overall glucan conversion in the substrate could be improved by using a two-stage hydrolysis strategy. Using 50 mg enzyme g^{-1} pulp at high total solids content >18.5% TS single-stage enzyme hydrolysis gave a maximum glucan conversion of 68%. It was found

that two-stage hydrolysis could give higher conversion if sugar inhibition was removed by an intermediate fermentation step between hydrolysis stages. This, however, was not as effective as direct removal of the sugar products, including xylose, by washing of the residual pulp at pH 5. This improved the water availability and allowed reactivation of the pulp-bound enzymes. Inhibition of enzyme activity could further be alleviated by replenishment of β-glucosidase which was shown to be removed during the wash step. The two-stage hydrolysis process developed could give an overall glucan conversion of 88%, with an average glucose concentration of 7.5 wt% in 4 days after combining the hydrolysates of the first and second stage of hydrolysis.

The residual washwater from the two-stage hydrolysis with intermediate wash step process contained a dilute amount of sugar. It was found that this washwater could be used as dilution water for a new batch of hydrolysis without any detriment to conversion efficiency. Thus, to further the work above a washwater recycle strategy was applied to the two-stage hydrolysis process. Washwater at various pHs and with or without the addition of PEG 6000 was used as dilution water for a subsequent round of hydrolysis, where up to 6 rounds of 48-hour hydrolysis were completed to reach a steady stage configuration. In these strategies the enzyme dose was reduced to 30 mg C-Tec3 g^{-1} pulp. Use of a pH 5 or pH 9 wash resulted in an increase in conversion of up to 5% in the first-stage hydrolysis rounds, indicating that enzyme carryover was occurring. The sugar augmentation and enzyme carryover consistently resulted in glucose yields above 7.0 wt% in the first stage hydrolysate when using this lower enzyme dose.

The best result achieved in this strategy was obtained when using 0.25 wt% PEG 6000 in the reaction medium and washwater. By reducing the amount of liquid in the second-stage of hydrolysis, it was found that an overall average glucan conversion of 81% could be achieved over the two hydrolysis stages with an average glucose concentration of over 8 wt% in a 4 or 5 day reaction period. This result is significant, as it meets the downstream processing requirements for bio-ethanol, a major bio-refinery product, and does this with a low enzyme loading. Furthermore, the waste discharge is minimised due to the high glucan conversion.

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Declaration of authorship

I Dhivya Jyoti Puri declare that this thesis and the work presented in it are my own and have been generated by me as the result of my own original research.

Title: Optimisation of the production of fermentable sugars using paper pulp derived from municipal solid waste.

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
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Puri, D. J., Heaven, S., Banks, C. J., (2013). "Improving the performance of enzymes in hydrolysis of high solids paper pulp derived from MSW." Biotechnology for Biofuels **6 : 107.**

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Definitions and Abbreviations

CBH – Cellobiohydrolase enzyme

CBM – Carbohydrate binding domain

DM – dry matter

EG - Endoglucanase

MSW – Municipal solid waste

RPM – rotations per minute

SED – substrate enzyme digestibility – equivalent to carbohydrate conversion

SHF – Separate hydrolysis and fermentation

SSF – Simultaneous saccharification and fermentation

TS – Total solids

Chapter 1: Introduction

1.1 Background

There is currently a pressing need to find alternative energy sources to fossil fuels, which contribute to rising greenhouse gas emissions, pollution, and resource depletion as well as being finite in extent. Within the liquid fuels sector, most of the world's crude oil resources lie in politically volatile countries, causing instability in both price and supply. Moreover, a recent report from the UN Intergovernmental Panel on Climate Change states that the world must move away from carbon intensive fuels with a very large shift towards renewable energy (McGrath, 2014). Thus it is desired that in the near future a larger proportion of so-called readily useable energy will have to be derived from renewable sources that are more secure and sustainable than fossil fuels. The trend over the last few decades has been towards research, development and implementation of biologically-derived fuels, including bioethanol, biodiesel and biogas. In the current fuel market the infrastructure already exists for the large-scale production and utilisation of bio-ethanol (Demirbas, 2007).

Currently bio-ethanol is made from primary food crops such as starch from corn or the sugar from sugar cane. A report by Chakrabortty, 2008 highlighted concerns raised in an Internal World Bank Study, and declared that primary biofuels (especially those made from corn) caused up to 75% increase in world food prices at that time. Although this figure was later re-evaluated in a follow-up study in 2010 (Baffes & Haniotis, 2010), the bio-ethanol market is still considered to affect crop prices. Aside from their effect on commodity prices, fuel crops produce large amounts of agricultural waste and also use arable land which then cannot be employed for the growth of food crops, thus reducing food supply. Although it is thought that the use of first generation bio-fuels has reduced greenhouse gas emissions compared to their equivalent fossil fuels, the extent to which this is the case is not clear (US Department of Energy, 2007). In view of these factors first generation bio-fuels have become increasingly contentious and many organisations

no longer support their use. This view has therefore led to the research and development of second generation biofuels made from sources such as corn stover, wood chips, other agricultural wastes or municipal solid waste (MSW).

The current research focuses on the precursor to second generation biofuels, sugar, which is made from the cellulosic fractions of the aforementioned wastes. These renewable resources are termed lignocellulosic feedstocks as they are primarily composed of cellulose, hemicellulose and lignin. Bio-fuels made from lignocellulosic waste have reduced greenhouse gas emissions over their whole life cycle as compared to primary fossil fuels, do not compete against food for land area, and if made from waste are exempt from the food vs. fuel debate. Lignocellulosic wastes are renewable, largely underexploited and abundantly available as raw materials for biofuel production.

A current market exists for bio-ethanol with modern engines having the ability to use petroleum containing up to 10% ethanol. It is possible to use higher ethanol blends and in Brazil many cars run on 85% ethanol (Balat, 2011; Hamelinck et al., 2005; Taherzadeh & Karimi, 2008). Ethanol fuel, however, is a low-value, high-volume product and facilities only producing this commodity often face a low return on investment (Bozell, 2008). Instead it is believed that integrating a fuel product into a bio-refinery concept is a more economically favourable option. In this process a number of bio-based products such as specialty chemicals, various fuels and pharmaceutical building blocks can be created from the renewable source of carbon. The diagram shown in figure 1.1-1, reprinted from the US National Renewable Energy Laboratory (NREL), demonstrates the potential products from an integrated sugar-lignin platform bio-refinery concept.

In reality this diagram could be expanded upon further to include processes such as pyrolysis and gasification for creating specialty fuels and other value added products from both the sugar and non-sugar components in the feedstock.

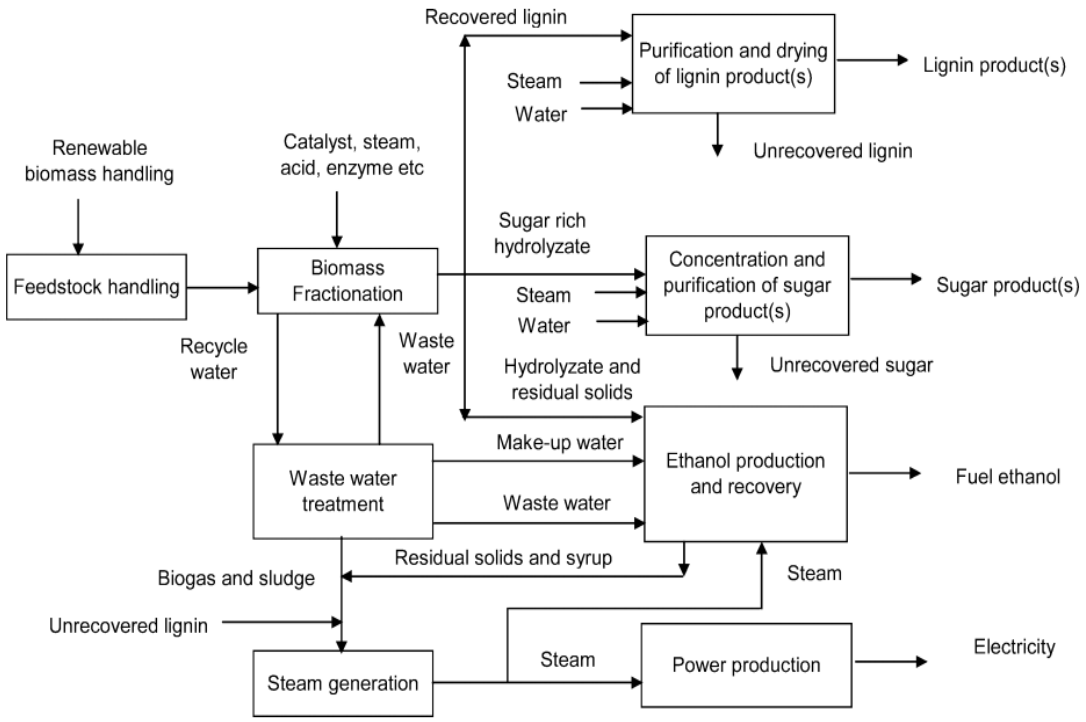


Figure 1.1-1. Concept for a sugar-lignin bio-refinery (McMillan, 2002)

Due to the low return on investment of bio-fuels it is important to optimise the way in which these are produced so that high sugar concentrations are obtained without the need for further, and sometimes costly, concentration steps. In addition a high substrate conversion is necessary to achieve the maximum value from the feedstock (Koppram et al., 2014; Modenbach & Nokes, 2013).

One of the major barriers in the bio-refinery concept is the technology gap that exists in converting the biomass into speciality chemicals that can replace those currently produced by the oil industry. As this concept continues to grow and commercialise, however, there will be a large incentive for increased research and development into this area. Furthermore, although specialty chemicals are required in conjunction to fuel to make the process economic it is the need for crude oil replacement that will help to drive development into bio-refineries (Bozell, 2008).

In this work the renewable lignocellulosic feedstock used was paper pulp derived from municipal solid waste (MSW). The material used was provided by Fiberight from their pilot plant in Lawrenceville, Virginia, USA. Locally collected MSW was processed to remove non-reactive products such as plastics, glass and metal

contaminants, thus increasing the cellulosic content of the material. The pulp was hydrolysed by the enzymatic route to produce monomeric sugars. The main focus of the research was to optimise enzyme usage and the obtainable sugar yield, with particular attention to methods that were potentially suitable for future large-scale application in industry within a bio-refinery concept.

1.2 Research aims

The aim of the work was to maximise the efficiency of producing a concentrated sugar solution from the cellulose (or glucan) component of municipal solid waste using commercial enzyme preparations.

Objectives:

- To determine the critical operational parameters that impact on enzyme performance and re-use potential.

- To develop methods for characterisation of waste material in terms of potentially extractable sugars.

- To carry out experimental work on hydrolysis with reference to its potential for further scale-up and industrial application, in particular at high solids concentrations

- To develop different approaches to maximising enzyme usage efficiency by exploring methods for enzyme recovery or minimising inhibition.

Chapter 2: Literature review

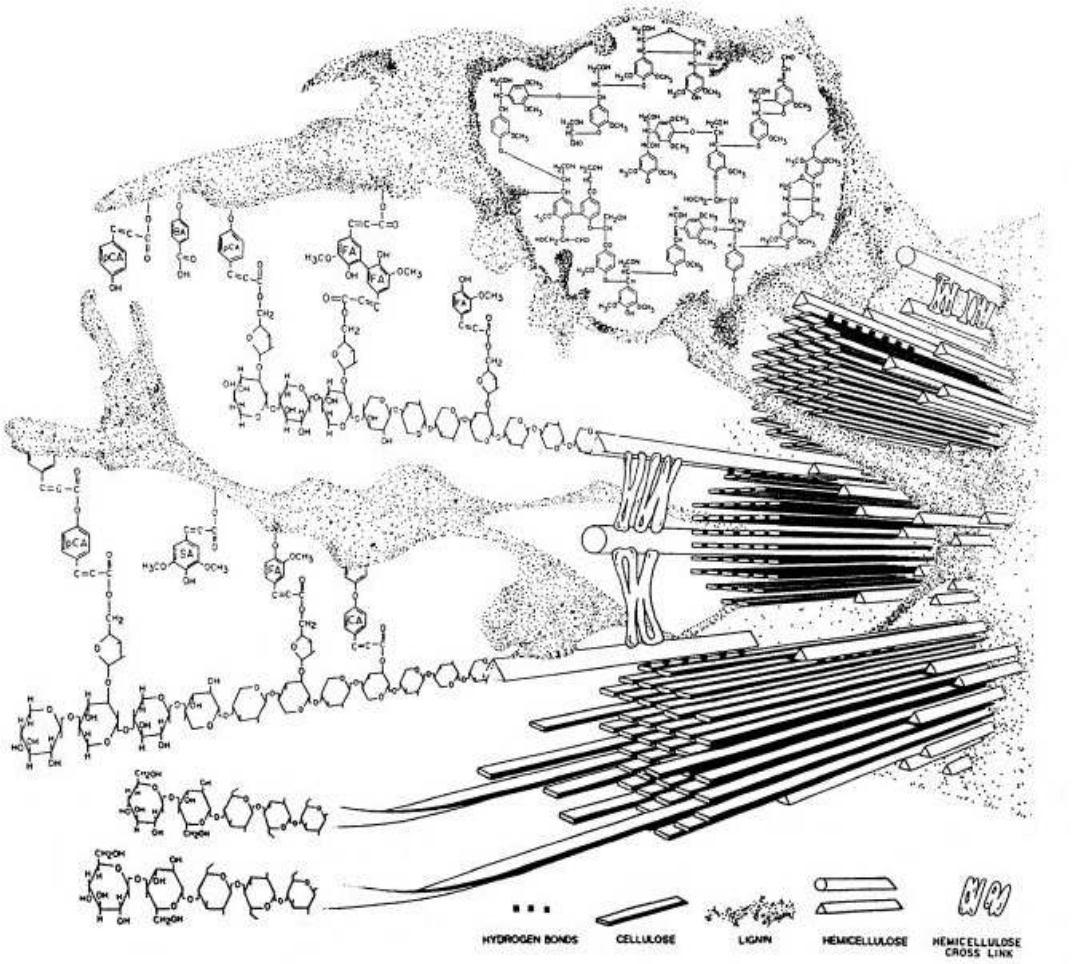
In a bio-refinery there are three processing steps analogous to petrochemical refineries, and these are: (i) inputs (supply) (ii) building blocks (separation) and (iii) outputs (conversion). The supply in a lignocellulosic bio-refinery process refers to the substrates used (e.g. MSW pulp). The separation step relates to the pre-treatment of the feedstock in order to access the key lignocellulosic components. This is followed by a breakdown of these components through enzymatic hydrolysis into the building blocks (i.e. sugars) which are then processed to produce the outputs (e.g. fuels and value-added chemicals).

In the introduction it was stated that the importance of the current work lies in optimising the production of sugars from lignocellulosic materials. Cellulose, the main component of lignocellulose, is the most abundant polymer on the Earth with an annual production of 10^{10} - 10^{11} tonnes (Walton & Blackwell, 1973). The potential feedstock for bio-refineries is thus enormous, and as such it is important to understand the composition of lignocellulosic material as presented below.

2.1 Bio-refinery Inputs

2.1.1 Composition of lignocellulosic material

Lignocellulosic materials contain a number of components from within the plant cell wall, including cellulose, hemicellulose, pectins, phenolics and proteins. The structure of the cell wall is depicted in Figure 2.1-1 (Bidlack et al., 1992).



Secondary cell-wall (CW) structure. Components are arranged so that the cellulose microfibrils and hemicellulosic chains are embedded in lignin. Specific linkages and components of non-core lignin are shown for a generalized grass secondary CW. Non-core lignin components include *p*-coumaric (*p*CA), ferulic (FA), *p*-hydroxybenzoic (BA), sinapic (SA), and cinnamic (CA) acids.

Figure 2.1-1. Structure of lignocellulose (Bidlack et al., 1992).

Cellulose microfilbrils are combined to make macrofibrils which are interspersed with hemicellulose and 'glued' together with lignin. The higher the lignin content of a material, the more rigid it is. For example, wood waste contains a large amount of lignin (approximately 22% for hardwood and 28% for softwood) and a comparatively small amount of hemicellulose (around 20%). Other materials such as wheat straw or barley straw contain less lignin (approximately 8%) but have a higher hemicellulose content (approximately 30%) (Olsson, 2005) and therefore have less rigidity. Cellulose fibres are chains of repeating glucose units and may be described as glucan chains. Hemicellulose is a branched polymer with either a xylan or a mannan backbone and branches containing glucose, galactose, arabinose,

xylose or mannose. Lignin is made up of a number of different polyphenols (Saha, 2003).

In addition to the carbohydrate component of the substrate, lignocellulosic materials can also contain extractives such as waxes, oils and proteins. These extractives may affect digestibility depending on their type and concentration. It has been shown that these compounds make it difficult to obtain a complete mass balance for compositional analysis on a range of forest residues (Burkhardt et al., 2013): it was observed, however, that mild alkali pre-treatment resulted in mass balance closures of 95%. Extractives have been removed using solvents such as acetone prior to lignin removal (Siqueira et al., 2013), and this has been shown to increase cellulose digestibility, possibly due to an increase in cellulose fibre accessibility of sugarcane bagasse used. In addition, solvent extraction may present a pathway for recovery of value added compounds from the biomass prior to it being hydrolysed.

2.1.2 Lignocellulosic wastes

Although the composition given above is typical of lignocellulosic plant material, it relates most closely to substrates such as agricultural waste. Due to the complex nature of MSW pulp and the many processing steps that the various components have been through, it is not clear whether this substrate will have the same type of structure, and it may be of interest to investigate this as it has not been reported to any significant degree within literature as explained in section 2.2.7.2.

2.2 Bio-refinery separation processes

The following section outlines the separation processes in a bio-refinery. Due to the complex nature of lignocellulosic materials many potential barriers need to be overcome to access the substrate. Furthermore, as there are a large variety of potential input materials, various pre-treatment steps can be undertaken in order to access the key components in the substrate.

2.2.1 Barriers to enzymatic hydrolysis from lignocellulosic material

Lignin and to some extent hemicellulose are barriers to obtaining cellulose, and in turn glucose, enzymatically from the cell wall structure. It has been observed that lignin and hemicellulose present physical barriers which stop enzymes from accessing the cellulose (Berlin et al., 2006; Rahikainen et al., 2011; Sewalt et al., 1997; Siqueira et al., 2013; Várnai et al., 2010). These 'roadblocks' along a cellulose fibre intercept the main cellobiohydrolase enzyme, which has a processive mode of action. In addition lignin is known to bind enzymes unproductively, with different types of lignin causing differing levels of inhibition (Nakagame et al., 2010; Rollin et al., 2011).

In recent years the role of hemicellulose as an inhibitor of enzymatic hydrolysis has become clearer. In hardwoods it has been found that xylan shows a two-fold interaction, one tightly associated with cellulose and one more loosely bound and easier to remove. In softwoods there tends to be a strong association of cellulose and gluco-mannan (Dammstrom et al., 2009; Penttilä et al., 2013). In woods and other agricultural crops this closely-associated xylan is believed to inhibit cellulose degradation, as hydrolysis becomes limited without the presence of xylanase enzymes. It has been shown in the hydrolysis of corn stover, sweet sourgum bagasse and steam pretreated lodgepole pine that when low cellulase loadings are used, the overall cellulose hydrolysis is low; however a marked improvement is seen with the addition of xylanases (Hu et al., 2011). It has also been shown that, even when the amount of xylan present in the lignocellulosic substrate is very small (0.34%), the addition of xylanases can result in a 12% increase in cellulose hydrolysis (Várnai et al., 2010). Addition of purified hemicellulose from birchwood to wheat straw also limited the efficiencies of the cellobiohydrolase enzymes, possibly due to unproductive binding of the enzymes. Soluble xylans presented slightly more inhibition than insoluble xylans (Zhang et al., 2012a) and xylose and its oligomers have been shown to inhibit the hydrolysis of pure glucan (Kumar & Wyman, 2009b).

Other factors that inhibit enzyme hydrolysis of lignocellulosic material are low surface area and high cellulose crystallinity (Hendriks & Zeeman, 2009; Sun &

Cheng, 2002b). The more crystalline the cellulose, the harder it is for the enzymes to access individual glucan chains (Arantes & Saddler, 2011). To overcome these barriers, pre-treatment processes are used to increase the number of amorphous regions in the cellulose, thus increasing the number of access points for the enzymes.

A number of pre-treatment options are currently used to achieve a decrease in crystallinity, removal of lignin and solubilisation of hemicellulose to produce a readily hydrolysable substrate. The following sections discuss the various pre-treatment options, followed by a review of enzyme hydrolysis.

2.2.2 Physical and physico-chemical pre-treatments

Lignocellulosic substrates are recalcitrant in nature due mainly to the resistance of lignin to biological degradation. As each substrate has its own unique chemical and physical properties, pre-treatments vary for each of the major classes of feedstock. The most common pre-treatment options are physical treatments such as milling and irradiation; and physico-chemical treatments such as explosion (either with or without catalysts such as sulphur dioxide or ammonia), alkali, acid, gas, liquid hot water (LHW) cooking, oxidising agents and solvent extraction treatments. Hemicellulose, unlike cellulose, is a branched polymer that is easily hydrolysed and this often occurs during a physico-chemical pre-treatment step (Bidlack et al., 1992; Brett & Waldron, 1996)

Physical treatments like milling can be used to reduce not only the size of particles but also the degree of crystallinity within cellulose. Examples include ball milling, hammer milling and colloid milling (Taherzadeh & Karimi, 2008). These treatments are normally used in conjunction with other pre-treatment options as they are not effective in solubilising hemicellulose or removing lignin.

Several studies have looked at physical refining techniques that can open up the fibres rather than simply decreasing the particle size. Milling techniques which have shown positive results are PFI milling (named after Papirindustriens Forskningsinstitutt – The Norwegian Pulp and Paper Research Institute) or valley

beating. These techniques aim to increase internal fibrillation and swelling or opening of fibres, rather than solely cutting the fibres. Due to the high energy costs associated with these techniques, however, they have only been carried out on a laboratory scale. Milling strategies of this type have been used on Kraft wood pulps of varying lignin contents (5-20%) where it was found that for hydrolysis of the higher lignin pulps the enzyme loading could be reduced by 32% to obtain a sugar conversion of 91% (Jones et al., 2013). Although this is a promising result, the hydrolysis was carried out at 5 % w/v solids content which is too low for an industrial-scale process. These laboratory-scale milling processes have a similar action to disk milling, which could be applied industrially: and some studies have used disk milling as a pre-treatment for a high solids hydrolysis process (Fujii et al., 2013). From the literature, however, it is currently not clear whether milling or disk refining would be a feasible prospect for increasing conversion in a high solids hydrolysis process, and whether the increased energy costs associated with milling are outweighed by the benefit it provides.

Irradiation has been shown to improve enzymatic digestibility of lignocellulosic materials, and can also be combined with other treatment options. As with milling, the cost of this treatment it quite high, although other energy-intensive techniques such as ultrasound are now being used as a pre-treatment in biogas production as the extra gas produced compensates for the increase in energy and financial costs (Cui & Jahng, 2006; Wang et al., 2005; Wang et al., 1999) .

Physico-chemical treatments such as steam and steam explosion are a popular mechanism for removing most of the hemicellulose in lignocellulosic waste and opening up the polymers (Kurabi et al., 2005; Varga et al., 2004). Steam explosion is often used in combination with various chemicals and gases to increase its efficiency, and addition of sulphur dioxide (SO_2), carbon dioxide (CO_2), dilute acids or carboxylic acids can improve yields of both cellulose and hemicellulose (Hendriks & Zeeman, 2009; Hoyer et al., 2010; Tu et al., 2009b). Another popular treatment is ammonia fibre explosion (AFEX) which uses alkaline liquid to lower or modify the lignin fraction of the feedstock while leaving the cellulose and hemicellulose intact. One major advantage of AFEX is that fermentation inhibition products such as

furans are not formed. The disadvantages include minimal solubilisation of hemicellulose, and the need to recycle ammonia to make this treatment cost effective (Lau & Dale, 2009; Sun & Cheng, 2002b; Taherzadeh & Karimi, 2008). As well as ammonia, other alkaline solutions such as sodium or calcium hydroxide have been successfully used (Carrillo et al., 2005; Zhang et al., 2011a). This treatment is popular in the paper pulping industry (Kraft process).

A simple yet effective method is a liquid hot water (LHW) cooking treatment, which has been used for several decades in the pulp industry. Water is used under high pressure, and helps to swell the cellulose and remove the hemicellulose and some lignin. This treatment uses no additional chemicals and there is no requirement for corrosion-resistant materials (Franceschin et al., 2010; Hendriks & Zeeman, 2009; Kim et al., 2009). The treatment can be combined with AFEX or alkaline cooking to increase lignin removal, or with an oxidation process in which lignin is cleaved and hemicellulose is converted to monomers (Taherzadeh & Karimi, 2008).

For high lignin feedstocks such as hardwoods, organic or aqueous-organic solvents such as alcohols, esters, ketones, glycols, organic acids, phenols and ethers can be utilised. Use of these solvents is known collectively as the organosolv process (Alvira et al., 2010; Taherzadeh & Karimi, 2008). This treatment is attractive as it produces a pure lignin by-product. It is currently expensive, however, due in part to the fact that the solvents used are highly volatile; and it is therefore important to ensure there are no leaks in the treatment vessels because of the inherent fire and explosion hazards. Also as the solvents are expensive they must be recycled and reused (Zhao et al., 2009).

Lignin has also been removed by the use of sodium chlorite and acetic acid in laboratory-scale experiments with a variety of feedstocks from sugar cane bagasse to softwood pulps (Mooney et al., 1998; Siqueira et al., 2013); this removal has shown positive effects in increasing hydrolysis yields. However the effectiveness of lignin removal and its positive correlation with enzymatic digestibility depends highly on the type lignin in question. Some lignins such as those found in hardwood

are not as recalcitrant as softwood lignins, and are therefore less detrimental (Ramos et al., 1992).

Another liquid treatment that has reported success in literature is ionic-liquid (IL) solvation. The use of ILs leads to the disruption of the 3D network structure of lignin, cellulose, and hemicellulose which can allow increased access to the fibres for enzyme hydrolysis. The uses of ILs can provide pure lignin as a bi-product for a bio-refinery. The IL used would most probably need to be removed after lignin extraction as it is known that ILs can denature proteins and thus, are detrimental for a subsequent cellulose hydrolysis (Mora-Pale et al., 2011). At high temperatures (90 ^{o}C +) there are a range of imidazolium-based ILs that can partially or completely dissolve whole wood pulps (Kilpeläinen et al., 2007; Zavrel et al., 2009). Due to the cost and extremely large volumes required for biomass pre-treatment the use of ILs is currently not feasible as a pre-processing option. There is, however, the potential for IL recycle and further reduction in costs as the technology develops which could lead to their commercial use in the future (Mora-Pale et al., 2011).

Often two treatment options are used in conjunction with each other to help decrease crystallinity and remove lignin, as many of the pre-treatment options do not simultaneously achieve both objectives.

Pre-treated substrates can be imaged and compared to the original feedstock using scanning electron microscopy (SEM) or atomic force microscopy (AFM) to determine their effectiveness. Studies have clearly shown the redistribution of lignin on wheat straw after hydrothermal processing followed by lignin removal by sodium chlorite (Kristensen et al., 2008).

2.2.3 Enzymes for hydrolysis

Once the cellulose has been treated and accessibility to it has been increased, it can be hydrolysed to monomeric sugars. Hydrolysis can be achieved by acid digestion (Taherzadeh & Karimi, 2007), although this can produce inhibitors in the conversion stages of a bio-refinery and is not regarded as an environmentally friendly option. Hydrolysis is therefore most commonly carried out using enzymes. Although this

process is currently expensive, advances in enzyme production and the possibility of enzyme reuse (Lu et al., 2002; Tu et al., 2007b) are reducing the costs, which are expected to fall further in future (Cherry & Fidantsef, 2003; Percival Zhang et al., 2006). The price of enzymes, however, is still estimated to be at least 15% of the total ethanol production cost (Humbird et al., 2011). Another strategy for reducing costs is on-site production of enzymes which would reduce the expense associated with purification and stabilisation of enzyme preparations, as well as allowing cultivation of enzymes mixtures that are better suited to the feedstock being used (Jørgensen et al., 2007). It is recognised that enzymatic hydrolysis is one of the major economic bottlenecks for the conversion of lignocellulose to monomeric sugars, and as such many efforts are being made to reduce its costs (Klein-Marcuschamer et al., 2011; Steele et al., 2005; Tu & Saddler, 2010; Tu et al., 2009a).

The most commonly used enzymes are those produced from the filamentous fungus *Trichoderma reesei*. This fungus, which excretes extracellular cellulolytic / hemicellulolytic enzymes, was discovered during World War II, in the south Pacific, where it was found to be responsible for breaking down cotton tents and clothing (Hamelinck et al., 2005). The fungus excretes a large variety of enzymes including cellulases (cellobiohydrolases, endoglucanases, β-glucosidases and gluco-oxidases) and hemicellulases (eg; xylanases and mannases). The cellulases work with a marked degree of synergy to break down crystalline cellulose, whereas the hemicellulases work to break down obstructing hemicellulose chains. *T. reesei* has genes that encode more than 39 cellulolytic and hemicellulolytic enzymes (Ouyang et al., 2006). The two most commonly used commercial preparations are those from Novozymes (Cellic C-Tec family) and Genencore (Accelerase).

T. reesei does not naturally produce a large amount of β-glucosidase and a lack of this can lead to an accumulation of cellobiose, which causes severe inhibition of cellulases. Therefore commercial mixtures are often supplemented with β-glucosidase from *Aspergillus Niger* and many researchers use β-glucosidase supplementation in their work (Kumar & Wyman, 2009b; Mesa et al., 2010; Qi et al., 2011).

The following section gives a description of the four types of cellulase enzymes and an overview of their structure and mechanism. The diagram in figure 2.2-2 shows how these all work together.

Endo and exo glucanases (EGs) work to break up the individual cellulose micro fibres so that there are more open ends for the cellobiohydrolases (CBHs) to work on. The two forms of EG have different structures to cut up a cellulose chain. The exoglucanases start at either end of a glucan chain and have a tunnel structure for the glucan chain to pass through. Endoglanases contain an open cleft and use a scissor type action (Davies & Henrissat, 1995). More recently, however, it has been suggested that the exoglucanases are actually forms of cellobiohydrolases as described below (Skovgaard et al., 2014; Sun & Cheng, 2002a).

There are several different cellobiohydrolases and these work with a processive mode of action. The enzyme has a binding region or Carbohydrate Binding Module (CBM) which is joined to the catalytic region by a glycosylated linker as shown in figure 2.2-1. This enzyme contains a large catalytic domain (left), linker (middle single strand), and cellulose binding module (right small domain). A cellodextrin (repeating cellobiose units) strand is shown peeled from the surface of the cellulose and is passing through the catalytic tunnel of Cel7A. The solvating water and the lower section of the cellulose fibre are not shown. Depending on the specific cellobiohydrolase it may bind to either the reducing or the non-reducing end of the cellulose fibre, using the CBM as a guide. The glucan chain passes through a tunnel in the catalytic region of the enzyme (Davies & Henrissat, 1995; Michael Crowley, 2011).

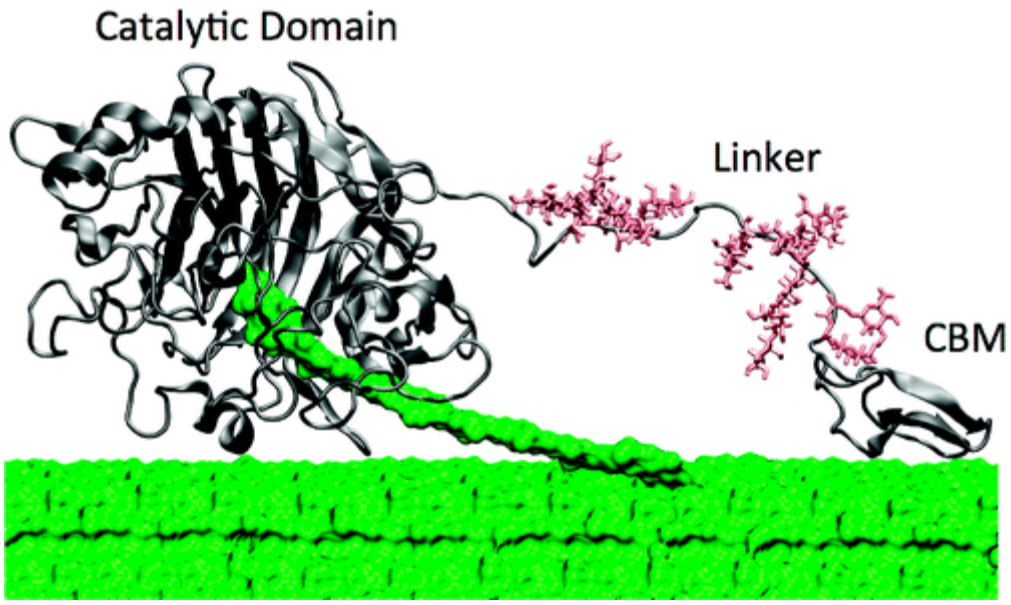


Figure 2.2-1. Model of Cellobiohydrolase I (Cel7A) from *Trichoderma reesei* on a cellulose surface. (Beckham et al., 2010)

The cellobiohydrolases produce cellobiose and cellotriose molecules, which are then cleaved to monomeric glucose by β-glucosidase. The β-glucosidase activity is extremely important as it is known that cellobiose has a large inhibitory effect on cellulase enzymes. Similarly β-glucosidase is inhibited by its product glucose, and product inhibition is therefore a significant factor to consider when producing high sugar concentration slurries (Andric et al., 2010; Smith et al., 2010; Xiao et al., 2004). β-glucosidase is known to lose its activity faster than the CBHs and EGs and it has been shown that after 144 hours of enzyme hydrolysis on avicel (pure cellulose) that β-glucosidase activity dropped by 75% (Varnai et al., 2011).

As β-glucosidases do not have a cellulose binding domain it has been thought that, of all the enzymes within the cellulase matrix, these would be easiest to recycle. A recent study by Haven & Jorgensen (2013) found that, while in earlier commercial enzyme preparations it was indeed the case that the β-glucosidases were free in solution, in newer preparations such as C-Tec2 there is a portion that is bound to the substrate rather than free. It was also shown that these β-glucosidase enzymes in C-Tec2 adsorb to lignin to a significant degree, but although the enzymes are bound they still seem to be catalytically active (Haven & Jorgensen, 2013).

In recent years a new enzyme complex known as GH 61 (or AA9) has been discovered. It was originally placed in the glycosyl-hydrolase family due to its weak hydrolytic activity, although it is now thought that this enzyme is an oxidative metallozyme and works as a catalyst to increase cellulase activity (Quinlan et al., 2011). This enzyme complex requires oxygen to operate and a redox potential of 1.2 eV. Using this complex, Harris et al., 2010 observed a 2-fold decrease in the amount of cellulase required to hydrolyse a given amount of substrate. Furthermore GH 61 has been shown to increase hydrolysis activity when used on pre-treated biomass, but not on pure substrates such as avicel due to the absence of redox-active co-factors (Harris et al., 2010; Horn et al., 2012). It is believed that lignin can act as this co-factor provided it is in a suitable form (Dimarogona et al., 2012). Ascorbic acid can also be added to a reaction medium as a co-factor (Cannella et al., 2012; Horn et al., 2012), to determine whether or not a substrate has a naturally occurring co-factor. Thus if the addition of ascorbic acid results in an increase in enzyme activity, this indicates that a suitable co-factor is not present.

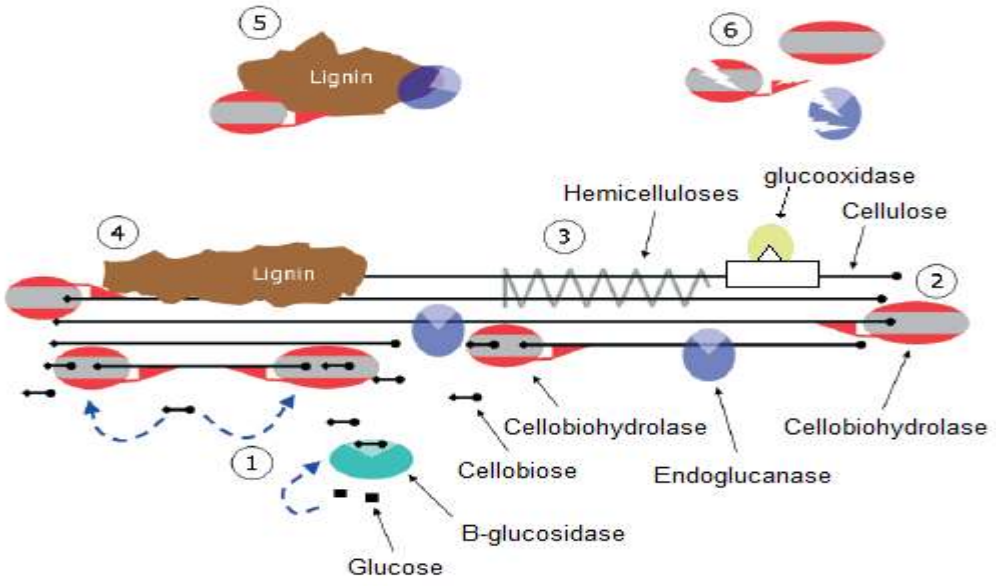


Figure 2.2-2. Simplified diagram for mechanisms of the various enzyme complexes (Jørgensen et al., 2007). 1) Product Inhibition of CBH and β-glucosidase by glucose and cellobiose. 2) Unproductive binding of CBH. 3 & 4) Enzyme path blockage by lignin and hemicellulose. 5) Non-specific binding of enzymes to lignin. 6) Denaturation and loss of enzyme activity caused by low thermal tolerance, mechanical shear or proteases.

Although advances in enzyme technology and cultivation are leading to more stable enzymes with higher specific activity, the efficiency of cellulases is still low. For example, amylase which is used to digest starch in primary bio-ethanol processes has an activity up to 100 x greater than the specific activity of cellulases (Bessler et al., 2003; de Castro et al., 2010).

2.2.4 Enzymatic hydrolysis

Enzyme hydrolysis of lignocellulosic material using a cellulase mixture containing the enzymes described above typically proceeds as shown in figure 2.2-3, where the rate of hydrolysis decreases with time and the process usually ends before complete hydrolysis of the substrate. This plateau in hydrolysis normally occurs within 24-72 hours depending on the substrate concentration used. Although it is possible to get 100% conversion at low solid concentrations (less than 5% TS), the percentage conversion decreases as the solids content increases.

Currently the most widely researched industrial bio-refinery product is bio-ethanol. In the production of this it is necessary to achieve high sugar concentrations from hydrolysis so that fermentation results in a high enough ethanol titre for economically feasible distillation. Larsson and Zacchi., 1995 showed that the costs of continuous distillation do not rise significantly when ethanol concentration increases above 40 g kg^{-1} or 4% wt (Larsson & Zacchi, 1996). It has been stated that a 4% ethanol solution requires an 8% sugar solution, which is equivalent to an initial feedstock total solids content of approximately 20% TS for most lignocellulosic substrates (Larsen et al., 2008). In addition to reduced distillation costs, working at high solids content is considered to also decrease the capital investment, heating demand and water requirement.

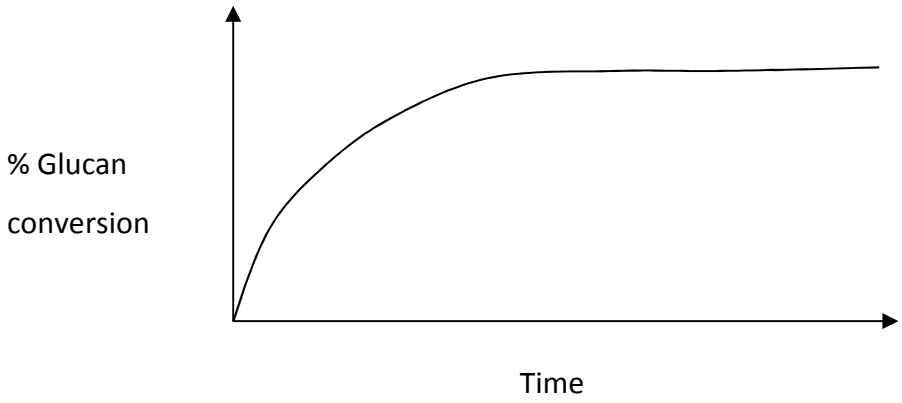


Figure 2.2-3. Typical hydrolysis curve where the initial rate of reaction is fast and conversion then plateaus to a maximum level.

As the solids concentration in a lignocellulosic in slurry increases there is less free water in the system and the materials can form networks and flocs that are hard to break and mix. Different mixing strategies have been proposed to solve this rheological problem. Zhang et al., (2009) demonstrated successful conversion of 20% TS slurries of an unbleached hardwood kraft pulp (alkali treated) and of an organosolv pre-treated poplar using a peg mixer. In a shake flask study they found that that the higher the substrate concentration (from 2%-20% TS), the longer the mixture took to liquefy in a shaking incubator. This finding is backed up by results from experiments conducted at the National Renewable Energy Laboratory (NREL) in the US (Roche et al., 2009), which showed very slow liquefaction for high TS slurries of pre-treated corn stover when subject to shaking alone. Freefall mixing was found to be a much more effective means for liquefaction, as this resulted in fewer mass transfer limitations and less localised product build-up. Most importantly they used a low enzyme dosing (20 mg protein g^{-1} cellulose) and demonstrated consistency in the results when scaling up the process from 125 ml to 2 L.

Traditional continuously-stirred tank reactors (CSTRs) have been widely used for mixing, with various impellor configurations including helical, Rushton, plate & frame and double curve blade. Helical impellers have been found to perform better than standard Rushton impellers, resulting in better ethanol productivity during simultaneous saccharification and fermentation and shorter liquefaction times

during hydrolysis (Modenbach & Nokes, 2013). There is evidence that fed batch operation in CSTRs can reduce the viscosity of the substrate, making mixing easier and thereby lowering the energy requirement. A number of fed batch studies have been carried out, with differing results. Studies performed with corn stover at 25% total solids found that fed batch and batch process produced the same sugar yield; however the energy input required for the fed batch process was lower (Hodge et al., 2009). Some studies show fed batch is a worse system for hydrolysis than batch (Chandra et al., 2011; Rosgaard et al., 2007), while others suggest it is more beneficial (Kuhad et al., 2010). It is likely that the effectiveness of fed-batch is substrate specific and is also dependent on when substrate and enzymes are fed into the system.

2.2.5 Inhibition of enzyme hydrolysis

Although higher substrate concentrations can give a higher sugar concentration in the hydrolysate, it has been shown that increasing the solids concentration results in a drop in conversion efficiency which is commonly referred to as 'the solids effect'. Observing this effect on filter paper suggests that it is not related to lignin or hemicellulose content. It has also been shown that product inhibition is not the only cause of the solids effect, and increasing the enzyme concentration does not necessarily alleviate the problem (Kristensen et al., 2009b). Instead it has been proposed that high solids concentrations prevent enzymes from adsorbing to the lignocellulosic material and this may be due to solids or product accumulation interfering with the CBM of the cellobiohydrolases (Kristensen et al., 2009b). In addition, the increase in viscosity of the high solids mixture may lead to a lower three-dimensional (3D) diffusion in the reaction mixture. Agitating a solution can increase 3D diffusion, and experiments by Wang et al. (2011a) showed that the solids effect can be somewhat alleviated by agitating a solution, although not completely overcome (Wang et al., 2011a).

This drop in conversion efficiency was partially counteracted by the approach tested by Xue et al., 2012 who used a split and thicken enzyme addition approach where half the enzyme loading was added at 5% substrate consistency, followed by

thickening through filter pressing and addition of the remainder of the enzymes when the substrate was at 20 % solids. This approach resulted in an increased sugar yield of 37 g L^{-1} sugar as compared to adding the enzyme directly to 20% initial solids which yielded a total sugar concentration of 84 g L^{-1} in the hydrolysate (Xue et al., 2012b). This study, however, was carried out with a relatively old enzyme preparation and thus it is not certain that this approach would work with newer enzyme preparations.

Product inhibition is a dominant factor during hydrolysis. It has been found that cellobiose is a strong inhibitor of the CBHs and it was thought that a build-up of glucose inhibits β-glucosidase, which results in the accumulation of cellobiose. Xiao et al., 2004 showed, however, that the whole enzyme system is affected by glucose and this could be related to synergistic effects rather than to the individual enzymes (Xiao et al., 2004). By removing glucose from the system, researchers have shown increased enzyme activity. Yang et al., 2010 & 2011 showed an 85% hydrolysis yield from corn stover in 30 hours using a 3-stage hydrolysis system in which the product sugars were removed by washing at 9, 18 and 30 hours to alleviate the effect of sugar inhibition. Fresh enzyme was added to compensate for the enzyme loss during the washing steps. Product inhibition has also been studied with C-14 labelled cellulose, and it has been shown that cellobiose inhibition decreases with increased temperature. This finding is important, as it suggests that separate hydrolysis and fermentation (SHF) is potentially a better strategy than simultaneous saccharification and fermentation (SSF) (Teugjas & Valjamae, 2013) as it is carried out at 50 ^{o}C rather than 30 ^{o}C, a more detailed desciption of SHF and SSF can be found in section 2.3-1.

It has been argued that the product inhibition effect is not solely caused by the sugars themselves, but also by the reduction in water availability due to the increase in soluble compounds. In high solids applications a large amount of sugar is produced; Roberts et al., 2011 showed that adding mannose, an isomer of glucose, to a pure cellulose hydrolysis resulted in a decrease in hydrolysis yield even though mannose is not an inhibitor of β-glucosidase activity. This suggests that hydrolysis is limited by a process other than end product inhibition. Similarly Selig et al., 2012

suggest that although insoluble components do constrain water they do not affect enzyme hydrolysis. Instead, the addition of soluble species has a much greater effect on water availability and this then constrains enzymatic hydrolysis. It was stated that water availability around soluble compounds is low and as the concentration of these species increases they draw water away from the insoluble species, causing a decrease in the water available for hydrolysis at the insoluble cellulosic solid (Selig et al., 2012).

Thus, it is important to keep the concentration of water soluble compounds low, especially during the initial part of hydrolysis when the enzyme activity is still high. Furthermore, in high solids hydrolysis the quantity of commercial enzyme used adds a significant amount of soluble compounds, such as stabilisers, to the liquid matrix. Increasing the enzyme loading is only effective in improving cellulose conversion up to a point, and it is believed that this is because of the increase in soluble compounds rather than jamming of the enzyme. Jamming is said to occur when the processive action of the cellulase enzymes is blocked by another enzyme in the same path (Bommarius et al., 2008; Xu & Ding, 2007).

It has been suggested that the rate limiting step of enzyme hydrolysis by CBHs is the release of the processive CBH from the end of a cellodextrin (glucan) strand. Insights from Bu et al., 2012 indicate that the CBH complex is most stable when a cellobiose is bound in the active site, hence limiting the processive action of bound cellulases (Bu et al., 2012). This could also explain why it has been found that a proportion of bound enzyme can only be unbound from the substrate when the pH is raised above 9, as this causes the enzyme to unfold resulting in its release from the substrate (Yu et al., 2012).

2.2.6 Increasing enzyme efficiency

2.2.6.1 **Preventing the unproductive binding of enzymes**

As cellulase enzymes are costly and have a relatively low specific activity in comparison to other similar enzymes (e.g. amylases) it is critical that the enzymes are used to their maximum capacity. Lignin is a major obstacle in enzymatic

hydrolysis as it not only presents a physical barrier, but has also been seen to form very strong non-specific bonds with enzymes which can cause them to lose their shape and activity, thereby rendering them ineffective (Berlin et al., 2006; Rahikainen et al., 2011).

This non-specific binding can be overcome to a degree by using compounds which interact or bind to the lignin, making it unavailable for the cellulase enzymes. It has been shown that nitrogen-containing compounds have strong affinity for lignin. Sewalt et al., 1997 demonstrated increased digestibility of lignocellulosic material after incubation with Polyethylene glycol (PEG), Polyvinylpyrrolidone (PVP), gelatin and ovalbumin. Similarly their experiments showed that treatment with ammonia increased the nitrogen (N) concentration within a lignocellulosic sample, but there was no observable increase of N in the non-lignin control cellulose. This ammonia addition also led to increased digestibility of the lignocellulosic substrate (Sewalt et al., 1997). Other studies advocating PEG as a means of increasing enzyme activity include Cannella & Jørgensen, (2013) who used PEG 3000 in conjunction with high solids hydrolysis and SSF of wheat straw at 30% dry matter with Cellic C-Tec2. Their results showed that ethanol yields were higher when the PEG additive was used in SHF, SSF and presaccharification SSF, where SSF is preceded with a short hydrolysis at the optimum hydrolysis temperature before adding yeast and dropping the temperature (Cannella & Jørgensen, 2013). Borjesson et al., 2007 also advocated PEG for enhancing hydrolysis efficiency with softwood substrates. The mechanism by which improvement is achieved is thought to be hydrophobic interactions between PEG and lignin, which prevent unproductive binding of enzymes to the lignin (Borjesson et al., 2007).

Experiments by Haven & Jorgensen, 2013 investigating the adsorption behaviour of β-glucosidase from Cellic C-Tec2 showed that the addition of PEG increased the amount of β-glucosidase free in solution, as the PEG reduced the hydrophobic interactions between lignin and β -glucosidase. It was noted that the plastic bottles used in the experiment also could produce hydrophobic interactions with the enzymes, and thus PEG addition also prevents this from occurring. Although there was more β-glucosidase free in solution with the addition of PEG, the paper is

unclear as to whether there was increased activity due to this. It was stated, however, that although much of the β-glucosidase activity was bound to hydrophobic surfaces without the addition of PEG the loss of catalytic activity was only 15%, suggesting that even though enzymes may be unproductively bound they still maintain a high degree of catalytic activity (Haven & Jorgensen, 2013). Further to this, work carried out by Le Costaouëc et al., 2013 showed that it was the hydrophobic region on the carbohydrate binding domain of the enzyme that is unproductively bound to lignin, rather than the core enzyme. It was also shown that PEG addition was of greater benefit to enzymes with a CBM than those without (Le Costaouëc et al., 2013).

Other surfactant additives such as Tween 20 and Tween 80 have also been used with success in achieving higher glucose conversion on softwood substrates (Eriksson et al., 2002; Tu et al., 2009b). A cost analysis carried out for the use of surfactants during enzyme hydrolysis indicated that there could be saving of up to 66% if both enzyme and surfactant recycling were used in a lignocellulosic bio-ethanol process (Tu & Saddler, 2010). Xue et al., 2012a found that although conversion of softwood substrates was increased with Tween addition, this was not the case for hardwood, indicating that surfactant action is substrate specific. In addition to single surfactant molecules, surfactant micelles have also been shown to increase enzyme recyclability with corn stover (Eckard et al., 2013)

Bovine serum albumin (BSA), another additive that has been used by researchers, has also been shown to increase substrate conversion. When BSA was added prior to hydrolysis with both avicel and lignocellulosic substrate, it was observed that hardly any BSA was adsorbed to the control avicel material which contained no lignin; whereas there was a large absorption on the lignocellulosic feedstock corn stover. This again indicates the potential of the additive in preventing unproductive binding to lignin. Yang & Wyman, 2006 found that addition of a 1% BSA solution increased cellulase activity 2-fold and β-glucosidase activity 14-fold, resulting in a reduced need for enzyme supplementation during subsequent simultaneous saccharification and fermentation (SSF) for the production of ethanol (Yang &

Wyman, 2006). This result also strengthens the argument that β-glucosidase has a higher affinity than the other cellulose enzymes for unproductive binding.

2.2.6.2 **Lignin recalcitrance**

As discussed above, the effectiveness of an additive is substrate and/or possibly lignin specific. Weiss et al., 2013 conducted solid recycling experiments to recycle the enzymes bound to the solid fraction of the hydrolysate. These were carried out on corn stover and, although each additional recycling round resulted in the amount of lignin increasing, this corn stover lignin did not seem to have a detrimental effect on hydrolysis (Weiss et al., 2013).

It has been shown that lignin associated with calcium reduces the propensity for enzymes to bind to that lignin (Barsberg et al., 2013). Non-productive binding has also been shown to decrease (thereby increasing enzyme activity) with the addition of calcium or magnesium ions, whereas heavy metals such as copper and iron decrease enzyme efficiency (Liu et al., 2010). The structure of lignin and the presence of specific ions in a substrate also affect enzyme binding (Nakagame et al., 2010).

It has been noted that various pre-treatments leave residual lignin in a specific form. Often, although alkali or LHW treatment may reduce the proportion of lignin present, this lignin is more detrimental to enzyme hydrolysis than residual lignin from acid treatment or steam explosion (Rollin et al., 2011). Pan et al., 2005 investigated this further to determine which treatments could selectively remove lignin with high affinity for cellulases, in order to enhance the enzymatic conversion of steam exploded Douglas fir. They performed cold and hot alkali treatment (4 ^{o}C and 100 ^{o}C respectively) with 1% sodium hydroxide and found that cold sodium hydroxide treatment removed the portion of lignin which had a strong negative impact on enzymatic hydrolysis.

Likewise Qi et al., 2011 evaluated the enzyme recycling potential of acid and alkali treated wheat straw. Using a recycling strategy akin to that of Tu et al., 2009a, where both bound and free cellulases were recovered, they showed that it was

possible to reuse enzymes for four rounds of hydrolysis. A 67% percentage conversion of alkali treated straw was achieved in the final round, whereas conversion for the acid treated sample was only 17% using 2 %TS in each round. This result again provides evidence that lignin is a major barrier for hydrolysis and enzyme recycling, but also that alkali treatment can remove the portion of lignin which readily associates with cellulases.

2.2.6.3 **Enzyme recycling**

It has been demonstrated that cellulases are quite robust and have been shown to give up to 80% yield after four rounds of hydrolysis at 2 %TS (based on cellulose) when used in conjunction with surfactants (Tu et al., 2007b). This experiment was carried out by recycling the liquid portion of the hydrolysate.

Using low substrate concentrations (2-6% TS) Ramos et al., (1993) showed that it was possible to carry out 5 hydrolysis rounds by using recycling enzymes. The cellulose enzyme showed good stability at 50 ^{o}C for hydrolysis rounds of 48 hours. In another study an 80% decrease in activity was observed after cellulases were incubated at 45 ^{o}C for 15 days (Ishihara et al., 1991). It should be noted that the quoted studies were carried out in 1991 and 1993, and since this time there have been vast improvements in the stability of commercial cellulase mixtures.

Enzyme recycling has also been achieved using filtrate recycling. Xue et al., 2012 studied various strategies to optimise enzyme recycling and carried out 6 recycle rounds, with the hydrolysate liquid, to reach a steady state system. By incubating fresh substrate at 5% TS with the liquid portion of the hydrolysate and then washing away excess sugars they were able to increase the weight loss of their softwood substrate in the final round by up to 15% using an enzyme dosing of 30 mg g^{-1} pulp with surfactant addition (Xue et al., 2012a). As with the experiments by Yang et al. (2010 & 2011) it was necessary to wash away sugars to observe a higher rate of enzyme activity (Yang et al., 2011). This wash, however, meant a loss of sugars which were not reclaimed.

Recovery of enzymes has been achieved using ultrafiltration, either after hydrolysis or continuously within a membrane bio-reactor set-up to remove inhibitory end products (Mores et al., 2001; Zhang et al., 2011a). The problem with many of these studies is that they use very low initial solids concentrations, which are not feasible at an industrial scale. Moreover only the enzymes in the supernatant can be recovered by ultrafiltration, not those bound to the substrate. It has been shown, however, that bound cellulases can be partly recovered by the addition of fresh substrate to a medium (Lee et al., 1995).

In a study by Weiss et al., 2013 the researchers used solids recycling to reduce the amount of fresh enzyme required per round of hydrolysis. As a large proportion of cellulase enzyme remains adhered to the solid fraction of the substrate after the apparent cessation of hydrolysis, it is necessary to re-utilise these enzymes if possible (Varnai et al., 2011). Using corn stover as a substrate various insoluble solids fractions were recycled by adding them to fresh substrate and the increase in conversion was evaluated. By recycling 95.6% of the residual solid and using a make-up enzyme concentration of 42 mg enzyme preparation (EP) g^{-1} cellulose it was possible to reduce the enzyme loading by 19%, giving a final hydrolysis yield of 82%. A control experiment where 51.4 mg EP g^{-1} cellulose was used for a 72 hour hydrolysis period achieved a hydrolysis yield of 76%. Furthermore by recycling 85% of the solid the enzyme loading could be decreased by 33% using an initial condition of 10% TS (Weiss et al., 2013).

The shortcoming of the above scheme for industrial application is the need for a large tank, as each round results in an increasing amount of insoluble solid. The whole slurry would also have to be wasted at some point, thereby diminishing the efficacy of the system; hence the ability to recycle enzymes is limited by the requirements of this process step. Furthermore, a more recent study by Pihlajaniemi et al., 2014 suggests that the apparent increase in enzymes in the above recycle scheme could be due to a misinterpretation of the results. Their study proposes that the increase in conversion was due to increased average incubation time of solids, and to product removal reducing the enzyme inhibition, rather than the old enzymes hydrolysing the fresh substrate (Pihlajaniemi et al., 2014).

Solids recycling was also found to be beneficial in the hydrolysis of biasulphite pulp (Ouyang et al., 2013). Lindedam et al., 2013 showed that whole slurry recycling of wheat straw hydrolysate increased the reusable enzyme activity with two different but current enzyme preparations. In their recycling experiment the second round of hydrolysis was carried out using filter paper rather than wheat straw (Lindedam et al., 2013). This demonstrated the residual enzyme activity, but this activity would probably not be recovered if the slurry was used on the actual substrate.

The affinity of cellulases to lignocellulosic material which causes them to remain bound to substrate after hydrolysis is mostly attributable to the carbohydrate binding module (CBM) of the cellobiohydrolases (CBH). Varnai et al., 2013 showed that at low substrate concentrations (1% TS) the CBM is necessary for high conversion. As the substrate concentration increases to 20%, however, cellulose conversion using enzymes with or without CBMs becomes equivalent, and more of the CBHs desorb into the liquid portion of the hydrolysate after hydrolysis if they do not contain a CBM. It was suggested that when the water content in the system reduces there is a close physical association between the enzymes and the substrate which promotes adsorption of both non CBM and CBM-containing enzymes, thereby reducing the advantage that CBM containing enzymes have at low substrate concentrations. As more CBHs desorb after hydrolysis if they do not have a CBM, they would be easier to recycle (Varnai et al., 2013).

2.2.6.4 **Enzyme affinity for lignocellulosic substrate**

A number of recent studies have focused on the factors that contribute to adsorption and desorption of cellulase enzymes, particularly the EGs and CBHs, onto a cellulosic substrate.

In the past, researchers mainly looked at monocomponent systems; but as cellulase enzymes work in synergy a monocomponent system is not a true representation of what actually occurs during hydrolysis. Pribowo et al., 2012 looked at enzyme adsorption profiles during a hydrolysis period of 72 hours and assessed the adsorption and activity characteristics of a commercial mixture of enzymes. Their results showed that the activity profiles of desorbed enzymes dropped with time,

and it was concluded that short rounds of hydrolysis (within 24 hours) should be undertaken to maximise enzyme efficiency if the enzymes are to be recycled (Pribowo et al., 2012).

Alkaline elution has proven to be an effective means of desorbing enzymes from lignocellulosic substrate. Adjusting the pH of a hydrolysate solution to pH 9 or 10 makes it possible to recover the enzyme adsorbed on the substrate (Rodrigues et al., 2012). It is speculated that the enzyme structure unfolds allowing it to desorb from the substrate, especially when it is unproductively bound. Furthermore it has been shown that the enzyme can regain its conformation and activity if the pH is readjusted to pH 5 after the alkali treatment. It was also found that although a greater amount of enzyme activity can be recovered if hydrolysis is run at 37 ^{o}C the yield suffers as this is not the optimal hydrolysis temperature (Du et al., 2012; Rodrigues et al., 2014; Rodrigues et al., 2012). These studies were carried out at 2-5 %TS.

A stepwise enzyme recovery strategy was developed by Xu & Chen, 2007 using a 3-step post hydrolysis wash process which involved washing the substrate twice with sodium acetate buffer followed by a pH 10 calcium hydroxide wash. Up to 98% of the adsorbed protein could be recovered using this process. On repeating the experiment with three different enzyme loadings, it was found that the absolute protein which could not be recovered by the buffer wash alone was almost the same for each of the loadings. This indicates a strong enzyme association with the substrate for a portion of the enzyme (as substrate load was the same in each case) possibly due to the cellulase binding domain of the cellobiohydrolase, which then required the conformational change induced by the pH 10 wash to be removed (Xu & Chen, 2007).

2.2.7 Enzymatic hydrolysis of feedstock

The feedstock used in this research was a paper-rich fraction of MSW, and the following is a short discussion of various other studies using paper pulp and MSW as their feedstock.

2.2.7.1 **Enzyme hydrolysis of paper pulp**

A number of studies have been carried out using paper and card as substrates for ethanol production. Paper is a good medium to use for hydrolysis studies as it has been through many 'pre-treatment' processes such as grinding, heating and de-lignification during the paper-making process, resulting in relatively low hemicellulose and lignin concentrations. The addition of chemicals during paper manufacturing, however, has been shown to have negative effects on hydrolysis (Wang et al., 2011b).

Calcium carbonate is one of the most commonly used fillers in the production of white paper. Chen et al., 2012 showed that by removing a large proportion of the calcium carbonate from paper the hydrolysis efficiency increased, indicating that the filler was interfering with hydrolysis. It is not known whether this effect is due to direct interference from the calcium carbonate binding the enzymes, or to the increase in hydrolysate pH caused by the presence of $CaCO_3$.

Wang et al., 2011b tested various acids to remove calcium carbonate from waste office paper. It was found that washing the substrate in sulphuric acid prior to hydrolysis was the most effective means of $CaCO_3$ removal, as opposed to hydrochloric, phosphoric and nitric acids. Nikolov et al., 2000 used varying concentrations of phosphoric acid and found that a wash with 0.25% concentration was most effective. In both studies the removal of calcium carbonate resulted in much higher levels of substrate degradation during hydrolysis (Nikolov et al., 2000; Wang et al., 2012a; Wang et al., 2011b).

Studies by Elliston et al., 2013 & 2014 have shown high glucose and ethanol yields for copy paper without the need for calcium carbonate removal. They used fed batch addition in SSF and observed that it was possible to obtain an ethanol yield of 11.6% v/v (Elliston et al., 2013). The shortcomings of this study were that a high enzyme load (20 FPU) was used, and the reaction medium was buffered to maintain the pH, thereby increasing costs. In a follow-up study aimed at obtaining high sugar concentrations rather than ethanol, a sugar concentration of 126 g L^{-1} was obtained using a fed batch system. Although this system used less enzyme (7 FPU), making it

industrially applicable, a process time of 10 days was required which would not be feasible for commercial application (Elliston et al., 2014).

Drying or hornification has also been shown to play a part in reducing the hydrolysis efficiency (Chen et al., 2012; Luo & Zhu, 2011; Luo et al., 2011). In the study quoted above, Chen et al., 2011 showed that the sugar yield was reduced up to 13% by drying the fibres. A dried sample could be transformed back to its never-been-dried state, however, by using a low intensity but high energy milling device such as a PFI mill. A PFI mill serves to sheer open up the fibres in lignocellulosic substrate rather than just cutting the fibres shorter. This indicates that hornification causes a collapse of pores in the substrate which limits the enzyme accessibility of the cellulose, and that this negative effect can be reversed by shearing open the fibres .

Other researchers have looked at how much drying decreases the substrate enzyme digestability (SED - referred to as carbohydrate conversion in the current research). The degree of drying has been quantified using instrumentation which can determine pore volume, such as differential scanning calorimetry (DSC) (Park et al., 2006); or through simpler tests such as water retention value (WRV) (Luo & Zhu, 2011; Luo et al., 2011). WRV is measured by centrifuging a wet sample and seeing how much water is retained as a percentage of the dry weight of the substrate. Luo et al., 2011 correlated cellulose accessibility to WRV and SED with WRV or Simons staining for pore measurement, and showed good correlation in both cases. Furthermore, it was found that wet pressing a fibre to a solids content of less than 40% has a negligible effect on carbohydrate conversion (Luo & Zhu, 2011; Luo et al., 2011).

The substrate used in this research is pulp recovered from MSW, which mostly consists of paper. The limiting factor of cellulose hydrolysis in lignocellulosic material has been shown to be cellulose accessibility which is proportional to the efficacy of the pre-treatment (Arantes & Saddler, 2011; Igarashi et al., 2011). As the pulp in MSW has already gone through a number of pre-treatment steps during formation of the original products, however, it is not necessary to undertake the energy intensive pre-treatments that are required with other substrates.

2.2.7.2 Enzyme hydrolysis of MSW

To date only a very small number of studies have focused on producing fermentable sugars using MSW as a substrate. Li et al., 2012 used an autoclave cooking process run at 165 ^{o}C to prepare their substrate. After collecting a paper-rich fraction they milled the material and found that (i) the highest conversion occurs with particles of the range 150-300µm; (ii) milling to ranges smaller than this did not increase carbohydrate conversion; and (iii) particles over this size had a lower carbohydrate conversion. The maximum hydrolysis conversion reached in this experiment was 53% using an enzyme concentration of 90 mg g^{-1} substrate (Li et al., 2012b).

A study by Jensen et al., 2011 investigated the use of a surfactant in increasing carbohydrate conversion of MSW, and the impact of contaminants such as calcium, potassium, sodium, chloride that are present in MSW wastewater. Their process involved thermal treatment of the MSW at 95 ^{o}C after hand-sorting of large plastic and metal items from the substrate. The addition of PEG as a surfactant had no effect in decreasing the particle size or reducing the viscosity of the substrate during enzymatic hydrolysis. They also hydrolysed filter paper in the presence of salts and metals in MSW process water and found that these did not affect hydrolysis. These results showcased the robustness of the cellulase enzyme system (Jensen et al., 2011).

2.2.8 Calculating enzymatic hydrolysis yield

The most commonly used formula for determining sugar yield and percentage conversion of hydrolysis is that from the National Renewable Energy Laboratory (NREL) (Brown & Torget, 1996). This formula, given below, works well for low solids concentrations (<5 % water insoluble solids).

NREL

$$\%Hydrolysis = \frac{[Glc] + 1.0526 \times [Cel]}{1.111 \times F_{cellulose} \times [Initial\ Solids]} \times 100 \qquad \text{Equation 2.2-1}$$

Where

[Glc] - glucose concentration in the supernatant of the slurry (g L^{-1})

[Cel] - cellobiose concentration in the supernatant of the slurry (g L^{-1})

$F_{cellulose}$ - fraction of cellulose in the substrate

[Initial Solids] - initial solids concentration (g L^{-1})

1.11 is the correction factor for the loss of water from the reaction medium which is used to form two glucose molecules from cellulose on a weight basis.

Increasing attention is being focused on methods of hydrolysing the high solids solutions that are required for commercial second generation lignocellulosic biofuel processes. However, the formula derived by NREL is not accurate for determining yields of high % total solids (TS) slurries. For high TS solutions, as the enzymatic hydrolysis reaction proceeds the mass of insoluble solids decreases, which in turn increases the density and volume of the liquid phase. Research has shown that when calculating the theoretical yield using the formula above there is overestimation of the yield when basing calculations on the initial volume. Kristensen et al., 2009 have developed a modified yield calculation for high solids content slurries (Equation 2.2-2) (Kristensen et al., 2009a).

$$\%Hydrolysis = \frac{\frac{m_{reaction} - m_{insoluble.solids}}{SG_{aq.phase}}[Glc] + 1.0526 \times [Cel]}{1.111 \times m_{substrate} F_{cellulose} \times TS} \times 100$$ **Equation 2.2-2**

Where

$m_{reaction}$ -mass of the whole reaction (g)

$m_{insoluble\ solids}$ - mass of insoluble solids after hydrolysis (g)

$m_{substrate}$ - mass of the substrate (g)

$SG_{aq.phase}$ - is the specific gravity of the aqueous phase (g L^{-1})

TS - initial dry total solids content (w/w)

2.2.9 Separation step obstacles

Although many pre-treatment options are available there is no clear consensus on which if any of these is the best. This is most probably due to the variation in lignocellulosic feedstocks and the differing quantities of cellulose, hemicellulose,

lignin, and extractives in these. In addition it is generally not known whether the increased cost associated with physical pre-treatments is justified by the enhancement of hydrolysis, especially under high solids hydrolysis conditions.

The role of hemicellulose as an inhibitor is not clear and it remains to be seen whether its interaction with cellulose is a purely physical association or whether it bio-chemical in nature. Thus it is not obvious how best to alleviate this inhibition i.e. by physical or enzymatic treatment.

Enzymes and enzyme hydrolysis are economic and process bottlenecks in a bio-refinery concept. The cellulase system has a low activity compared to similar enzymes and the best methods for optimising its performance on a range of substrates are yet to be identified. A wide variety of options are available for enhancing the activity but most of these come at a cost, whether it be (i) monetary e.g. in the case of using additives; or (ii) in decreasing process efficiency e.g. through decreased glucose concentrations in the final product which then create the need for extra and potentially costly concentration steps following hydrolysis.

One of the biggest gaps in knowledge identified from this section on separations is whether many of the treatments and process options presented would work under high solids conditions. Most studies detailed in this section were carried out at low total solids (< 5 %TS); however higher concentrations will be needed for industrial-scale application.

2.3 Bio-refinery outputs

As stated above, much of the current research into bio-refineries relates to the production of bio-ethanol, and this is a relatively easy commodity to produce and can be used within the current liquid fuel infrastructure. A short discussion on fermentation to produce ethanol and its effects on hydrolysis is presented below. As technology develops, however, sugar building blocks could be used to make various other fuels such as butanol and farnesane (Connor & Liao, 2009; Peralta-Yahya et al., 2012), as well as chemical building blocks such as sorbitol and xylitol

(Fernando et al., 2006). Residual low concentration sugars could also go into energy production process such as anaerobic digestion (Dererie et al., 2011).

2.3.1 Fermentation to make bio-ethanol

Once hydrolysed, the sugar solution can be fermented to ethanol. Currently there are two main strategies used for fermentation: Separate Hydrolysis and Fermentation (SHF) and Simultaneous Saccharification and Fermentation (SSF). In SHF the substrate is hydrolysed to monomeric sugars in one vessel and then transferred to a separate vessel for fermentation. In SSF the two steps are carried out simultaneously in one vessel. Each method has its advantages and disadvantages, as listed in table 2.3-1.

Table 2.3-1. Advantages and disadvantages of SHF and SSF.

SHF		**SSF**	
Advantages	*Disadvantages*	*Advantages*	*Disadvantages*
- Enzyme hydrolysis and Fermentation steps performed at optimum pH and temperature	- Multiple vessels required - Increased costs in moving slurry between tanks -May cause end product inhibition of cellulases	- Only one vessel needed -Reduced/No end product inhibition of cellulases	- Sacrifice optimum operating conditions for enzymatic hydrolysis -Rheological problems with high TS feedstocks

Another issue associated with fermentation is hexose vs. hexose and pentose fermentation. For substrates with a high hemicellulose content, consisting largely of xylose and to a lesser extent arabinose, it is often necessary to convert both types of sugars to obtain enough product (bio-ethanol) from the process. Recent advances have seen the development of pentose-utilising micro-organisms. Frequently these are recombinant yeasts that have one of two pentose-utilising pathways. The first pathway is from fungi which convert xylose to xylitol, xylitol to D-xylulose and lastly D-xylulose to Xylulose-5P, after which the compound goes into the pentose phosphate pathway and finally enters glycolysis (glucose to ethanol pathway). The other pentose-utilising pathway is from bacteria (*P. stipites*), in which the xylose is converted to D-Xylulose and then follows the same steps as the fungal pathway. Although a number of different micoorganisms have been genetically engineered to incorporate a pentose-utilising pathway, low tolerance for products

or inhibitors can lead to unsuccessful results. As genetic engineering progresses, stable recombinants are now being produced that can yield high amounts of ethanol and can utilise both C6 and C5 sugars (Hahn-Hägerdal et al., 2007; Wisselink et al., 2009; Young et al., 2010). This technology has been commercialised, and recombinant bacterial/yeast stains are available from companies such as Terranol (http://www.terranol.com), which is now a subsidiary of Novozymes.

A recent article by Fox et al., 2012 looked at an engineered strain of *S. cerevisiae*, DA24-16 which could consume cellobiose whilst also consuming xylose at a rate similar to *Pichia stipitis*, wild type yeast that grows on xylose. Their findings suggested that this recombinant yeast is much more suited to SSF. This type of yeast could alleviate the problem of glucose inhibition of xylose uptake which occurs in many recombinant strains (Fox et al., 2012).

In addition to pentose utilisation, yeast strains are also being engineered to increase their tolerance to inhibitors and ethanol (Hahn-Hägerdal et al., 2007). Yeast has some capability to convert certain toxic compounds such as furans to furan alcohols that are much less toxic to the organism (Liu et al., 2005; Tu et al., 2009b): however this process is slow and requires a lag time before ethanol production can begin.

2.3.1.1 **Ethanol inhibition of enzymes**

Product inhibition is a major obstacle in enzymatic hydrolysis of cellulose, and SSF was developed to alleviate this problem. Ethanol can also inhibit enzyme activity, however, Wu and Lee., 1997 showed that there is a decrease in carbohydrate conversion in the presence of ethanol and that this effect is more pronounced at 50 ^{o}C than at 38 ^{o}C. Over a 72-hour hydrolysis period the carbohydrate conversion decreased ~10% at 38 ^{o}C and ~20% at 50 ^{o}C (Wu & Lee, 1997).

Skovgard et al., 2013 studied the effect of high temperature and ethanol on the inactivation of thermostable enzymes, and showed that enzymes would remain stable under vacuum distillation conditions between 55 ^{o}C and 65 ^{o}C. As the ethanol concentration in the enzyme mixture increased, however, the inactivation rate of enzymes exposed to their upper temperature limit also increased (Skovgaard

& Jorgensen, 2013), indicating that it is probably not ideal to recycle enzymes in high ethanol concentrations after distillation. Using SDS PAGE analysis it was found that the inactivation of the cellobiohydrolases was due to degradation of the enzyme, whereas the inactivation of the endoglucanases and β-glucosidase was due to structural changes in the enzymes.

Podkaminer et al., 2012 used themostable yeast, *thermoanaerobacterium saccharolyticum*, to determine the type of inhibition experienced by commercial cellulases in a thermophilic SSF set-up. At high solids concentrations and relatively high ethanol titres (32.17 g L^{-1}) a substantial decrease in conversion was observed, far more than predicted by a model developed for low initial solids concentrations. Their findings suggested that the anaerobic conditions required for fermentation hamper the cellulase activity; and that this decrease in activity could be recovered by re-exposing the reaction medium to aerobic conditions e.g. by adding air. They postulated that the anaerobic conditions possibly prevent the activity of the GH61 (AA9) oxidative complex which increases the activity of the cellulase system (Podkaminer et al., 2012).

2.4 Conclusions from the literature review

2.4.1 Comments & Discussion

From the literature review it can be seen that a high volume of research is currently being conducted on sugar-platform lignocellulosic bio-refineries. The interest into this area serves to indicate that there is a large potential for the bio-refinery concept to be part of a sustainable future for the production of fuels and chemicals. Although the first steps are being made at converting this technology into practice at industrial scale (Advanced Ethanol Council, 2012; BiofuelsDigest, 2012); the process relies quite heavily on government subsidies. Thus, major process optimisations and improvements are still required in order for the technology to be deployed at large-scale in the future. At present the most extensively researched bio-refinery product is bio-ethanol. The process of making this economic at large scale is related to obtaining high enough glucose yields (≥ 8 wt%) to match

downstream processing requirements for ethanol extraction (Larsen et al., 2008; Larsson & Zacchi, 1996). To achieve this outcome economically, the enzymes in the system need to be utilised with maximum efficiency to reduce their costs which can be significant (Humbird et al., 2011). In response a number of strategies have been devised by a various researchers who have investigated enzyme recovery and reuse post hydrolysis from both the hydrolysate liquid and the residual solid (Tu et al., 2007b; Weiss et al., 2013; Xue et al., 2012a).

Although a number of studies have shown some success, they were generally conducted in small laboratory-scale experiments, making it hard to determine whether or not they will work at industry-relevant scales and substrate concentrations. Other studies that have shown promising results at high substrate concentration have shortcomings which would limit their efficacy in a commercial situation.

Examples include: (i) The use of surfactants to increase enzyme recycling efficiency, which brings in an additional cost and some studies suggest that this will only be feasible if both the surfactant and enzyme were recycled. This poses problems as it introduces the need for expensive ultrafiltration technologies to separate the enzymes and surfactants from the product sugars. (ii) Residual solids recycling, which works best when a large proportion of the solid is recycled. This means that the solid content and thus the required tank volume increases in each round of hydrolysis. In order to make this process economic the solid residue would need to be wasted every 'x' number of rounds, diminishing the optimal conditions required for the recovered enzyme activity. (iii) Nearly all promising enzyme recycle strategies are conducted at low solids concentrations so it is not clear if these could or would work at high solids. (iv) Most enzyme recycling strategies focus on recycling the liquid portion of the hydrolysate alone which is not optimal as there is enzyme activity that remains associated with the solid residue post hydrolysis. (v) Relatively low overall glucan conversions are achieved, increasing the amount of waste discharged and decreasing the value obtained from the product. Strategies that have been used to increase overall substrate conversion can include a wash steps which is introduced to reduce the time taken for cellulosic conversion by

eliminating product inhibition. The wash step is performed early in the process, before the sugar concentration is sufficiently high enough for downstream processing; this in turn introduces the need for concentration steps which can be expensive or energy intensive. (vi) Many developed pre-treatment and enzyme enhancing strategies are substrate specific.

Furthermore, only a very limited number of studies have been carried out on MSW which is a readily available waste feedstock. These studies have only looked at the typical hydrolysis behaviour of this material and have not investigated in depth its potential as a lignocellulosic substrate for the bio-refinery concept.

2.4.2 Key Points

- Lignocellulosic material is composed of three main fibre components – cellulose, hemicellulose and lignin
- Cellulose and hemicellulose can be broken into monomeric sugars by cellulase and hemicellulase enzymes. In a cellulase enzyme mixture there are 4 main classes of enzyme

    1) Endoglucanases – These enzymes cut up an insoluble cellulose fibres into smaller chains

    2) Cellobiohydrolases – These enzymes move along the cut insoluble chains to produce soluble cellobiose and cellotriose

    3) β-glucosidase – this enzyme breaks the cellobiose and triose into glucose monomers

    4) GH 61 (AA9) – This complex helps to increase enzyme activity by using a redox mechanism
- Lignocellulosic material is recalcitrant mostly due to the lignin component. Lignin can affect hydrolysis in two ways: 1) it can unproductively bind enzymes; and 2) it can cause steric hindrance which prevents enzyme binding on a cellulose fibril. Hemicellulose can also cause this steric hindrance.
- For successful hydrolysis both cellulases and hemicellulases must be used, as the enzymes in these mixtures work in synergy.

- This synergistic action may be required due to the structure of lignocellulosic material. It is believed that cellulose is associated with two fractions of hemicellulose which are either loosely or strongly bound and thus affect hydrolysis in different way.
- The unproductive binding and steric hindrance caused by lignin and hemicellulose can be partially overcome with the addition of various compounds including proteins and surfactants.
- For the commercialisation of lignocellulosic ethanol it is required that a fermentation broth be produced containing > 4% wt ethanol. This means that a >8% w/w sugar solution is required which is equivalent to a starting solid concentration of > 20% TS.
- When working at high solids concentrations a decrease in overall substrate conversion is seen compared to hydrolysis at low solids.
- This decrease in conversion is known as the solids effect and explanations for this include: insufficient mixing; product inhibition as a result of increasing sugar concentration; decreased water availability; irreversible binding of adsorbed enzyme to the substrate, including non-productive binding to lignin; inhibition of enzyme adsorption; and enzyme denaturation. There may also be other, as yet unidentified, reasons for decreased conversion.
- In order to overcome these limitations researchers have employed a variety of recycling strategies which include; surfactants to decrease binding, changes in pH to desorb enzyme from substrate, washing strategies, filtrate recycling, solids recycling, and membrane reactors for product removal.
- Most studies have been conducted at low solids concentrations which means that there is no interference from the solids effect, no mass transfer limitations and no issues with substrate accessibility as would be the case in industrially relevant scales.

2.4.3 Conclusion

The current work builds upon the knowledge gained by other researchers to propose strategies that are intended not only to allow recycling and reuse of enzymes in the liquid portion of the hydrolysate but also to increase the conversion of the solid substrate, whilst still obtaining a sugar solution that can be used for downstream processing without the need for further concentration. Thus making a significant contribution to the overall viability of a sugar platform bio-refinery.

Chapter 3: Research methodology

This research methodology section only contains methods that were used multiple times in the current research or those that were very lengthy. For one off individual experiments please refer to the results & discussion chapters.

Note: All water used in the analytic methods within this research was de-ionised water at a conductivity of 18 MΩ - cm resistivity (Thermo, Barnstead, UK).

3.1 Substrate characterisation

The substrate used in this research was processed MSW pulp obtained from the Fiberight pilot plant in Lawrenceville, Virginia, USA. Locally collected MSW was processed to remove non-reactive products such as plastics, glass and metal contaminants, thus increasing the cellulosic content of the material. The material was autoclaved at 80-125 ^{o}C prior to plastic and metal removal. These contaminants were removed through screening and the pulp was washed through a Regenex washing system then cooked at pH 5 (pH adjusted with phosphoric acid) at a temperature of 125 ^{o}C. The resultant material was filter cake pressed to a solids content between 30 and 50 % TS. The pulp was then frozen and sent by air to Southampton where it was either dried or used as provided.

As can be expected from a crude product, the wet pulp obtained from the Fiberight pilot plant in Lawrenceville, Virginia showed slight batch to batch variations, due in part on process modifications which occurred in the plant through the duration of this work, and in part perhaps on variability in the input material. Thus there were slight variations in the hydrolysis results, with some batches showing better performance than others. As a consequence not all results are directly comparable, and experiments were therefore carried out alongside a control hydrolysis in order to allow for these variations.

3.1.1 Substrates used

Municipal solid waste (MSW) pulp.

This was provided by Fiberight Ltd from its pilot plant in Lawrenceville, Virginia, USA. The MSW was first autoclaved and then washed to remove plastic, metals and mineral contaminants. On NREL acid hydrolysis the pulp typically yielded 56: 10 : 6 : 24 : 4 of glucan : xylan : araban/galactan/mannan : lignin : ash respectively. This was as supplied, at a total solids (TS) content of 30-50% after mechanical dewatering.

Municipal solid waste-dried from 2010

Dried substrate was recovered from the provided MSW pulp which was air dried to 92% TS. On NREL acid hydrolysis the pulp typically yielded 63 : 19 : 12 : 4, cellulose : hemicellulose : lignin : ash.

Control substrate

Fisher Brand filter paper (cat no. FB59035, Fisher Scientific, Loughborough, UK), was used as a defined source of paper cellulose without lignin and ash, which on NREL acid hydrolysis yielded a 84:14:2 mix of glucan : xylan : araban/galactan/mannan.

3.1.2 Total solids and ash

TS and VS determination was based on Standard Method 2540 G (APHA, 2005). Weights were determined to an accuracy of ± 0.001 g (Sartorius LC6215 balance, Sartorius AG, Gottingen Germany). After thorough mixing, approximately 10 g of sample was transferred into a weighed crucible which was placed in an oven (LTE Scientific Ltd., Oldham UK) for drying overnight at 105 ± 1 ^{o}C. After drying the samples were transferred to a desiccator to cool for at least 40 minutes. Samples were then weighed again with the same balance, transferred to an ashing furnace (Carbolite Furnace 201, Carbolite, UK) and heated to 550 ± 10 ^{o}C for two hours. After this ashing step, samples were again cooled in a desiccator for at least one hour before weighing a third time.

Total and volatile solids were calculated according to the following equations:

$$\%\, TS\ = \frac{W_3 - W_1}{W_2 - W_1} x\ 100$$ **Equation 3.1-1**

$$\%\ VS\ (on\ a\ wet\ weight\ basis) = \frac{W_3 - W_4}{W_2 - W_1} x\ 100$$ **Equation 3.1-2**

$$\%\ VS\ (on\ a\ TS\ basis) = \frac{W_3 - W_4}{W_3 - W_1} x\ 100$$ **Equation 3.1-3**

Where:

W_1 = weight of empty crucible (g)

W_2 = weight of crucible containing fresh sample (g)

W_3 = weight of crucible and sample after drying at 105 ^{o}C (g)

W_4 = weight of crucible and sample after heating to 550 ^{o}C (g)

3.1.2.1 Total suspended solids

Total suspended solids (TSS) content was measured by passing a sample of known volume through a 0.45 µm pore size glass fibre filter paper (GF/C, Whatman, UK) of known dry weight (± 0.1 mg). After drying at 105 ^{o}C for 24 hours the paper was again weighed and the TSS content determined according to the following equation:

$$TSS = \frac{(W_2 - W_1)\ x\ 1000}{V_s}$$ **Equation 3.1-4**

Where:

TSS = total suspended solids (mg l^{-1})

W_1 = weight of clean filter paper (mg)

W_2 = weight of filter paper + sample (mg)

V_s = sample volume (ml)

3.1.2.2 Soluble solids and soluble ash analysis

Soluble solids and soluble ash content were measured using the same method as TS, but the sample was first filtered through a 0.45 µm nylon membrane into the weighed crucible to remove the insoluble solids.

3.1.3 Calcium carbonate analysis

The method for calcium carbonate analysis is taken from the Technical Association of the Paper and Pulp Industries (TAPPI) standard method T-413 om-11 (TAPPI, 1980). Following the ashing procedure described above the pulp was placed in a furnace at 900 ^{o}C for 1 hour in order to convert the calcium carbonate present in the ash to calcium oxide and carbon dioxide according to the following equation.

$$CaCO_3 \xrightarrow{heat} CaO + CO_2$$ **Equation 3.1-5**

The change in weight of the sample between ashing at 550 ^{o}C and 900 ^{o}C can be attributed to carbon dioxide liberation. Stoichiometry is then used to calculate the initial calcium carbonate content of the ash. If no change in weight is detected between ashing at 500 ^{o}C and 900 ^{o}C it is assumed that no calcium carbonate is present.

3.1.4 NREL method – Sugars, Lignin, Total Solids and Ash

The structural carbohydrates, lignin, ash and solids content of the material were determined using a slightly modified version of the standard NREL procedure (Sluiter et al., 2011).

The biomass was hydrolysed using a 2-step acid hydrolysis procedure. 300 mg of biomass was weighed and placed into a shake flask or glass schott bottle and incubated at 30 ^{o}C in an orbital shaking incubator (Gallenkamp S/N: 101400YY2C) at 200 rpm for 1 hour with 7 ml of 72 wt% Sulphuric acid (H_2SO_4). Following incubation the acid was diluted to 4 wt% with the addition of 119 ml of water and the sample was placed into an autoclave for 1 hour at 121 ^{o}C. After cooling a Buckner funnel was used in conjunction with a pre-weighed Whatman GFC filter paper, which had been previously placed in the furnace to burn off any organic residue, to separate the insoluble solids from the liquid fraction. The liquid fraction was collected and its sugar content was measured using the sugar analysis methods in section 3.23.2 below. The insoluble solids remaining on the filter paper were considered to be

composed of acid-insoluble lignin and ash and were placed into a pre-weighed crucible and dried overnight at 105 ^{o}C to determine the total insoluble solids content. After weighing the crucible and its contents were placed into a furnace at 550 ^{o}C for 2 hours, cooled and weighed to determine the ash content as in section 3.1.2.1 above.

The acid soluble lignin was determined using TAPPI method T-222 om-02 (TAPPI, 1991). The filtered sugar solution (liquid fraction) from a sample was diluted until it gave a reading of between 0.2 and 0.7 on a spectrophotometer (Cecil 3000, Cambridge) at a wavelength of 205 nm, compared to deionised water at a conductivity of 1 MΩ as a zero blank.

$$ASL\% = \frac{\frac{A \times D \times V}{a \times b \times M}.1000mg/g}{10} \qquad \textbf{Equation 3.1-6}$$

Where

ASL% = Acid soluble lignin (% of total solids)

A - Absorption at 205nm

D - Dilution Factor

V - Volume of filtrate (L)

a - Extinction coefficient of lignin (g L^{-1} cm^{-1})

b - Cuvette path length (cm)

M - Weight of Sample [100% TS] before acid hydrolysis (g)

The acid-insoluble lignin was calculated by difference:

$$AIL\% = \frac{(W_3 - W_1 - W_{FP})}{Totalsolids_{initial}} \times 100 \qquad \textbf{Equation 3.1-7}$$

Where

AIL% = acid insoluble lignin (% of total solids)

W_3 - Weight of crucible, solids and filter paper after drying at 105 ^{o}C (g)

W_1 - Weigh of crucible (g)

W_{FP} - Weigh of filter paper (g)

Where Totalsolids is measured in grams.

Hence, the total lignin content is calculated from the sum of the soluble and insoluble lignin

3.1.5 Fibercap analysis

The Fibrecap procedure (Kitcherside et al., 2000) required the following reagents:

Reagents:

(1) Neutral Detergent Solution (ND):

Disodium ethylene diaminetetraacetate EDTA (dehydrate) 18.61 g
Sodium Borate 6.81 g
Sodium lauryl sulphate 30 g
2-ethoxyethanol 10 ml
Disodium hydrogen phosphate (anhydrous) 4.56 g
Alfa-Amylase solution – Termamyl 300L, type DX available from Foss Tecator

(2) Acid Detergent Fibre Solution (AD):

1.00N H_2SO_4 with CTAB made from;
Concentrated sulfuric acid 49.04 g
Cetyl trimethylammonium bromide CTAB 20 g

(3) Acid Detergent Lignin (ADL) Solution:

Sulfuric acid, 72 wt% made from; Concentrated sulfuric acid, 98 wt%

Fibrecap was carried out using a FOSS kit Fibretec TM 2023 (Hillerød, Denmark) which included an 18-capsule tray, an extraction beaker and proprietary capsules. The capsule dimensions were 58 mm length and 23 mm diameter with a simple hydrolphilic snap on lid. The capsules were composed of polypropylene with the same filtration characteristics as Whatman 541 filter paper (quick filtration, 22 µm)

to ensure free flow of reagent through the sample during the analysis, and no sample transfer.

The Fibercap procedure consisted of three steps.

(1) Neutral detergent fibre (NDF)

ND washing was used to remove all the non-fibrous matter from the sample so that theoretically only cellulose, hemicellulose, lignin and ash were left in the sample following this step. In a paper by Van Stoeset et al., (1991) it is states that ND removes the extractive components and some nitrogenous matter like proteins: it also chelates calcium and removes pectins and some non-fibrous matter. 0.3-0.7 (± 0.0001) g of sample was weighed into a pre-weighed capsule BP110S (Sartorius, New York, USA). The samples were then placed into the capsule tray which was immersed in 1L of boiling ND for 30 minutes. The capsules were then removed, half the ND was replaced with new ND, and the procedure was repeated. Following ND washing the capsules were washed five times in deionised water at 80 ^{o}C in order to remove the ND from the sample. The capsules were then oven dried overnight at 105 ^{o}C, cooled and weighed before being placed in ceramic crucibles for ashing in a furnace at 600 ^{o}C for four hours (Carbolite Furnace 201, Carbolite, UK). The ashed sample was then cooled and weighed. Following this procedure the neutral detergent fibre was calculated using equation 3.1-9.

The capsules can lose a small amount of weight during reaction with the reagents. A correction factor (C) to compensate for this loss is used in the formula for calculation of analytical results. Typically the correction factor (C) is >0.9990, corresponding to ~3 mg weight loss of a capsule during processing:

$$C = \frac{\textit{Blank capsule weight after extractions}}{\textit{Blank capsule weight at start}}$$ **Equation 3.1-8**

$$NDF = \frac{W_3 - (W_1 * C) - (W_5 - W_4 - D)}{W_2} * 100$$ Equation 3.1-9

Where

W1 - Initial capsule weight (g)

W2 - Sample weight (g)

W3 - Capsule + residue weight (g)

W4 - Empty ashing crucible (g)

W5 - Total ash (including ashing crucible) (g)

C - Blank correction for capsule solubility

D - Capsule ash (g)

(2) Acid detergent fibre (ADF)

The acid detergent washing step was used to remove hemicellulose from the neutral detergent fibre. Step 1 was repeated using AD instead of ND and the capsules were boiled in AD for 1 hour rather than 30 minutes. The acid detergent fibre was calculated using equation 3.1-10.

$$\%ADF = Cellulose + Lignin = \frac{W_3 - (W_1 \times C) - (W_5 - W_4 - D)}{W_2} \times 100$$ Equation 3.1-10

Where

W_1 - Initial capsule weight, g;

W_2 - Sample weight, g;

W_3 - Weight of capsule + residue sample after extracting and drying, g;

W_4 - Weight of empty ashing crucible, g;

W_5 - Weight of total ash and ashing crucible, g;

(3) Acid detergent lignin fibre (ADLF)

The last step in the Fibrecap analyses allowed for the determination of the lignin content in the sample. The capsules with sample were placed in the ADL for 4 hours

at ambient temperature. The capsules were then removed, oven dried overnight and weighed. The samples were then ashed at 600 ^{o}C for 4 hours.

$$\%ADL = Lignin = \frac{W_3 - (W_1 \times C) - (W_5 - W_4 - D)}{W_2} \times 100 \qquad \text{Equation 3.1-11}$$

where

W_1 - Initial capsule weight, g;

W_2 - Sample weight, g;

W_3 - Weight of capsule + residue sample after extracting and drying, g;

W_4 - Weight of empty ashing crucible, g;

W_5 - Weight of total ash and ashing crucible, g;

C - Blank correction for capsule solubility;

D - Capsule ash, g.

Using the values calculated in the equations above the percentage of each fibre component in the sample could be calculated:

Hemicellulose + cellulose + lignin = NDF

Hemicellulose = NDF-ADF

Cellulose = ADF-ADLF

Lignin = ADLF.

The ash content is taken as the ash in the NDF.

3.1.6 Elemental analysis

Carbon, hydrogen, nitrogen and sulphur contents of samples were determined using a FlashEA 1112 Elemental Analyser (Thermo Finnigan, Italy). Samples were air dried and milled to obtain a homogenous sample. Sub-samples of approximately 3-4 mg were weighed into standard weight tin disks using a five decimal place analytical scale (Radwig, XA110/X, Poland). These were placed in a combustion/reduction reactor held at 900 °C then flash combusted in a gas flow temporarily enriched with

oxygen resulting in a temperature greater than 1700 ^{o}C and the release of N_xO_x, CO_2, H_2O and SO_2 (depending on the composition of the sample). The gas mixture was then analysed by GC with the different components are measured by appropriate detectors. The working conditions of the elemental analyser were as described in the manufacturer's technical literature and method sheets. Standards used in this analysis were atropine, nicotinamide and birch leaf. The TS and VS of dried and milled samples were also analysed to obtain the most accuracy values.

3.2 Sugar analysis – High performance anion exchange chromatography

High performance anion exchange chromatography with pulsed amperometric detection (HPAEC – PAD). Samples for sugars determination were placed on ice as soon as they were taken, and if not analysed immediately were frozen. Before analysis both fresh and defrosted samples were centrifuged (Galaxy 16HD, VWR, Leicestershire, UK) at 13000 g for 7 minutes. The supernatant was diluted and placed in a 5 ml sample vial with a 0.45 µm nylon filter cap. Sugar analysis was carried out on a Dionex DX-500 system using a method adapted from that of Davis., 1998. In this glucose, xylose, galactose, arabinose, mannose and cellobiose were separated at 30 ^{o}C on a CarboPac PA1 column (250 x 4 mm) in combination with a CarboPac guard column (25 x 4 mm) (Dionex, Sunnyvale, CA, USA). The mobile phase components were 200 mM sodium hydroxide (A), distilled water (18.2 M Ω - cm resistivity, Thermo Barnstead) (B) and 170 mM sodium acetate in 200 mM sodium hydroxide (C). The system set up used a 2.5 µL sample loop and 450 mM sodium hydroxide post column eluent at a pressure of 2.76 bar to aid sugar detection. The PAD settings were E_1 = 0.1, E_2 = 0.1, E_3 = 0.1, E_4 = -0.2, E_5 = -0.2, E_6 = 0.6, E_7 = -0.1, E_8 = -0.1 applied for duration T_1 = 0 s, T_2 = 0.20 s, T_3 = 0.40 s, T_4 = 0.41 s, T_5 = 0.42 s, T_6 =0.43 s, T_7 = 0.44 s, T_8 = 0.5 s respectively, at a sensitivity of 1uC.

The detailed gradient eluent set up for Dionex ion chromatography of sugars is shown in Table 3.2-1.

Table 3.2-1. Eluent gradient and flow conditions for HPAEC-PAD analysis of monomeric sugars.

Time	A (200 mM NaOH)	B (DI H_2O)	C (170 mM NaOAc in 200 mM NaOH)	Flow Rate, ml min^{-1}
0	2	98	0	1.3
5	2	98	0	
7	2	98	0	
9	0	100	0	
11	0	100	0	
12	0	10	90	
15	0	10	90	
16	70	30	0	
18	70	30	0	
19	2	98	0	
24.5	2	98	0	

The numbers in the eluent columns A, B and C refer to the % total.

3.3 Cation analysis – Ion chromatography

Calcium (Ca), Magnesium (Mg) and Sodium (Na) were measured by ion chromatography using a Metrohm 882 Compact IC plus 1 fitted with direct conductivity detector, autosampler and Metrosep C4 column (4x250mm) and guard column. The eluent (mobile phase) used was 1.7 mM L^{-1} nitric acid and 0.7 mM L^{-1} Dipicolinic acid at a flow rate of 0.9 mL min^{-1}. The eluent was prepared using a standard stock solution obtained from Thermofisher Scientific (Loughborough, UK) as was a standard stock solution containing other cations including calcium and magnesium. Samples were filtered (cellulose nitrate membrane filter, 0.45 µm pore size x 25mm) followed by dilution to the correct concentration range with the eluent. All reagents were made up to the required volume using deionised water (18.2 M Ω - cm resistivity, Thermo Barnstead). Results integration was carried out using Magic IC net software.

3.4 Anion analysis – Ion chromatography

Phosphate, Nitrate and Sulphate were measured by ion chromatography using a Metrohm 882 Compact IC plus fitted with a direct conductivity detector, autosampler and Metrosep A Supp 5 column (4x150mm) and guard column. The eluent (mobile phase) used was carbonate buffer (1 mM L^{-1}) in $NaHCO_3$, 3.2mM L^{-1}

Na_2CO_3 and 2% acetonitrile) at a flow rate of 0.7 ml min^{-1}. Samples were filtered (cellulose nitrate membrane filter, 0.45µm pore size x 25mm) followed by dilution to the correct concentration range with the eluent. All reagents were made up to the required volume using deionised water (18.2 M Ω - cm resistivity, Thermo Barnstead). Results integration was carried out using Magic IC net software.

3.5 Scanning electron microscopy (SEM) imaging

SEM analysis was performed with a JEOL JSM5910 scanning electron microscope with a tungsten filament electron source. The samples were coated with gold to a thickness of 10-20 nm with a SC7640 Suto/Manual High Resolution Sputter Coater (Quorum Technologies, Newhaven, UK).

3.6 Enzyme hydrolysis

3.6.1 Water and enzymes used

All water used in the hydrolysis experiments in this research was de-ionised water at a conductivity of 1MΩ - cm resistivity (Thermo, Barnstead, UK). This water was used to make up the hydrolysis reaction medium of either; (i) pH 5 citric acid buffer or (ii) water adjusted to pH 5 with phosphoric acid.

The commercial cellulase mixtures Cellic (C-Tec2 or C-Tec3) and/or H-Tec3 (Novozymes, Copenhagen, Denmark) were used for hydrolysis.

Note: H-Tec3 is a hemicellulase preparation with a mixture of various hemicallulases including xylanases, mannases and β-xylosidase.

3.6.2 Enzyme hydrolysis – low solids (dried MSW)

Enzyme, reaction mixture and conditions. The reaction medium was either 7 mM citric acid buffer or water adjusted to pH 5 with phosphoric acid. Hydrolysis was initially carried out in 250 ml Erlenmeyer flasks, and in 250 ml Nalgene™ PPCO centrifuge bottles from section 4.4 onwards. The substrate was added to the bottle

and diluted to concentrations just above the desired solids content (between 2 - 12% TS) by adding an additional 5 ml of water than required to compensate for water loss in the autoclave. The mixture was then autoclaved at 121 ^{o}C for 15 minutes. Once cooled the substrate was charged with Cellic C-Tec2 or C-Tec3 enzyme at a concentration of 10-50 mg g^{-1} substrate. The mix was then incubated at 50 ^{o}C in an orbital shaker (Gallenkamp S/N: 101400YY2C), hybridisation oven (Hybrid Maxi 14m Thermo, Barnstead, UK) or tumbling mixer (Associated Design & Manufacturing Co., Alexandria, Virginia, US) at 33 RPM.

For the hydrolysis experiments carried out in this research the reaction was allowed to proceed for periods of up to 8 days, with 0.15 ml samples being taken at regular intervals after centrifugation at 5000 g for 5 minutes (Sorwall Legend XTR S/N: 41125528, Thermo Scientific, Waltham, MA USA). The samples were placed in eppendorf tubes and centrifuged at 13,000 g (Galaxy 16DH centrifuge, VWR, UK) the supernatant was removed and the sugar content of the samples was determined by high performance anion exchange chromatography.

Calculation of Glucan conversion

The glucan conversion is the amount of glucan converted during hydrolysis. This was calculated on a total solids basis using the formulae in equation 3.6-1

$$\% \text{ Hydrolysis} = \frac{[Glu] * (A - B + C + D)}{\text{Glucan in sample} * 1.11} \qquad \textbf{Equation 3.6-1}$$

Where

[Glc] - Glucose concentration in the supernatant of the slurry (g g^{-1})

A - Initial water content (g)

B - Water added to cellulose/hemicellulose to make monomeric sugars (g)

C - Monomeric sugars released (g)

D - Enzyme preparation added (g)

Where glucan in sample is measured in grams.

The formula is an iterative one as in order to calculate 'B' and 'C' the % hydrolysis is required. Therefore the formula is iterated until these input and outputs are balanced.

B and C are based on the glucose and xylose liberated as the other monomeric sugar are present in negligible quantities.

3.6.3 Enzyme hydrolysis – high solids (wet MSW)*

Enzyme, reaction mixture and conditions. The commercial cellulase mixture Cellic C-Tec3 (Novozymes, Copenhagen, Denmark) was used for hydrolysis. The reaction medium was water adjusted to pH 5 with phosphoric acid. Hydrolysis was carried out in Nalgene™ PPCO centrifuge bottles to which the substrate was added at the original concentration as provided by Fiberight and diluted to 18.5 or 20% TS before being autoclaved at 121 ^{o}C for 15 minutes. Once cooled the substrate was charged with Cellic C-Tec3 enzyme at a concentration of 30, 50 or 55 mg per g of substrate. The mix was then incubated at 50 ^{o}C in a tumbling mixer at 33 RPM. Hydrolysis was allowed to proceed for periods of up to 8 days, with 0.2 ml samples being taken at regular intervals after centrifugation at 5000 g for 5 minutes (Sorwall Legend XTR S/N: 41125528, Thermo Scientific, Waltham, MA USA). The sugar content of the samples was determined by high performance anion exchange chromatography.

3.7 Substrate treatments

3.7.1 Milling

Dried pulp was milled to ≤0.5 mm in a centrifugal shearing mill (ZM1, Glen Creston, London, UK).

3.7.2 Acetone extraction

Wet pulp at 38.5% TS was refluxed with acetone using a soxhlet extractor. The extraction was carried out with 8 g of pulp for 4 hours using 400 ml acetone. After extraction the pulp was left to air dry for 48 hours, and then placed in an oven at 105 ^{o}C overnight.

3.7.3 1 % NaOH extraction

Wet pulp at 38.5% TS was refluxed with 1% NaOH using a soxhlet extractor. The extraction was carried out with 8 g of pulp for 8 hours using 400 ml 1% NaOH. After extraction the pulp was washed thoroughly in deionised water at 1 MΩ and filtered through a pre-dried Whatman GFC filter paper. The pulp was then oven dried overnight at 105 ^{o}C.

3.7.4 Lignin extraction

The method used for lignin extraction was taken from Siqueira et al., 2013. Dried pulp, both acetone extracted and not extracted, was placed in a 250 ml Erlenmeyer flask. 32 ml of deionised water was used for each gram of pulp and the flask was placed in an orbital shaker at 75 ^{o}C. For each gram of solid the pulp was charged with 0.3 g sodium chlorite and 0.1 ml acetic acid every half an hour for a 2-hour period. After this the pulp was washed thoroughly in deionised water at 1 MΩ to remove any soluble residue and neutralise the pH of the pulp. The pulp was then oven dried overnight at 105 ^{o}C.

3.7.5 Neutral detergent washing

This procedure was adapted from the neutral detergent washing procedure used in Fibercap analysis (Kitcherside et al., 2000). Neutral detergent was made up using the method below

(1) Neutral Detergent Solution (ND):

Disodium ethylene diaminetetraacetate EDTA (dehydrate) 18.61 g
Sodium Borate 6.81 g
Sodium lauryl sulphate 30 g
2-ethoxyethanol 10 ml
Disodium hydrogen phosphate (anhydrous) 4.56 g
Alfa-Amylase solution – Termamyl 300L, type DX available from Foss Tecator

18.61 g of EDTA (Disodium ethylene diaminetetraacetate, $C_{10}H_{14}N_2Na_2O_8x2H_2O$) and 6.81 g of Sodium Borate decahydrate, ($Na_2B_4O_7x10H_2O$), was placed in a beaker with some distilled water, this was heated until the solid dissolved. 30 g Sodium Lauryl Sulphate, ($C_{12}H_{25}OSO_3Na$), 10 ml of 2-ethoxyethanol, ($C_4H_{10}O_2$) and 4.56 g Disodium Hydrogen phosphate, (Na_2HPO_4) were then added with more water, this was placed back on the heat until the contents dissolved. The mixture was then diluted to 1000 ml. The pH was checked to ensure it ranged between 6.9-7.1; if this was not the case the solution was adjusted with NaOH.

Neutral detergent washing procedure

Wet pulp at 38.5% TS was placed in soxhlet cellulose crucibles within a large beaker containing neutral detergent (ND). The pulp was boiled for half an hour in ND, the ND was then removed and replaced with fresh ND for a further 30 minute period of boiling. This second lot of ND was then removed and the pulp was boiled 4 times (for half an hour) in deionised water. The pulp was then placed in a jam cloth and washed further in cold deionised water until a pH of 7 was reached. The pulp was then oven dried overnight at 105 ^{o}C.

3.7.6 Control substrate

After each substrate treatment the treated pulp was hydrolysed alongside a control substrate, the exact conditions for which are given in the results sections. The control substrate was milled to 0.5 mm (unless stated otherwise in the results) and oven dried overnight at 105 ^{o}C. The composition of this dried pulp is given in the results section for each individual experiment.

3.8 Enzyme efficiency enhancement

Note: enzyme efficiency refers not only the amount of sugar produced by a given amount of enzyme but also the concentration of sugar produced in the hydrolysate and the degree of substrate conversion obtained.

The detailed methods for this section can be found in the results & discussion sections 5.3 and 5.4. In order to achieve enzyme efficiency enhancement the following approaches were trialled:

- Glycine addition

- pH adjustment with phosphoric acid as compared to citric acid buffer

- PEG 6000 addition

- Ascorbic acid addition

- Fed-batch substrate addition

- Split-batch substrate addition

- Enzyme dilution

3.9 High solids hydrolysis study

The detailed methods for this section can be found in the results & discussion sections 5.3.3 and 6.2. In order to achieve enzyme efficiency enhancement and determine the limitations of the substrate digestibility at high solids the following approaches were trialled:

- Hydrolysis at high solids with PEG 6000
- Maximum conversion capability

3.9.1 Two-stage hydrolysis

A series of two or multiple stage hydrolyses were carried out where the hydrolysis was split into two 48-hour segments with a sugar removal step in between the hydrolysis. This sugar removal was carried out by either fermentation or liquid removal through centrifugation. The methods in this section refer to the results outlined in the section 6.5 - 6.8.

3.9.1.1 Two stage hydrolysis with intermediate fermentation

20% TS pulp was hydrolysed for 24 or 48 hours followed by a 24-hour fermentation that was sufficient to consume most of the sugar produced. After fermentation the reaction medium was readjusted to pH 5 to give optimum conditions for hydrolysis, and incubated with tumbling at 50 ^{o}C for a further period of between 2 to 5 days. During all stages samples were taken for quantification of the type and yield of individual sugars, from which ethanol yield could be calculated. The experiment was repeated twice, on both occasions in duplicate.

3.9.1.2 Fermentation

All ethanol fermentations used Youngs' super wine yeast compound (Youngs, Bilston, UK). 1 g of yeast was cultured for 24-72 hours in 200 ml of pH 5 basal medium containing 30 g L^{-1} glucose and 10 g L^{-1} yeast extract. The yeast was harvested by centrifuging a portion of the fermentation broth for 10 minutes at 5000 g. Once centrifuged the supernatant was removed leaving a wet yeast pellet of 1.7 g which was re-suspended in 2 ml of pH 5 solution. This suspension was then added to centrifuge bottles containing hydrolysate following a purge of the headspace with nitrogen. Fermentation was carried out over 24 hours at 30 ^{o}C with orbital shaking.

3.9.1.3 Two stage hydrolysis with intermediate product removal

25% TS dried pulp was hydrolysed for periods of 48 hours with 50 mg C-Tec2 or C-Tec3 g^{-1} pulp. After hydrolysis the hydrolysate was centrifuged for 10 minutes and the supernatant removed. The supernatant was replaced with the same volume of water adjusted to pH 5 with phosphoric acid. The mixture was then set to continue hydrolysis for a further 2-4 days.

3.9.1.4 Enzyme recovery after hydrolysis – Wash step

After hydrolysis of MSW pulp for 48 hours the hydrolysate was centrifuged and the supernatant removed. The residual centrifuged pulp was then washed once for 1 hour in 100 ml of water at either pH 5 or pH 9. The enzyme activity of the

supernatants was determined by first separating the enzymes from the sugars by ultrafiltration and then using the filter paper and β-glucosidase assay methods (see section 3.10).

The washed residue was re-suspended in pH 5 water (volume equivalent to the removed sugar solution) and, without the addition of further enzyme, was then incubated at 50 ^{o}C and tumbled to promote second-stage hydrolysis. Samples were removed from the hydrolysis reaction mixture at regular intervals and assayed for their sugar concentration using HPLC. Experiments were carried out in duplicate and repeated twice.

β-glucosidase addition. After centrifugation, washing and re-suspension of hydrolysed pulp in pH 5 solution, β-glucosidase was added at concentrations of 25 or 12.5 mg enzyme g^{-1} pulp (original weight) and hydrolysis conditions restored. Samples were then taken for sugar analysis over the second hydrolysis period of up to 4 days. The experiment was carried out in duplicate and repeated twice.

These methods refer to the results outlined in the section 6.6 & 6.7.

3.9.2 Washwater recycle experiments

Enzyme, reaction mixture and conditions. The commercial cellulase mixture Cellic C-Tec3 (Novozymes, Copenhagen, Denmark) was used for hydrolysis. The reaction medium was water adjusted to pH 5 with phosphoric acid either with or without PEG 6000. Hydrolysis was carried out in 250 ml Nalgene™ PPCO centrifuge bottles to which wet MSW pulp from Fiberight was added. The pulp was provided at 45-50% TS and was diluted to 40% TS with the pH 5 water before being autoclaved at 121 ^{o}C for 15 minutes: the quantity of pulp solids used in each experiment was 20 g. Once cooled the substrate was charged with Cellic C-Tec3 enzyme at a concentration of 30 mg g^{-1} of substrate. The mix was then incubated at 50 ^{o}C in a tumbling mixer at 33 RPM for 1.5 hours before being diluted with pH 5 water (adjusted with phosphoric acid) in the first round or with washwater adjusted to pH 5 from a previous round of hydrolysis. The hydrolysis was carried out for 48 hours at which point the hydrolysate was centrifuged at 5000 g (Sorwall Legend XTR S/N:

41125528, Thermo Scientific, Waltham, MA USA) for 10 minutes. The supernatant sugar solution was removed and replaced with 100 ml of water adjusted to the selected pH value either with or without PEG 6000 (see individual experiments in chapter 7) and then left to mix in an orbital shaker (Gallenkamp S/N: 101400YY2C), at 50 rpm and 25 ^{o}C for 1 hour. After the wash, the water was removed by centrifugation at 5000 g (Sorwall Legend XTR S/N: 41125528, Thermo Scientific, Waltham, MA USA) for 10 minutes. The pH of this washwater was measured and adjusted to pH 5 (unless otherwise stated) and used as the dilution water for the subsequent round of hydrolysis. All experiments were carried out in duplicate.

In all experiments when the pH was adjusted manually pH reduction was achieved by adding phosphoric acid and pH increase by adding sodium hydroxide.

3.9.2.1 **Second stage hydrolysis from washwater recycle experiments**

The residual solid obtained after concentrated sugar removal and washing was left in the 250 ml Nalgene™ PPCO centrifuge bottles to undergo a second round of hydrolysis. This hydrolysis was undertaken with differing quantities of washwater. The wash slurry (i.e. residual solid and remaining washwater) was adjusted to pH 5 with phosphoric acid. The washwater used was that from the wash after the first stage of hydrolysis for that particular round (see figure 3.9-1). No additional enzyme was added and hydrolysis was allowed to proceed for 3 days at 50 ^{o}C in a tumbling mixer at 33 RPM. The full first and second stage washwater procedure is shown in the schematic diagram below.

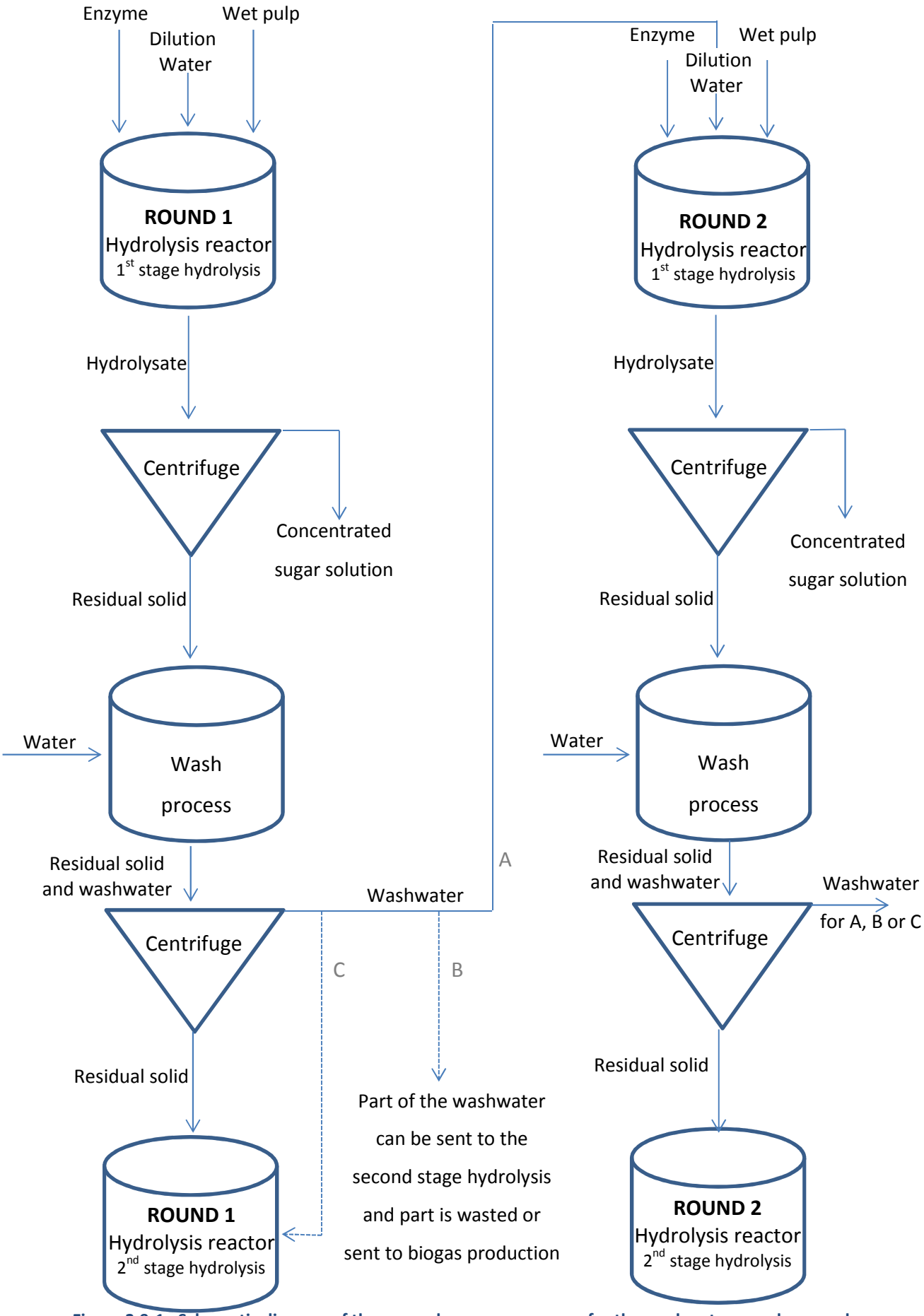


Figure 3.9-1. Schematic diagram of the general process sequence for the washwater recycle procedure

3.10 Enzyme Activity Assays

Enzyme assays. Filter paper activity (FPA), and β-glucosidase activity were measured using methods adapted from that of Ghose, 1987.

Filter paper activity. This was used to determine cellulase activity of recovered enzymes from hydrolysate after separation by ultrafiltration. Whatman No. 1 filter paper was cut into strips weighing 50 mg. A strip was placed into a test tube with 1 ml of water adjusted to pH 5 with phosphoric acid and equilibrated to 50 ^{o}C. 0.5 ml of the ultrafiltered test solution was then added and the mixture was incubated at the same temperature for 1 hour. Further reaction was then stopped by placing the tubes in boiling water for 5 minutes, and the glucose concentration was measured by HPAEC-PAD. All assays were carried out in triplicate. The test was standardised by comparison to the FPA of C-Tec3 at a dilution of 1 g enzyme preparation per 80 g pH 5 water, as this was the enzyme concentration used in the hydrolysis experiment in this work.

B-glucosidase assay. The β-glucosidase activity was measure by adding 1 ml of a 19.6 mM cellobiose solution at 50 ^{o}C to 1 ml of enzyme solution at 50 ^{o}C. The mixture was incubated in test tubes at 50 ^{o}C for 1 hour. The reaction was quenched by placing the tubes in boiling water for 5 minutes. The liberated glucose was measured by HPAEC. All experiments were performed in triplicate.

3.11 Ultrafiltration for the separation of sugar and enzymes

This was carried out using an AKTA crossflow automated filtration system (GE Healthcare Life Sciences, Uppsala, Sweden). Samples were first filtered through a mesh with a pore diameter of 0.5 mm. The filtered liquid was then run through a 500 kD hollow fibre with a 1 mm lumen i.d. The flow was shear controlled at 8000 s^{-1} with a transmembrane pressure (TMP) of 0.5 bar. The permeate product was collected and used as the feed for a Kvickstart cassette with a 10 kD cut off. This cassette was used to separate the enzymes from the sugars. The cassette was flux controlled at a TMP of 1 bar.

Chapter 4: Results & Discussion - Compositional analysis, enzymes and hydrolysis

4.1 Introduction

The purpose of this part of the research was to characterise the substrate in detail, including its behaviour during hydrolysis. Characterisation was carried out in terms of fibre, sugar potential and non-fibre components. The typical hydrolysis behaviour of the substrate was then analysed and compared to other control lignocellulosic materials. Various mixing strategies were also trialled to observe how these affected hydrolysis. Lastly a comparison was made between the two enzymes preparations used in this research, Cellic C-Tec2 and 3. This was done to determine their differences and to identify any improvement offered by the newer preparation C-Tec3.

4.2 Compositional analysis

Objective: To determine the composition of the substrates used in this work.

Method summary: The composition of the substrate as received and after acetone washing to remove extractives was determined using the standard NREL analysis. In addition Fibercap, total solids and ash content, and elemental analysis were also conducted. See section 3.1 for detailed methods.

A control substrate of Fisher brand filter paper (Cat no. FB59035, Fisher Scientific, Loughborough, UK) was used in some experiments and the composition of this was also analysed.

Results: The research used a washed and sorted fraction of MSW as the lignocellulosic feedstock for sugar and/or ethanol production, obtained from Fiberight, Lawrenceville, Virginia. Each batch of pulp varied slightly in composition due to non-homogeneous nature of the substrate and the variability in the Fiberight MSW pulp production process. The composition given here is for a batch of pulp

which is representative of that used in the research. The compositional analysis of this MSW pulp sample, given in Table 4.2-1, indicated that as expected the substrate is high in sugars, with a compositional ratio of 56:12:26: 5 of Glucan: Hemicellulosic sugar chains other than glucan : Lignin & pseudo lignin : Ash.

Note: Pseudo lignin refers to all unclassified and acid insoluble material which is not ash.

Table 4.2-1. Average compositional analysis of MSW pulp

Method	Celluose* Or Glucan	Hemicellulose* Or Xylan, Mannan Arabinan & Galactan	Lignin (Acid insoluble and acid soluble)	Ash	Missing mass	Mass closure	Proportion of original mass
NREL	55.5 ± 0.4	12.0 ± 0.1	26.3 ± 0.7	1.8 ± 0.0	4.1 ± 0.5	95.9 ± 0.5	N/A
NREL of AEP	60.4 ± 2.5	16.9 ± 0.5	14.7 ± 0.1	1.7 ± 0.1	8.0 ± 3.4	92.0 ± 3.4	92.3 ± 2
Fibercap*	63.6 ± 0.0	10.9 ± 0.0	9.6 ± 0.0	4.1 ± 0.0	11.8	88.2	N/A

Compositional analysis was performed using 3 methods 1. NREL compositional analysis, 2) an acetone extraction of pulp followed by NREL compositional analysis and 3) Fibrecap compositional analysis. All values in the table are given as percentage (%) on total oven dried solids (TS). Nomenclature; AEP – acetone extracted pulp; (*) refers to the fact that the fibrecap analysis calculates the composition based on cellulose and hemicellulose rather than the sugar chains which is the case with the NREL analysis

Table 4.2-2. Organic solids analysis of MSW pulp.

Organic component of solid	Ash on solid	Calcium carbonate on ash
95.4	4.6	4.2

The values in the table are percentages of total oven dried solids (TS)

Table 4.2-3. Carbon, nitrogen, hydrogen and sulphur content of MSW pulp.

Carbon (C)	Nitrogen (N)	Hydrogen (H)	Sulphur (S)	Total weight in (C, H, N, S)
44.5 ± 1.1	0.0	6.0 ± 0.2	0.1 ± 0.0	50.6

All values in the table are given as percentage (%) on total oven dried solids (TS)

Table 4.2-4. NREL compositional analysis of Fisher brand filter paper.

Substrate	Glucose	Xylose	Arabinose Galactose Mannose	Lignin	Ash	Mass closure
Fisher brand filter paper	83.5 ± 0.4	14.1 ± 0.1	2.0 ± 0.1	0.0	0	99.6 ± 0.4

All values in the table are given as percentage (%) on total oven dried solids (TS)

Discussion: Due to the non-homogenous nature of the feedstock it is difficult to provide an exact definition of the pulp composition, but the methods used allowed quantification of the average and most important characteristics. The most

important component analysed was the glucan content. This was typically 55% of total solids (TS); however, a higher glucan content of 58% was measured in one batch of wet pulp. A dried pulp substrate was used at the beginning of this research and this contained a glucan content of 63%.

For the most part in this research the standard NREL method for compositional analysis was used (see section 3.1.4). It was found that although this method was suitable for quantifying the available sugar content it was not sufficient for closure of the mass balance. The other shortcoming of this method is that it quantifies the pulp in terms of available monomeric sugars rather than fibre fractions i.e. cellulose and hemicellulose. As a result some of the glucose which is present as part of the hemicellulose is accounted for as glucose potential or glucan, which is not strictly the case, and this material would not be broken down by cellulase enzymes but would require hemicellulases for its release.

A modification of the NREL method was also performed where acetone extraction was carried out prior to the acid hydrolysis to remove extractives found in the feedstock that can interfere with compositional analysis. Other studies using acetone or 1% sodium hydroxide to remove extractives on whole wood pulps suggest that it is possible to obtain mass closure of materials that contain fats, oils and waxes once these are removed by methods such as solvent extraction (Burkhardt et al., 2013). In the current research it was found that using the NREL method alone tended to overestimate the amount of lignin present in the sample, as many of the extractives or other unclassified organic matter were counted as lignin (known as pseudo lignin) (see table 4.2-1). Acetone extraction allowed removal of these compounds which reduced this overestimation. After the removal of (or part of) the pseudo lignin the actual lignin content was more closely related to that obtained in the Fibrecap method (see table 4.2-1).

The ash content given by the NREL method was also slightly low as some of the ash components, such as paper fillers (including calcium carbonate, kaolin and clays) and other acid soluble components, can dissolve in the acid and are therefore counted as ‘missing mass’ rather than ash. In order to determine the true ash

content of the sample a total and volatile solids analysis was carried out. This was done using the following staged process: (i) oven dry the pulp at 105 ^{o}C overnight and weigh; (ii) ash at 550 ^{o}C for 2 hours and weigh; and (iii) re-ash the sample at 900 ^{o}C. This last step converts any calcium carbonate to calcium oxide which enables the determination of calcium carbonate in the ash.

Comparison of the results from this total solids analysis (table 4.2-2) to the ash content from the NREL method shows that the real ash content is almost 3% higher than the NREL determined ash content. This difference accounts for most of the missing mass as determined by the NREL method.

The Fibercap method is widely used in the determination of fibre content in foodstuffs and animal feed. The method involves calculation of the cellulose, hemicellulose and lignin content by weight difference after a series of washes with various detergents and acids. Due to the complex nature of the MSW substrate it is likely that each step may not remove all the extraneous compounds present in the material, which could lead to overestimation of the fibre content. The method is a 3-step process. In the first step the material is washed in neutral detergent to remove the extractive components and some nitrogenous matter like proteins: it also chelates calcium and removes pectins and some non-fibrous matter (Van Soest et al., 1991). Following this step the substrate is ashed and this is the ash content given in table 4.2.1. It is thought that this ash step removes some of the calcium carbonate as well as other oils, waxes and extractives in the material. Hence, this accounts for the difference in the ash content between the fibercap analysis in table 4.2.1 and total solids analysis in table 4.2.1. The ash content as given by the NREL method is any inorganic material that is remaining after an acid hydrolysis at 72%. This step will solubilise a range of compounds including perhaps certain plastics, clays and organics which have undergone structural changes due to the various processing steps carried out on the MSW. Thus, the ash content in the NREL will be much lower than the actual ash content when using a complex matrix such as MSW.

The remaining material is assumed to be composed purely of cellulose, hemicellulose and lignin. In the second step this fibre-rich material is washed in acid

detergent to remove the hemicellulose, leaving behind the cellulose and lignin. If small particles of plastics, metal or paper fillers such as clays and kaolin are present, these may not be removed in either the first or second step, and thus their weight will be counted as lignin or cellulose. The third step which involves treatment with 72% acid can solubilise a number of these extraneous compounds. This could lead to overestimation of cellulose, however, as the strong acid step is designed to remove cellulose and leave behind the lignin. There is also a component of lignin called the acid soluble lignin which will then be counted towards the cellulose fraction. Furthermore, processing steps employed either in the primary product formation or secondarily by Fiberight could result in structural changes in the lignin, which in turn could increase its acid solubility. This would also result in overestimation of the cellulose fraction. Thus the 'by weight' determination of the fibre fractions used in the Fibercap method is not considered to be the most suitable compositional analysis method for this substrate.

From the above analytical results and discussion it is clear that various inorganic or organic components (pseudo lignin) may be present in the material. A discussion on how these may influence hydrolysis is presented in section 5.2.2 below.

It is also evident from these different compositional analyses that glucan is the major sugar component of this substrate, with a much lower proportion of the other neutral sugars found in hemicellulose. Thus most of the research presented here focused on improving glucan conversion rather than on the overall sugar yield. In reality the small amount of other sugars released aside from glucose would also contribute to product concentration and in turn to potential ethanol yield or other bio-refinery product. However as these other neutral sugars are composed mostly of xylose, which is a C5 sugar, recombinant yeast/bacteria would be required for fermentation.

In addition to the pulp substrate a control filter paper substrate of Fisher brand filter paper was sometimes used. The compositional analysis of this confirmed that it contains both cellulose and hemicellulose, but no lignin or ash (see table 4.2-4).

4.3 Enzyme Hydrolysis

4.3.1 Testing typical hydrolysis parameters

Objective: To determine the hydrolysis behaviour of the MSW derived paper pulp when using the cellulase enzyme preparation Cellic C-Tec 2.

Method summary: Enzymatic hydrolysis was carried out using dried MSW paper pulp at an enzyme loading of 5-13% C-Tec2 g^{-1} pulp. Hydrolysis was carried out at low total solids contents (between 2 – 7% TS) using orbital shaking in Erlenmeyer flasks.

Results & Discussion: At a low total solids content of 2% TS, it was possible to obtain a high sugar conversion of ~80% in a period of 48 hours as shown in figure 4.3-1. The hydrolysis followed a typical cellulosic hydrolysis curve where most of the substrate is converted in the first 24 hours, after which the rate decreases with time. It is thought that a conversion yield of less than 100% is due to the use of dried pulp (see section 2.2.4 of literature review) and the use of C-Tec2 rather than the newer enzyme C-Tec3 (see section 4.4).

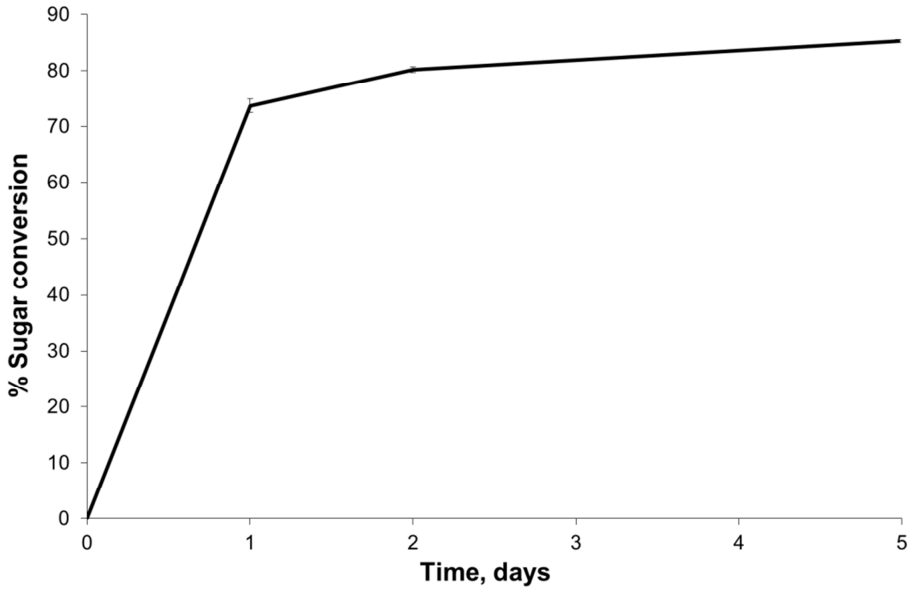


Figure 4.3-1. Percentage of total sugar conversion for hydrolysis performed at of low total solids (~2%) using 50 mg C-Tec2 g^{-1} pulp

Figure 4.3-2 shows the results at different solids contents and enzyme doses, and two important hydrolysis trends can be seen. Firstly, an increase in enzyme dose i.e. doubling of the enzyme dose, does not give a proportional increase in conversion, and this result is in agreement with the findings of other researchers (Kristensen et al., 2009; Roche et al., 2009; Shen et al., 2011). It was found that an increase in dosing of C-Tec2 from 6% enzyme to 12% enzyme at a solids content of 2.5% TS increased conversion from ~45% to ~67% over a 48-hour hydrolysis period (figure 4.3-2 A).

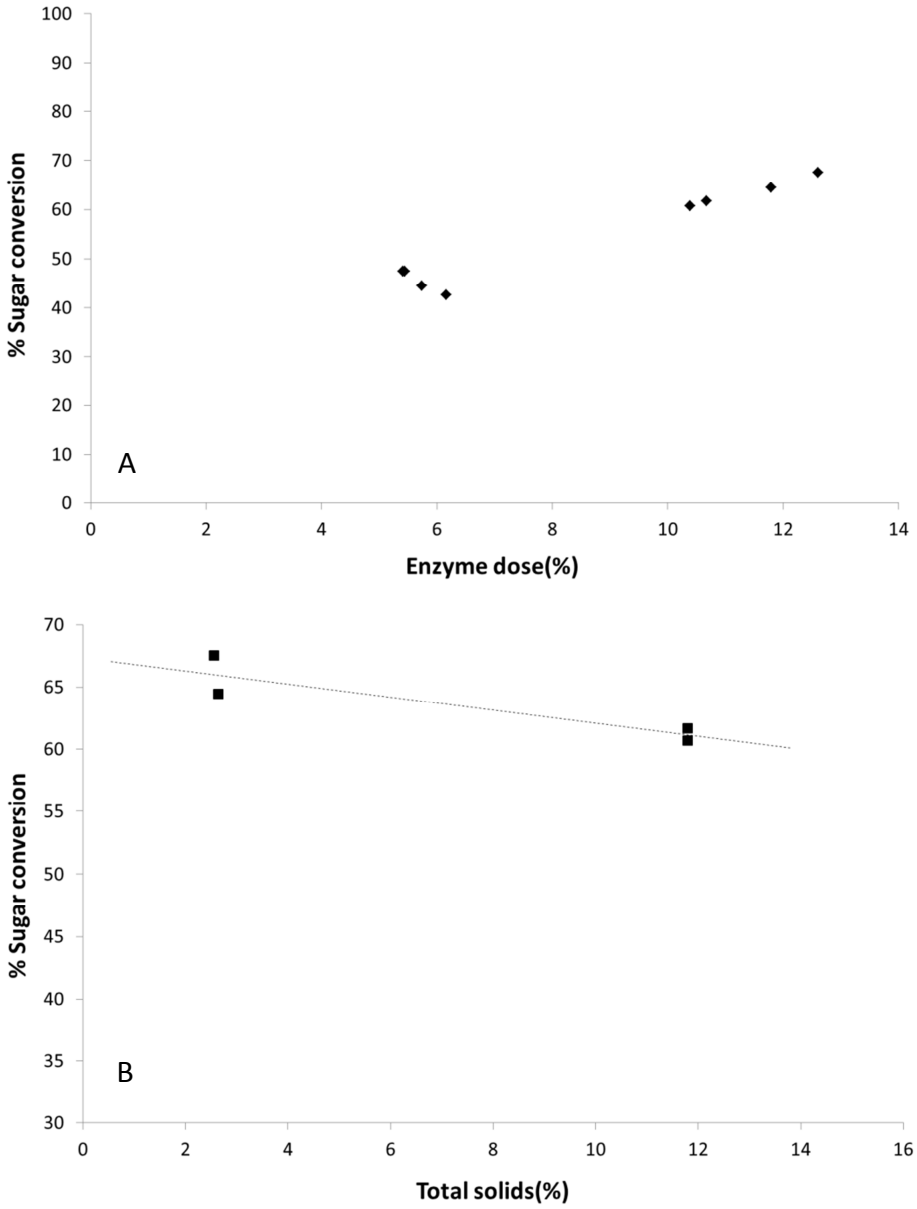


Figure 4.3-2. (A) Sugar conversion (%) in relation to enzyme dose on total solids (%); (B) Sugar conversion in relation to solids content (%)

Secondly, it appears that the substrate is subject to the solids effect where an increase in solids content leads to a decrease in the proportion converted. At 2% solids an enzyme dosing of 12% g^{-1} pulp led to a 48-hour sugar conversion of 67%; at 7% solids this same enzyme dosing gave a conversion of 61%. Thus, an increase of the dry matter content from 2 - 7 % led to a decrease in conversion of 6% (figure 4.3-2 B). This reduction in conversion is quite pronounced due to the dried substrate used and the fact that total sugar was analysed not just glucan conversion. As noted in the literature review this effect is thought to be due to a variety of causes including inhibition by reaction products and/or solids interfering with enzyme adsorption onto the substrate (Kristensen et al., 2009; Wang et al., 2011). These other studies also reported that as the solids content increases further this decrease in percentage conversion becomes even more pronounced.

4.3.2 Mixing Configuration method development

Objective: To determine the most appropriate mixing strategy for the hydrolysis of MSW pulp.

Method summary: Mixing of dried MSW pulp at low and high solids contents was carried out using various mixing set-ups: 1) orbital shaking; 2) rotational mixing in a rotisserie-style mixer (figure 4.3-3); and 3) gravity mixing using a tumbler (figure 4.3-4). The hydrolysis was carried out with enzyme dose ranging from 10 -50 mg g^{-1} pulp for up to 3 days.

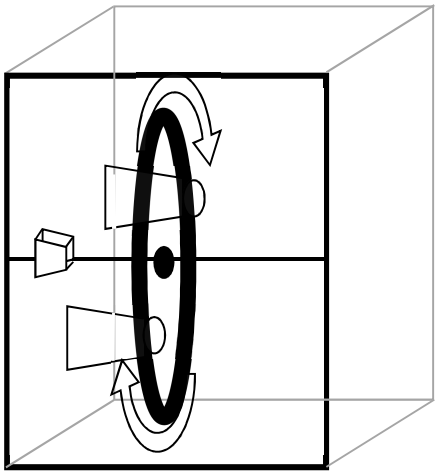


Figure 4.3-3. Schematic representation of rotational mixing using a rotisserie-type mixer from a hybridisation oven

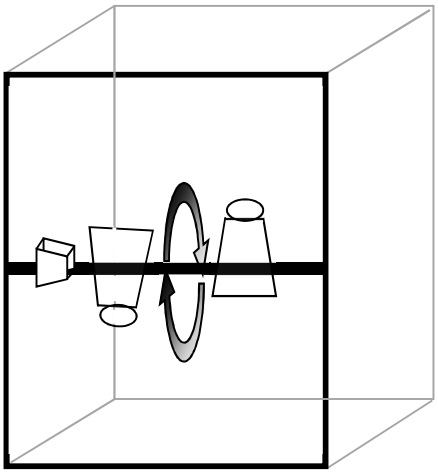


Figure 4.3-4. Schematic representation of the tumbling mixing scheme. The tumbler used in this work rotated at 33 rpm. The bottles were placed in the tumbler as depicted allowing gravity or free fall mixing.

Results & Discussion: The slurries in the experiments described in section 4.3.1 above were mixed using orbital shaking. Other mixing methods such as CSTR or gravitational mixing have been shown by other researchers to be superior to this mixing set-up, however, especially at high solids concentrations (Ludwig et al., 2014; Roche et al., 2009).

Two different mixing strategies were trialled here and it was found that for slurries of up to 16% TS the rotational mixing strategy (figure 4.3-3) achieved observable liquefaction more rapidly than the orbital mixing strategy. Experiments showed that with rotational mixing liquefaction could be achieved within 12-36 hours using 20 mg C-Tec2 g^{-1} pulp, whereas when using orbital shaking no liquefaction was observed in the same time period.

Further experiments were carried out to investigate whether liquefaction could be achieved with an even higher solids concentrations and lower enzyme dose using rotational mixing. It was observed that for slurries with a total solids content greater than 17% there was little liquefaction within a 48-hour period when using ≤ 20 mg C-Tec2 g^{-1} pulp. For an 18 %TS solution there was no observable liquefaction when using 10 mg C-Tec2 g^{-1} pulp.

This result is thought to be due to two factors, namely (i) the use of a dried substrate which is harder to wet and mix and (ii) formation of large fibre flocs or networks by the paper pulp which are difficult to break up. The results suggested that a tumbling action with free fall mixing would be better than rotational mixing. Accordingly a tumbling mixer was set up (figure 4.3-4) and this was readily able to liquefy samples with solids contents of up to 25% TS within 24 hours at enzyme dosages between 25 and 50 mg enzyme g^{-1} pulp.

These results are in accordance with Roche et al., 2009 who demonstrated that gravitational mixing was a better way to obtain liquefaction in a shorter period of time using a hydrothermally pre-treated corn stover. Ludwig et al., 2014 observed a significant influence of the solids effect when using shake flasks for hydrolysis at above 13% TS for alkaline treated wheat straw. The large uptake of water with this substrate made the mixture very viscous even at lower fibre loadings (< 15%) (Ludwig et al., 2014). The fast water uptake and the production of a highly viscous material is similar to that seen with the MSW pulp used in this research.

Conclusion: Tumbling was found to be superior as a mixing strategy to rotational mixing and orbital shaking. Thus it was decided that this free fall mixing would be used for the remainder of the research.

4.4 Cellic C-Tec2 vs C-Tec3

The initial experiments described in this work were carried out with C-Tec2. Later experiments, however, were completed using the newer enzyme C-Tec3 once it became available. It was therefore considered important to study the difference between these two enzymes to investigate whether the new generation of enzymes offered any improvement in performance and if so how much.

Objectives: To compare the performance of C-Tec2 and C-Tec3 in hydrolysis of the test substrates.

Method Summary: Hydrolysis was carried out on Fisher brand filter paper (Cat no. FB59035, Fisher Scientific, Loughborough, UK) and on wet MSW pulp using both the C-Tec2 and C-Tec3 enzyme preparations. Hydrolysis was carried out at 20% TS using 30 mg enzyme g^{-1} filter paper over 6 days, and at 10% TS using 50 mg enzyme g^{-1} MSW pulp for 2 days. For more details on the standard enzymatic hydrolysis process see section 3.6.

Results: Figure 4.4-1 shows that hydrolysis of filter paper using C-Tec3 yielded 10.9 wt% glucose in hydrolysate while C-Tec2 yielded 9.8% over a 6 day hydrolysis period. Overall this corresponds to a 4 ± 1 % improvement in glucan conversion using C-Tec3: this improvement was seen at 2 days and maintained until day 6. The overall glucan conversion for filter paper using 30 mg g^{-1} C-Tec3 was 40%.

A similar experiment was carried out over 3 days using MSW pulp at 10% TS and 50 mg g^{-1} C-Tec3 and C-Tec2. In this experiment a 4 ± 1% improvement was also seen over the hydrolysis period when using C-Tec3 over C-Tec2 . In this later experiment glucose content was only measured on day 3 hence graphical results are not presented here.

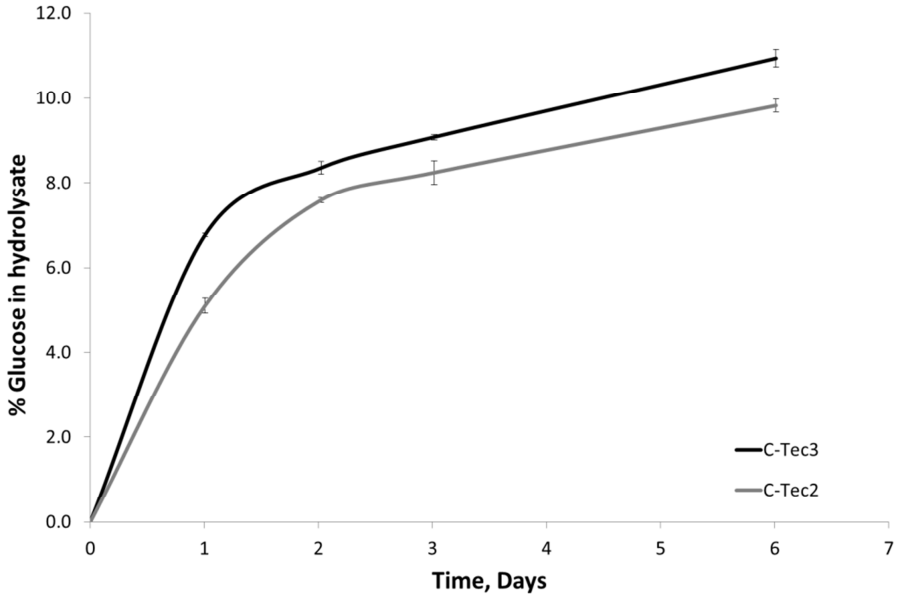


Figure 4.4-1. Enzyme hydrolysis on filter paper using the enzyme preparations C-Tec2 & C-Tec3. 30 mg g^{-1} enzyme

The data in figure 4.4-1 shows that the rate at which hydrolysis occurs, as indicated by the amount of sugar produced in a given time, improves with C-Tec3. It is noted however that both enzyme preparations still follow the same typical hydrolysis curve, indicating that the mode of action of these enzyme remains the same, although the properties of the enzyme system such as product tolerance and rate of reaction have been improved.

Discussion: Novozymes enzyme complexes were chosen for use in this research as the company is one of the leading corporations in the field making state of the art products and investing heavily in development. In addition, they have a collaboration agreement with Fiberight which made it easier to obtain various enzyme preparation. Upon initiation of this research in May 2011 the enzyme preparation on the market was Cellic C-Tec2 with Cellic C-Tec3 only becoming available for use in March 2012. The initial experiments were therefore carried out with C-Tec2, but it was decided to change to C-Tec3 when this became available as this preparation is likely to be used commercially. In this experiment and in later work, it quickly became evident that Novozymes had significantly improved on some of the limitations of C-Tec2 in the new product.

The improvements seen in C-Tec3 were attributed by the company to 'proprietary enzyme activities, including advanced GH61/AA9 compounds, improved β-glucosidase as well as a new array of hemicellulase activities' (Novozymes, 2012). The main cellulase enzyme system was still produced from the fungi *Trichoderma Resei*, however, and thus the structure and function of the main cellulose degrading enzymes remained the same, given that the cellobiohydrolase still contained a carbohydrate binding domain which enabled it to move processively along a cellulose fibril. Thus strategies which utilised and manipulated the enzymes' mechanisms and their binding capacity were still relevant to the newer enzyme preparation. In this experiment the newer enzyme was found to have greater product tolerance and a faster rate of reaction. Thus strategies that enhanced the performance of C-Tec2 when tested in subsequent work were sometimes not as effective when the newer preparation, C-Tec3, was used, and as a result new pathways had to be investigated.

In terms of substrate limitations, processes or reactions that are substrate specific should affect both enzyme preparations in a similar way as the enzyme system had not changed, nor had its ability to form unproductive hydrophobic bonds with various non-cellulosic components within the system. Thus it was considered that results from experiments looking at substrate properties and their effects on hydrolysis using C-Tec2 should translate to similar results if C-Tec3 was used. Some experiments, however, were repeated with C-Tec3 when the substrate properties had a significant impact and/or it was unclear how the new enzyme would behave. Furthermore as new aspects of the enzyme system became evident from the literature or through experimentation, simple verification tests were performed with the newer enzyme preparation only.

4.4.1 Enzyme loading analysis

Objective: To determine an optimum loading and/or a relevant range of enzyme loadings for each enzyme preparation.

Method summary: MSW pulp was hydrolysed for 48-hours at 6.5% TS using either C-Tec2 or C-Tec3 at enzyme doses between 10 and 100 mg g^{-1} pulp.

Results: Figure 4.4-2 shows dose dependence curves for C-Tec2 and C-Tec3. Although there is an increase in conversion with an increase in enzyme loading, it is not directly proportional to the increased dosage in either case. This result suggests that it is preferable to use dosages of ≤ 50 mg g^{-1} pulp for C-Tec2 and ≤ 30 mg g^{-1} pulp for C-Tec3.

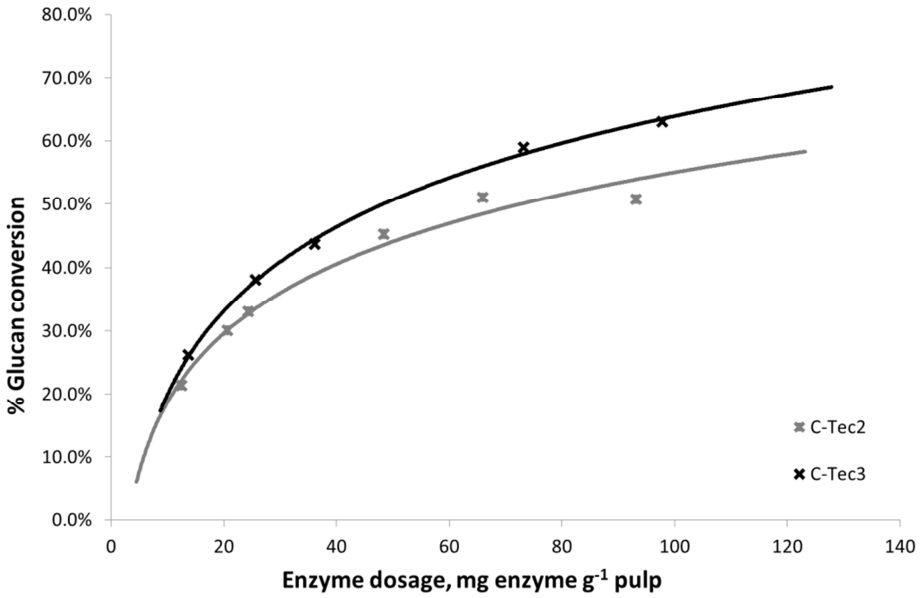


Figure 4.4-2. Conversion of MSW pulp over 2 days with 6.5% total solids using both C-Tec2 and C-Tec3 at enzyme doses between 10 and 100 mg g^{-1} pulp.

Discussion: Pulp at 6.5% TS was used in this experiment as this solids concentration is only marginally affected by the solids effect and the performance of the enzyme can thus be seen without interference from various other inhibitory parameters.

Figure 4.4-2 shows that the amount of conversion per mg enzyme is higher at lower enzyme doses (≤30 mg g^{-1} pulp) than at higher doses. It is not practical to use enzyme loadings of 10-20 mg enzyme g^{-1} pulp, however, because of the long hydrolysis times required at these low doses. Although it is likely that an enzyme dosage of 30 mg g^{-1} pulp would be sufficient for hydrolysis at low solids it was decided to use a loading of 50 mg g^{-1} pulp in most of the following experiments to ensure that the hydrolysis systems were not enzyme limited and any effects from

changes in substrate could be related to the substrate rather than an enzyme deficiency.

4.5 Conclusions from chapter 4

In this chapter it was found that the standard NREL method was an adequate method for the compositional analysis of MSW pulp. It was noted, however, that the method underestimated the ash content and overestimated the lignin content in the sample. This overestimation could be corrected by performing an acetone extraction of the pulp prior to the NREL compositional analysis. The actual ash value was taken from performing a standard total solids and ash content analysis of the MSW pulp without any other treatments. It was found that the substrate contained a NREL compositional ratio of 56:12:26: 1.8 of Glucan: Hemicellulosic sugar chains other than glucan: Lignin & pseudo lignin: Ash. From consideration of the acetone extraction results, the total and volatile solids and data from Fibercap analysis it is believed that the true lignin content of the material is between 9-14% and the true ash content is on average ~4.5% on a total solids basis.

The hydrolysis behaviour of the MSW pulp was found to be similar to that of other lignocellulosic substrates in that the hydrolysis rate is initially rapid but decreases with time. This substrate is subject to the solids effect where an increase in solids content leads to an overall decrease in percentage sugar conversion. Also, an increase in enzyme dosing does not lead to a proportional increase in substrate conversion. At low total solids (2% TS) an overall sugar conversion of >80% was achievable with dried MSW pulp and Cellic C-Tec2; however it is believed that this would be higher with a wet pulp direct from the Fiberight process plant and the use of the newer enzyme C-Tec3

Investigation of 3 mixing strategies (orbital shaking, rotational mixing and tumbling mixing) showed that tumbling was the most effective. Using this approach it was possibly to liquefy a mixture with high solids (>20%) in 24 hours whereas this was not possible with the other mixing strategies and with enzyme doses less than 50 mg g^{-1} pulp.

Hydrolysis was performed using the Cellic range of enzyme from Novozymes. At the start of the project only C-Tec2 was available; however in March 2012 it was possible to access C-Tec3. Thus a comparison of activity was carried out for these different preparations and it was found that use of the newer enzyme preparation Cellic C-Tec3 resulted in higher glucose yields and faster initial rates of reaction. For both enzyme preparations it was found that a low enzyme dose ≤30 mg g^{-1} pulp gave the best conversion per mg of enzyme; however these low doses mean that longer process times are required to achieve adequate sugar yields. Thus, in the next chapter an enzyme loading of 50 mg g^{-1} pulp was used so that the experiments were not enzyme limited.

Chapter 5: Results & Discussion - Substrate limitations and enzyme activity enhancements

5.1 Introduction

The MSW pulp used in this research is a complex substrate and contains many compounds that may be beneficial or inhibitory to hydrolysis. This section of the work looked at parameters which may limit conversion, and whether it is possible to manipulate the substrate or use additives to improve conversion yields. It also investigated enzyme and substrate addition strategies to help determine the optimum hydrolysis set up.

5.2 Substrate limitations

From the results in section 4.3.1 it was clear that the substrate used in this research shows similar behaviour to other lignocellulosic materials even though the matrix is possibly a little more complex. It was considered important to investigate whether use of this substrate posed any particular limitations which could lead to decreased hydrolysis yields.

A number of factors were manipulated to observe their effect on hydrolysis performance. These included: (i) particle size or fibre length; (ii) the presence of extractives such as oils, waxes and other solvent soluble compounds; (iii) the presence of lignin; and (iv) the presence of calcium carbonate. Manipulation strategies trialled to address these factors were: (i) milling to ≤0.5 mm to reduce particle size and floc forming capacity and to decrease crystallinity; (ii) extractives removal with 1% sodium hydroxide or acetone to increase cellulose accessibility; (iii) lignin removal with sodium chlorite and acetic acid to improve substrate accessibility; (iv) calcium carbonate removal with sulphuric acid or neutral detergent washing.

5.2.1 Milling

Objective: To assess the effect on hydrolysis of increasing substrate accessibility by reducing particle size.

Method Summary: Dried pulp was milled to 0.5 mm in a centrifugal shearing mill, and the resulting pulp was hydrolysed at 8% TS with 35 mg C-Tec2 g^{-1} pulp for 3 days in 7 mM citric acid buffer to maintain the pH of the hydrolysis system at 5. Unmilled dried MSW pulp was run under the same conditions to act as a control (methods section 3.7.1 and 3.7.6). The composition of the milled and unmilled substrate was 63 : 9 : 7 : 10 : 2 of glucan : xylan : mannan / araban / galactan : lignin : ash.

Results: As can be seen in figure 5.2-1 the glucan conversion over a 3-day hydrolysis period of pulp milled to less than 0.5 mm was 57.5%, while unmilled pulp achieved a conversion of 64.4%.

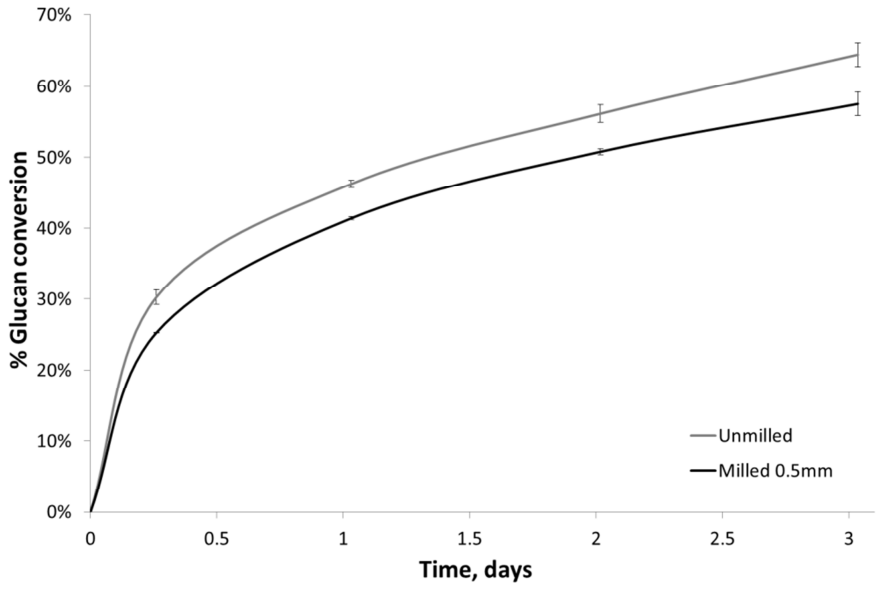


Figure 5.2-1. Enzyme hydrolysis of dried milled MSW pulp (black) and unmilled pulp (grey) performed at 8% TS using 35 mg g^{-1} C-Tec2

Discussion: As can be seen the milling treatment used in this work did not improve the hydrolysability of the pulp and in fact decreased the hydrolysis yield (figure 5.2-1). There are a number of possible reasons for this outcome, one being that milling

to such an extent could release inhibitory compounds which would otherwise be confined within the matrix, and these could have detrimental effects on hydrolysis.

It should be noted that the type of milling used here only reduces the fibre length, theoretically increasing the number of available cellulose binding sites. Thus another possible reason for the decrease in hydrolysis yield could be that much of the enzyme remains bound to the solid after the apparent cessation of hydrolysis (a concept which is explored further in chapter 6 and 7). A further possibility is that the reduced fibre length leads to shorter cellulose chains for the enzyme to move along, resulting in the production of a smaller quantity of glucose from a single fibril strand. The enzyme moves processively along the fibre, and once it reaches the end it needs to desorb and reattach to another fibre. It is possible, however, that at this point a considerable amount of glucose is already present in the system due to the shortened fibres. This may inhibit desorption and re-adsorption of the enzyme onto new cellulose fibres, thereby limiting the final hydrolysis potential. The initial rate at which hydrolysis proceeds in both samples would, however, be the same as the speed of the enzymes is constant regardless of fibre length of the cellulose.

Disc milling is an alternative milling strategy that was not trialled in this work but that can serve to open up the fibres. Fiberight has reported using this strategy with some success (personal communication), however, and the potential of disc milling could be investigated in future work.

Furthermore, other milling strategies such as blending in a food processor and shearing in a mortar and pestle type mill was trialled but by visual observation these milling strategies did not break up the pulp as it seemed too light to process.

Conclusion: The milling strategy used here did not have a beneficial impact on hydrolysis yield. Moreover there are high energy requirements (and therefore high costs) associated with this pre-treatment (Hendriks & Zeeman, 2009). As a result it was not pursued any further in the current work.

5.2.2 Removal of extractives or lignin as a means of improving hydrolysis potential

In this part of the research a number of pre-treatments were carried out to alter the substrate by removing compounds such as extractives or lignin, which could potentially hinder hydrolysis (Kemppainen et al., 2014; Nakagame et al., 2010). After removal of these compounds the substrate was hydrolysed to see the effect of the various treatments. Some samples were also imaged by scanning electron microscopy (SEM) to see whether any physical changes in substrate structure could be observed.

Note: All experiment in this section were carried out with 50 mg enzyme g^{-1} pulp so that the systems were not enzyme limited and it was possible to determine what effects the various treatments had on the hydrolysis potential.

5.2.2.1 **Extractives removal**

Objective: To determine the effect on the hydrolysis behaviour of the MSW pulp of removal of extractives identified as forming a component of the pseudo lignin in the compositional analysis.

Method summary: Extractive removal was trialled using acetone and 1% NaOH extraction. The MSW pulp was dried and milled to ≤0.5 mm to increase the accessible surface area. Extractives were first removed using a soxhlet extractor (section 3.7.2 & 3.7.3) in combination with either a solvent (acetone) or a weak alkali (1% NaOH). After extraction the substrates were washed thoroughly in deionised water to return the pH to neutral. Hydrolysis was carried out on two pulps and on an unextracted but milled control. The treated and untreated pulps were oven dried at 105 ^{o}C prior to hydrolysis and then diluted to 11% TS in water adjusted to pH 5 with phosphoric acid. The enzyme dose was based on total solids not glucan content and the enzyme loading was 50 mg C-Tec3 g^{-1} (residual pulp i.e, after treatment). The samples were also analysed by scanning electron microscopy to determine whether any structural changes could be identified as a result of the use of the extraction process.

Results: The compositional analysis of the two pulps and the unextracted control are given in table 5.2-1.

Results for glucan conversion in the data below refer to the glucan content in the treated samples, not as a proportion of original glucose. For 1% NaOH extraction it was not possible to determine the proportion of solid lost during the treatment; for acetone extraction however this value is given in table 4.2-1.

Table 5.2-1. Substrate composition of unextracted, acetone extracted and 1% NaOH extracted MSW derived paper pulp.

Substrate treatment	Glucan	Xylose	Arabinan Galactan Mannan	NREL Lignin	NREL Ash	Mass closure
Unextracted Pulp	55.5 ± 0.4	7.0 ± 0.1	5.5 ± 0.0	26.3 ± 0.7	1.8 ± 0.0	95.9 ± 0.5
Acetone Extraction	60.4 ± 2.5	9.1 ± 0.3	6.8 ± 0.5	14.0 ± 0.0	1.7 ± 0.1	92.0 ± 3.4
1% NaOH extraction	61.3 ± 1.7	8.0 ± 0.1	5.7 ± 0.0	16.2 ± 0.8	1.2 ± 0.2	92.4 ± 1.5

Compositional analysis was performed using the standard NREL analysis. The values given in the table are percentage of TS.

From figure 5.2-2 and figure 5.2-3 it can be seen that the glucan conversion of the un-extracted and acetone extracted pulp over the 3-day hydrolysis period was 48 ± 2% whereas conversion of the 1% NaOH extracted pulp was much lower at 11 ± 2%. The glucose concentrations in the hydrolysate of the three pulps were 3.6 wt%, 3.8 ± 0.1 wt%, 1.0 ± 0.1 wt% for the un-extracted, acetone extracted and 1% NaOH extracted pulps respectively.

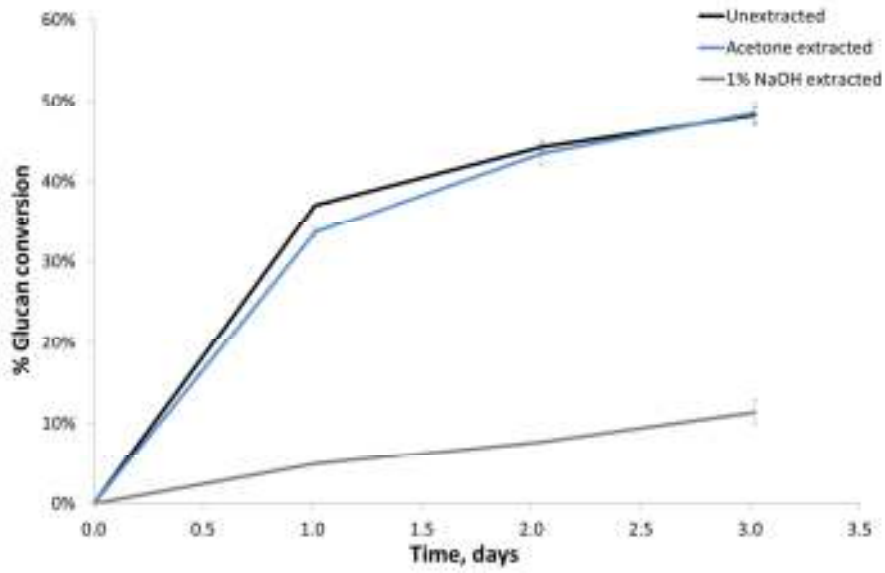


Figure 5.2-2. Glucan conversion of MSW pulp. The pulp was either untreated (black) extracted with acetone (blue) or extracted with 1% NaOH (grey) and then hydrolysed at 11% TS with 50 mg g-1 C-Tec3 . Error bars represent range.

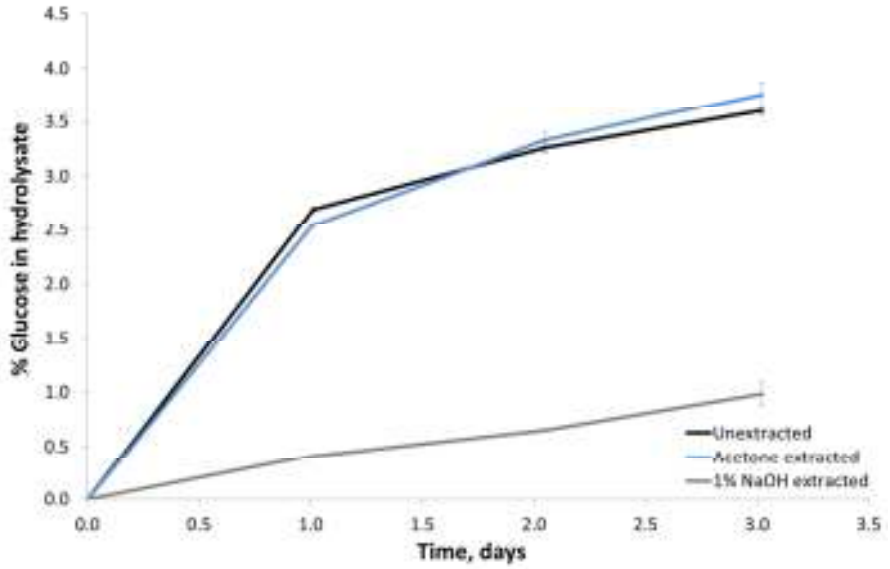


Figure 5.2-3. Percentage glucose in hydrolysate. MSW pulp was either untreated (black) extracted with acetone (blue) or extracted with 1% NaOH (grey) and then hydrolysed at 11% TS with 50 mg g-1 C-Tec3. Error bars represent range.

Figures 5.2-4 to 5.2-6 show SEM images of the unextracted and extracted pulp. The strands of unextracted pulp appear smooth, although some of the cellulose fibrils are visible (Figure 5.2-4 B & C). These fibrils became much clearer after acetone extraction as can be seen in Figure 5.2-5 A & B. This could indicate the removal of surface deposits which in turn could be the reason for improved conversion when using the acetone extracted pulp.

Acetone extraction was repeated on another batch of pulp and in this case the extracted pulp showed a 6% increase in glucan conversion compared to un-extracted pulp over a 2-day hydrolysis period (graph not shown as single measurements were taken at 48 hours only) These results indicate that some acetone soluble extractives are present which cause a slight interference in enzymatic hydrolysis; however this is pulp and/or batch specific.

Unextracted pulp

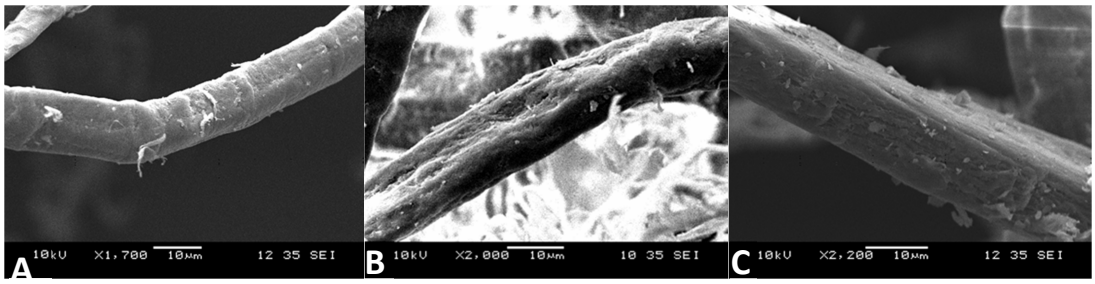


Figure 5.2-4. SEM images of milled and dried MSW pulp

Acetone extracted

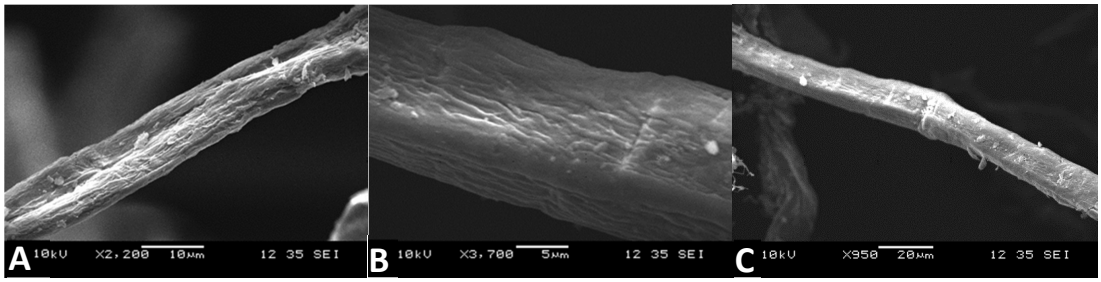


Figure 5.2-5. SEM images of acetone extracted dried milled MSW pulp

1% NaOH extracted

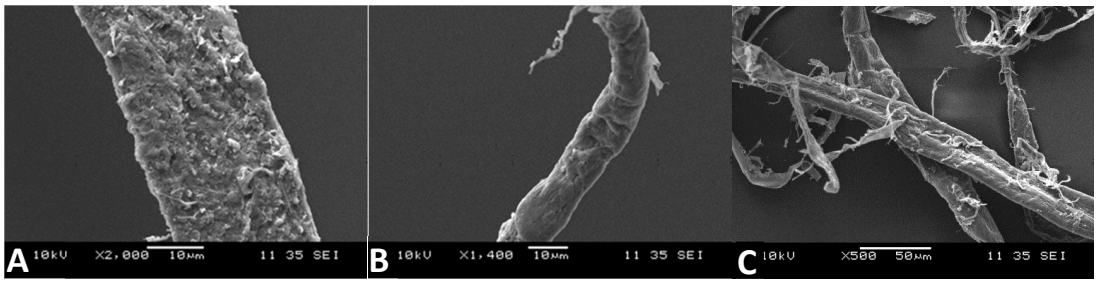


Figure 5.2-6. SEM images of 1% NaOH extracted dried milled MSW pulp.

Discussion: The two solutions used for extraction were chosen as they have previously shown promising results at removing extractives such as waxes, oils, and some inorganic components in other wood substrates and sugar cane bagasse (Burkhardt et al., 2013; Siqueira et al., 2013).

Compositional analysis of the acetone and 1% sodium hydroxide extracted pulps showed a decrease in lignin content, indicating that extraction probably removed certain compounds which were not acid soluble and therefore were mistakenly counted as lignin in the NREL compositional analysis method (i.e; pseudo lignin). Both extraction procedures led to increased glucose potential (Table 5.2-1).

Although the two extracted pulps seemed to have similar composition profiles their hydrolysis profile was quite different.

As noted above, the untreated and acetone treated samples reached a conversion of 48% over a 3-day period and the hydrolysis profiles for these pulps were similar. The percentage absolute glucose yield for the acetone extracted pulp was marginally higher, however, as the pulp had a higher glucose potential and less 'lignin', this possibly indicates a reduction in non-productive binding sites for the enzymes. Furthermore, it was found that acetone extraction was pulp specific as a later batch showed a 6% increase in glucan conversion after extraction with the solvent. Although acetone extraction can show some promising results, it would not necessarily be a desirable process option in industry as acetone is a very volatile and flammable liquid which means extreme safety measures would need to be undertaken to prevent leaks and minimize fire and explosion risks. As such a detailed analysis of potential costs and cost improvement using this solvent was not carried out as is done in some parts of chapters 5,6,& 7.

Although the compositional analysis of the 1% NaOH treated pulp seemed to indicate the removal of extraneous compounds, this pulp did not hydrolyse as well as expected. Alkali pre-treatment is known for solubilising and removing hemicellulose as well as possibly leading to delignification and changes in cellulose structure and crystallinity (Hendriks & Zeeman, 2009; Taherzadeh & Karimi, 2008). The decreased hydrolysability of this alkali-treated pulp is possibly due to solubilisation and re-deposition of lignin or hemicellulose onto the surface of the cellulose fibrils, thereby decreasing cellulose accessibility. This may have occurred if the lignin was precipitated from the fibre but not solubilised and washed out through the cellulose soxhlet crucible used in the extraction procedure. Following extraction the pulp was washed thoroughly with deionised water and filtered through Whatman GFC filter paper, and this would also have prevented any large precipitates being removed from the pulp. Another possible explanation could be that leaving the pulp to extract for an extended period may have had a detrimental effect on the fibre structure. SEM images of the alkali extracted fibre showed no clear cellulose fibrils, and the pulp also seemed to have deposits on it and appears

more bulbous in outline (Figure 5.2-6). This observation could indicate that there were structural changes in the fibre.

The results obtained here indicate that any improvement resulting from extraction is not significant enough to justify the additional costs associated with the procedure. It would be of interest to conduct further experiments on high solids hydrolysis with acetone-extracted pulp to investigate whether this leads to increased hydrolysis yields; these experiments, however, were beyond the scope of the present work.

5.2.2.2 **Lignin removal**

Objective: To determine whether the lignin present in the substrate can be removed and to identify the effect of this removal on hydrolysis performance.

Method summary: Lignin removal was performed using acetic acid and sodium chlorite (section 3.7.4) on pulp 'as is' and on acetone-extracted pulp. In both cases the pulp was milled to ≤0.5 mm prior to the various treatments in order to increase the surface area. Following extraction and lignin removal the pulps were washed thoroughly in deionised water and oven dried at 105 ^{o}C overnight. The treated pulp and the control were hydrolysed with 50 mg C-Tec3 g^{-1} residual pulp. SEM images were taken of the lignin-extracted pulp only (i.e. not of the acetone & lignin extracted pulp).

Results & Discussion: Table 5.2-2 gives the compositional analysis of the lignin extracted pulps and the control. Figure 5.2-7 shows results for a 3-day hydrolysis of the unextracted and lignin extracted pulps. From this it can been seen that the overall glucan conversion for the unextracted, acetone & lignin extracted and lignin extracted pulps was 48%, 11%, and 3% respectively.

Table 5.2-2. Substrate composition of lignin extracted and acetone & lignin extracted MSW derived paper pulp.

Substrate treatment	Glucan	Xylan	Arabinan Galactan Mannan	NREL Lignin	NREL Ash	Mass closure
Unextracted	55.5 ± 0.4	7.0 ± 0.1	5.5 ± 0.0	26.3 ± 0.7	1.8 ± 0.0	95.9 ± 0.5
Lignin extracted	65.7 ± 1.0	9.2 ± 0.3	5.4 ± 0.1	14.2 ± 0.1	1.4 ± 0.1	95.8 ± 1.1
Acetone & lignin extracted	65.1 ± 0.3	9.6 ± 0.2	6.7 ± 0.1	17.4 ± 0.4	1.5 ± 0.2	100.3 ± 0.5

Compositional analysis was performed using the standard NREL method. The values given in the table are percentage of TS. The lignin content is a combination of acid soluble and acid insoluble lignin.

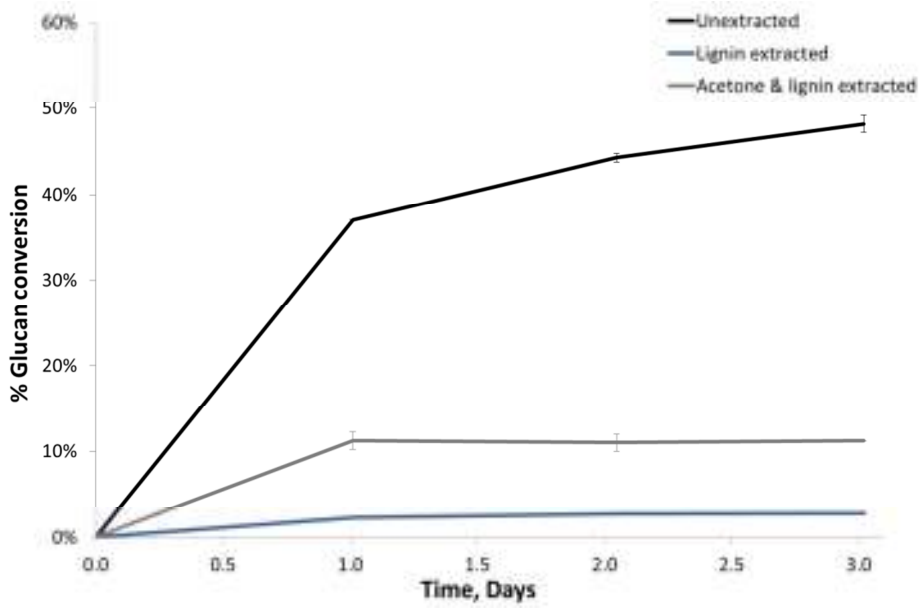


Figure 5.2-7. Hydrolysis conversion of unextracted pulp (black), acetone & lignin extracted pulp (grey) and lignin only extracted pulp (blue) against time. Hydrolysis was performed on these three substrates at 11% TS with 50 mg C-Tec3 g^{-1} pulp. Error bars represent range.

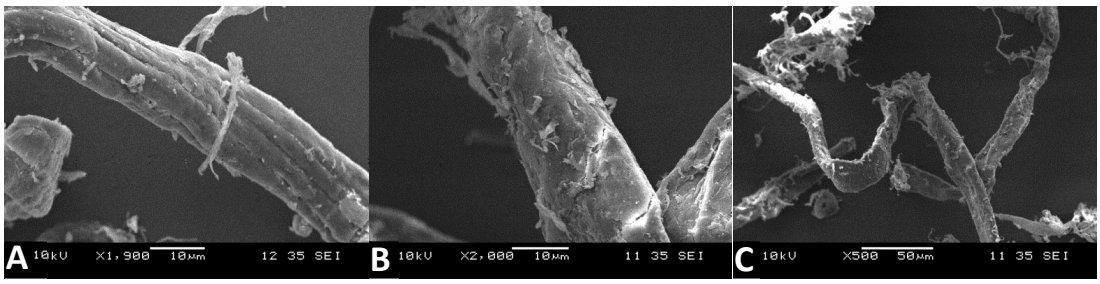


Figure 5.2-8. SEM images of dried milled and lignin extracted MSW pulp

From the NREL compositional analysis in table 5.2-2 the lignin removal on both pulps did not appear highly effective, although it is unclear whether the remaining

material is really lignin or pseudo lignin: it is possible that lignin was removed and what remained was pseudo lignin. Hydrolysis was carried out at 11% TS on both pulps and, as can be seen, they hydrolysed to a much lesser extent than the un-extracted 'as is' pulp. The acetone & lignin extracted pulp did perform slightly better than the solely lignin-extracted pulp, again indicating that acetone may be removing compounds that hinder hydrolysis.

It is noteworthy that the glucan conversion of the lignin extracted pulps did not increase at all after 24 hours of hydrolysis possibly due to lignin re-deposition limiting hydrolysis. Furthermore lignin extraction appeared to be more detrimental than alkali extraction in section 5.2.2.1 as the latter pulp continued to hydrolyse, albeit very slowly, as time increased even though both treatments were performed on the same batch of pulp.

SEM images of the solely lignin extracted pulp (figure 5.2-8) were similar to those for the alkali extracted pulp, in that clear cellulose fibrils could not be seen and a large amount of debris seemed to be present on the surface of the fibres, perhaps indicating decreased cellulose accessibility.

As discussed in the literature review, lignin can be detrimental to hydrolysis due to the unproductive binding of enzymes and also from steric hindrance preventing enzyme access to cellulose or hemicellulose (Berlin et al., 2006; Rahikainen et al., 2011; Sewalt et al., 1997; Siqueira et al., 2013; Várnai et al., 2010). Lignin removal carried out with acetic acid and sodium chlorite is known to work well on other substrates (Schwald et al., 1988; Siqueira et al., 2013). It is thought however that the substrate matrix used here was not susceptible to lignin removal by this method. Also, the many processing steps the pulp has been through before being used in this work may perhaps have led to structural changes in the lignin which meant it could not be removed using this method.

5.2.3 Calcium carbonate as a potential hydrolysis inhibitor or enhancer

Objective: To determine the effect of the calcium carbonate in the pulp on enzyme hydrolysis.

Methods summary: Two methods were tested for removal of calcium carbonate from samples of pulp milled to ≤0.5 mm: a) a sulphuric acid wash and b) a neutral detergent (ND) wash. The sulphuric acid pulp washing procedure was taken from Wang et al., (2011b). Acid washing involved mixing 50 g of MSW pulp with 20 mL acid solution (H_2SO_4) at the hydrogen ion concentration 0.1 mol L^{-1}. 500 mL of deionized water was then added and the mixture was stirred for 10 minutes. The acid solution was removed by squeezing by hand. The procedure was repeated three times, and then the pulp was washed in deionized water until the pH was 5.0. The pulp was then oven dried overnight at 105 ^{o}C. The second treatment used neutral detergent as in the Fibercap fibre analysis (Kitcherside et al., 2000). The pulp was boiled in neutral detergent and subsequently washed thoroughly until pH 7 was reached, then dried (see ND part of section 3.1.5). Once dried, both pulps underwent compositional analysis and hydrolysis at 10% TS with 50 mg C-Tec3 g^{-1} residual pulp in water adjusted to pH 5 with phosphoric acid. An untreated and unmilled pulp was used as the control (see section 3.7.6).

Results: The NREL compositional analysis results for organic solids and calcium carbonate content are given in table 5.2-3 & 4 with results for untreated pulp for comparison.

The hydrolysis results (Figure 5.2-9) show that the MSW pulp, sulphuric acid washed pulp and neutral detergent washed pulp had glucan conversions of 54 ± 1%, 53 ± 2%, and 36 ± 1% respectively. In terms of glucose yield in hydrolysate this corresponded to 3.6 ± 0.1, 3.3 ± 0.1, and 2.6 ± 0.1 wt%.

Although the conversions of the MSW pulp and the sulphuric acid washed pulp were the same, the compositional analysis of the control pulp showed a slightly

higher glucose content. Thus the absolute glucose yield from control pulp was higher than from the H_2SO_4 washed pulp.

Table 5.2-3. Composition of Sulphuric acid and neutral detergent washed pulp.

Pulp treatment	Glucan	Xylan	Arabinnan Galactan Mannan	Lignin	Ash	Mass closure
H_2SO_4 washed pulp	52.9 ± 1.5	6.4 ± 0.1	4.6 ± 0.2	19.4 ± 0.1	1.4 ± 0.1	84.7 ± 0.1
ND washed pulp	60.6 ± 1.5	8.3 ± 0.2	7.1 ± 0.3	17.5 ± 1.2	1.8 ± 0.3	95.0 ± 2.2

The results in the table were obtained by NREL compositional analysis after each respective substrate treatment. The values given are percentages of total dried treated solid.

Table 5.2-4. Organic solids and ash analysis of as is MSW pulp vs Sulphuric acid washed and Neutral detergent washed pulp

Pulp treatment	Organic component of solid	Ash on solid	Calcium carbonate on ash
MSW pulp control	95.4	4.6	4.2
H_2SO_4 washed pulp	97.0	3.0	0.7
ND washed pulp	98.0	1.2	4.3

The values given in the table are percentages on total dried treated solid.

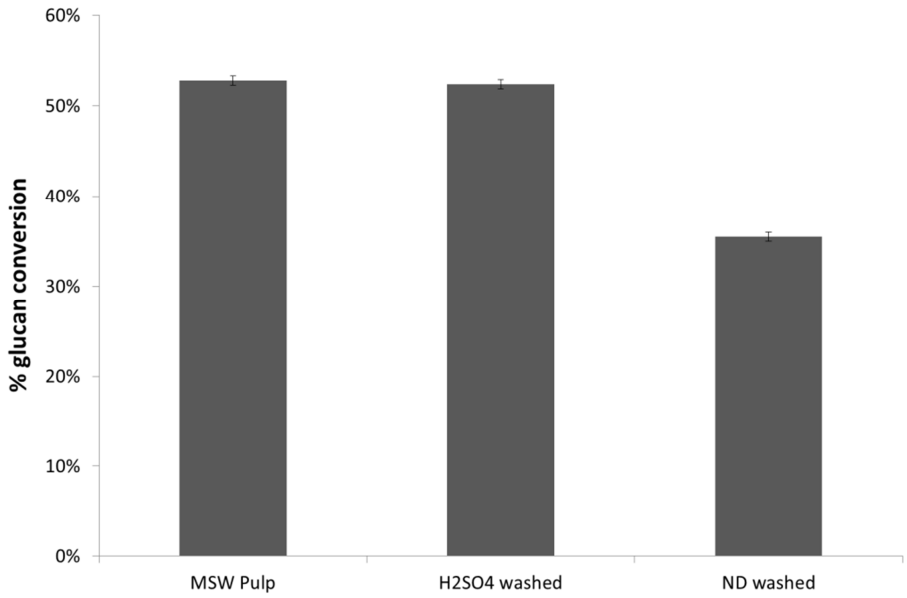


Figure 5.2-9. Glucan conversion for MSW pulp (untreated, sulphuric acid washed and neutral detergent washed) after hydrolysis at 10% TS for 3 days with 50 mg C-Tec3 g^{-1} pulp. Error bars represent range.

Discussion: These two methods for calcium carbonate removal as from literature it was thought they would be successful (Wang et al., 2012a; Wang et al., 2011b; Van Soest et al., 1991). In the studies by Wang et al., calcium carbonate was removed by washing copy paper in various acids including sulphuric, hydrochloric and nitric acid,

and this was shown to increase the hydrolysability of this substrate. As noted in section 4.2 neutral detergent solubilises proteins and removes some nitrogenous matter, pectins, and some non-fibrous matter; and chelates calcium (Van Soest et al., 1991). Elemental analysis of the MSW pulp (table 4.2-3) showed that there was no nitrogen and hence no protein present. No galacturonic acid (the main component of pectin) was detected during compositional analysis. The lipid content was not measured, but was thought to be low due to the extensive washing of the pulp performed by Fiberight. Thus it was believed that neutral detergent would act to solubilise the calcium carbonate and potentially any waxes or oils present, whilst keeping the fibre intact, and hence it was chosen as a washing agent. It was thought that this solubilised calcium would then be removed in the water rinsing step post washing.

As can be seen, in the current research both washing treatments did reduce the ash content and the calcium carbonate content in the pulp. The sulphuric acid washing reduced the calcium carbonate content more than the neutral detergent washing, whereas the neutral washing removed more ash components. From visual observation, the fibres in the sulphuric acid washed pulp looked as if they had been cut and the liquefaction of this substrate was much faster than for the other two pulps. A possible change in fibre structure may thus have prevented a further increase in conversion, despite the potential for calcium carbonate removal to enhance hydrolysis. Washing with neutral detergent should not have caused any major structural changes to the fibre. A significant portion of the ash was removed during ND washing, however, and it is possible that this may have increased the number of pores within the pulp which would then have collapsed upon drying, thereby reducing the substrate accessibility. This in turn could reduce the hydrolysis yield, as hornification reduces enzyme digestibility of a substrate (Luo & Zhu, 2011).

In studies with copy paper or office paper it has been shown that calcium carbonate can contribute to decreased hydrolysis yields. Calcium carbonate can be present as a significant portion of the material in various paper products, with office paper, newspaper and cardboard containing up to 20% of this filler (Min et al., 2013). The presence of calcium carbonate during enzymatic hydrolysis is significant because it

dissolves into the liquid medium and acts to increase the pH, which reduces the enzymes' efficiency. Furthermore it has been shown that enzymes may preferentially bind to calcium carbonate instead of cellulosic fibres from hardwood pulp (Chen et al., 2012).

Conversely it has also been shown that calcium and magnesium at concentrations of 10 mM (400 mg L^{-1} or 240 mg L^{-1} respectively) can increase enzyme activity by the formation of lignin-metal complexes which prevent unproductive binding of the cellulases (Liu et al., 2010).

5.3 Enzyme enhancement

The literature review identified some reports of enzyme enhancement by the use of additives. This has been attributed to a range of mechanisms including preventing unproductive binding of enzymes, stabilising the enzymes, preventing enzyme jamming along a cellulose fibril and catalysing the enzyme system. As also noted, water availability in the hydrolysis system can also affect enzyme performance. The following experiments were carried out to determine whether employing various additives or changing the reaction medium could have a positive effect on hydrolysis. The following compounds were added to hydrolysis: (i) Nitrogen addition in the form of glycine to help reduce any unproductive binding of enzymes; (ii) pH control with buffer vs no buffer to determine whether the buffer caused a decrease in substrate accessibility, enzyme performance or water availability; (iii) surfactant/polymer additive in the form of PEG 6000 to help reduce any unproductive binding of enzymes and to increase enzyme activity; and (iv) addition of ascorbic acid to determine whether the substrate had a sufficient redox factor to help catalyse hydrolysis.

5.3.1 Nitrogen additive

Objective: To determine whether addition of a nitrogen containing compound can increase enzymatic hydrolysis yield.

Method Summary: Low solids enzyme hydrolysis was performed as described in section (3.6.2), however glycine was added to pH 5 citric acid buffer with phosphoric acid, at 1, 2 and 5 wt% prior to substrate dilution and hydrolysis. The pH of the solutions was also checked and if required re-adjusted to pH 5 after glycine addition. The hydrolysis was run at 12 %TS and the reaction mixture was autoclaved for 15 minutes at 121 ^{o}C prior to enzyme addition of 50 mg enzyme g^{-1} pulp. The hydrolysis was carried out over a 48-hour period.

Results: As shown in Figure 5.3-1 the addition of glycine at 1-2 wt% in the reaction medium increased the percentage glucan conversion by 3-5%, whereas addition of 5% glycine concentration caused a dramatic 9% decrease in glucan conversion.

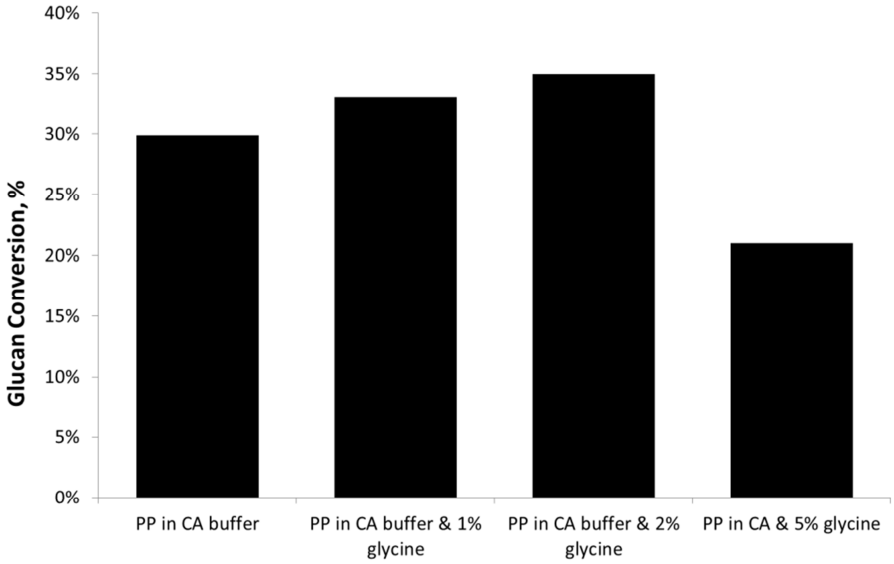


Figure 5.3-1. Glucan conversion of dried MSW pulp (PP) with the addition of glycine in citric acid buffer (CA).The hydrolysis was carried out using 11.5 %TS and 50 mg C-Tec2 g^{-1} pulp. over 3 days

Discussion: Here it can be seen that low concentrations of glycine (1-2 wt%) did give improved substrate conversion, indicating that a single amino acid can have a positive effect and a whole protein molecule is not needed, as discussed in the literature review section 2.2.6.1. It can also be seen that slightly more glycine (5 wt%) caused a decrease in conversion. This may be due to the decrease in water availability, which has been shown to limit enzyme activity (Roberts et al., 2011; Selig et al., 2012). The potential role of decreased water availability as a significant contributor to enzyme inhibition is discussed in more detail in the literature review

and in section 2.2-5. Here the use of glycine, a component which is in no way related to sugar or the stabilisers used in the enzyme preparation, was shown to have the same effect in decreasing water-availability as sugar compounds indicating that most probably any soluble compound will effect enzyme hydrolysis and thus efforts should be made to reduce the soluble content in a hydrolysis reaction medium prior to the initiation of hydrolysis.

A range of compounds have been tested by other researchers in an attempt to overcome problems of non-specific binding of the enzymes to the residual lignin. The tested compounds include those which preferentially interact with or bind to lignin, thereby making the lignin binding sites unavailable for binding to cellulase enzymes. The compounds can be assayed by their ability to increase carbohydrate conversion, and to date the promising compounds fall into two groups: (i) nitrogen-containing compounds, e.g. polyvinylpyrrolidone (PVP), gelatin, ammonia, ovalbumin, and bovine serum albumin (BSA) (Sewalt et al., 1997; Yang & Wyman, 2006); and (ii) surfactants, e.g. polyethylene glycol (PEG) and the non-ionic detergent Tween which has also been used in recovery and recycling of enzymes after hydrolysis (Tu et al., 2007a; Tu et al., 2007b; Tu et al., 2009a; Tu et al., 2009b). Many of these compounds have been shown to increase the digestibility of lignocellulosic substrates, although not of pure cellulosic substrates such as Avicel. This strongly supports the theory that the mechanism involved is through limiting the number of lignin binding sites to which cellulases can attach and thus lose their activity (Eriksson et al., 2002). The current result is in accordance with other researchers who report increased glucan conversion when using nitrogen containing compounds during hydrolysis. Previous studies have not, however, demonstrated the fact the same increase in conversion can be achieved with a small amount of a single amino acid as opposed to a whole molecule. This result indicates that perhaps binding sites on lignin only need to adsorb a small section of an enzyme for it to then become unproductively bound.

Conclusion: In this experiment glycine at low doses (1-2%) improved the hydrolysis yield but the extent was not considered significant enough to justify further detailed investigation

5.3.2 Phosphoric acid as compared to citric acid buffer to adjust the pH of the reaction medium

Phosphoric acid is commonly used in industry to adjust the pH of a reaction medium. The use of an acid is also preferable to a buffer for processing large quantities of material, as buffers can be expensive and require a range of chemicals. In this instance it is not crucial to maintain the pH at 5. A pH range between 4.8-5.2 is acceptable for maximal hydrolysis (Novozymes, 2012), and as such a buffer is not strictly required. For these reasons it was of interest to see if there was any difference in hydrolysis yield when using phosphoric acid for pH adjustment as compared to citric acid buffer.

Objective: To compare hydrolysis yield from MSW pulp with pH adjustment by phosphoric acid and citric acid buffer.

Method Summary: Hydrolysis was carried out in either 7 mM citric acid buffer at pH 5 or water adjusted to pH 5 with phosphoric acid. Dried MSW pulp was diluted to 12.5% TS and autoclaved at 121 ^{o}C for 15 minutes after which the temperature of the wet pulp was brought down to 50 ^{o}C. The pulp was charged with 50 mg C-Tec2 g^{-1} pulp and left to hydrolyse for 3 days. For other general conditions of hydrolysis see section 3.6

Results: A 5% increase in glucan conversion (overall conversion 35%) was seen for the MSW pulp hydrolysed using phosphoric acid for pH control compared to using citric acid buffer (30% glucan conversion) (figure 5.3-2).

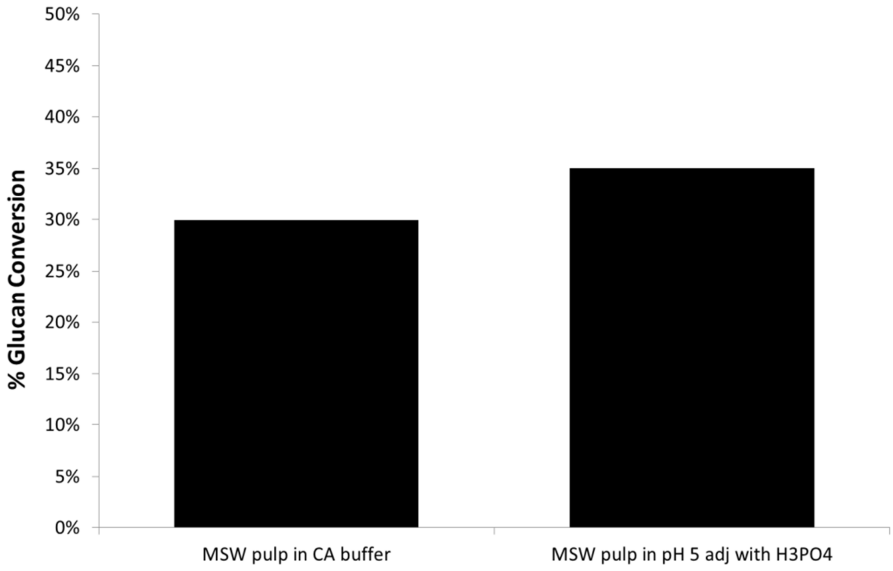


Figure 5.3-2. Glucan conversion of MSW pulp in either 7mM citric acid buffer at pH 5 or water adjusted to pH 5 with phosphoric acid. Hydrolysis was carried out for 3 days with 12.5% solids using 50 mg C-Tec2 g^{-1} pulp.

Discussion: Phosphoric acid was chosen as the acid for pH adjustment as it has less potential to solubilise calcium carbonate, which can be detrimental for hydrolysis (Wang et al., 2011b).

When phosphoric acid was used to adjust the pH of the dilution water there was an increase in conversion efficiency compared to using the enzyme in citric acid buffer at pH 5. The amount of acid used was too small to consider that it was working by direct hydrolysis of the hemicellulose or by decreasing the crystallinity of the substrate. It is likely that the phosphoric acid acts to increase pore volume, which reduces upon drying and has been shown to affect conversion (Luo & Zhu, 2011; Luo et al., 2011). Another possible explanation for the increase in conversion for the phosphoric acid hydrolysis is that the citric acid buffer may reduce the water availability due to the ions present in the buffer. A detailed discussion of water availability and its effect on hydrolysis can be found in section 2.2.5 of the literature review. Decreased conversion in citric acid buffer compared to hydrolysis in water has also been shown in a very recent study by (Hsieh et al., 2014).

Conclusion: As a result of this finding all further experiments were performed in water adjusted to pH 5 with phosphoric acid.

5.3.3 Hydrolysis at high solids with PEG 6000 as an additive

As glycine showed a positive effect on a low solids hydrolysis it was of interest to determine whether a more widely used additive, PEG 6000, would also give an increase in yield, especially at high solids as this is the working condition used in chapters 6 and 7.

Objective: To determine whether addition of PEG 6000 can increase enzymatic hydrolysis yield.

Method Summary: Hydrolysis was carried out 20% TS with 30 mg C-Tec3 g^{-1} pulp in pH 5 water containing 0.6 wt% PEG 6000. Wet MSW pulp was diluted from 40% TS with pH 5 dilution water containing 1% PEG to give a final concentration of 0.6% PEG in the reaction medium. The enzyme was added directly after dilution and hydrolysis was carried out for 6 days.

Results: Figure 5.3-3 shows the glucose concentrations for MSW pulp hydrolysed with 30 mg C-Tec3 g^{-1} pulp in the presence of 0.6 % PEG, and MSW pulp hydrolysed with 50 mg C-Tec3 g^{-1} pulp without PEG addition. From these results it is clear that the use of PEG results in a greater substrate conversion with less enzyme than hydrolysis of pulp without PEG. Over 6 days the hydrolysate containing PEG and a lower enzyme dosage achieved a conversion of 76 ± 1%, whereas the control with the higher enzyme dose and no PEG achieved a conversion of 74 ± 1%, equivalent to glucose yields in the hydrolysate of 10.1 ± 0.1 and 10.5 ± 0.2 wt% respectively. This shows that the use of PEG allows a 40% decrease in the enzyme requirement.

A further experiment was conducted in which the same amount of enzyme (30 mg g^{-1} pulp) was used with and without the addition of PEG. Results showed that the addition of PEG resulted in a 15 ± 1% increase in conversion over 48 hours. The glucose yields and glucan conversions from the experiment without PEG and that with the additive were 6.4 ± 0.0 wt% (44 ± 1%) and 8.3 ± 0.3 wt% (59 ± 2%) respectively. The results is not presented graphically as the experiment was only run for 48 hours and was part of the subset of experiments in chapter 7.

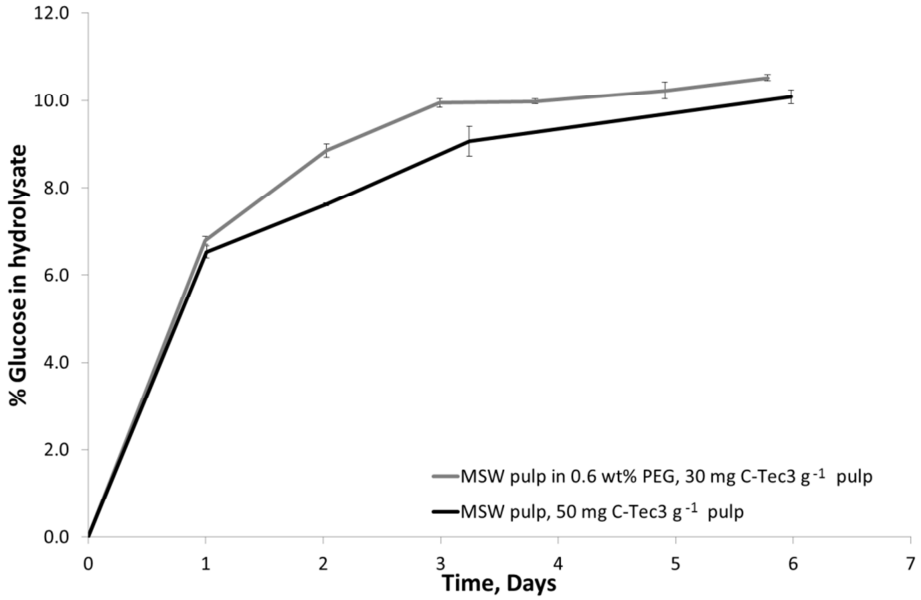


Figure 5.3-3. Hydrolysate glucose concentrations over time from hydrolysis of 20% TS pulp with 30 mg C-Tec3 g^{-1} pulp and 0.6% PEG (grey) and with 50 mg C-Tec3 g^{-1} pulp without PEG (black). Error bars represent range.

Discussion: The result shown here is contradictory to that from other MSW studies where PEG has been used. Here it is clear that PEG enhances conversion but in other studies this has not been the case (Jensen et al., 2011; Kemppainen et al., 2014), perhaps indicating that specific conditions are required for its positive activity. The role of PEG and the MSW pulp used is discussed further in chapter 6 and 7.

PEG 6000 was chosen as it is a common additive that has been used in several recent studies, and has shown a positive effect on different substrates such as corn stover, wheat straw, and wood substrates at both high and low solids (Borjesson et al., 2007; Cannella & Jørgensen, 2013; Kumar & Wyman, 2009a; Lindedam et al., 2013; Xue et al., 2012a; Zhang et al., 2011b). There are also conflicting results regarding the effect of of using surfactants: for example Xue et al., 2012 found that surfactants increasew conversion and enzyme recycling efficiency on softwood substrates but not on hardwoods (Xue et al., 2012a). This result is probably related to differing levels of substrate recalcitrance which is partly due to the various forms of lignin and their affinity for non-productively binding enzymes (Ramos et al., 1992). Furthermore, although surfactants such as PEG and Tween appear to lead to

increased numbers of free unbound enzymes, this does not have a detrimental impact on conversion (Yu et al., 2013).

In this experiment the PEG concentration used was approximately 2% per g dry substrate. This parameter was not optimised here but it is possible that a lower concentration, between 0.5 – 2% per g dry matter, would be sufficient as has been found in other studies (Cannella & Jørgensen, 2013; Li et al., 2012). PEG 6000 costs between US$ 900-1800 per tonne (Alibaba, 2014). For 1 tonne of dried MSW pulp the amount of PEG required would thus be 10 kg, costing US$ 9-18. From personal correspondence (confidential) it is known that the price of C-Tec3 is approximately $3.5 per kg. A 3% or 5% enzyme loading would require 30 or 50 kg of enzyme per tonne of MSW pulp, equating to US$ 105 or 175 per tonne, respectively. Thus, the 40% decrease in enzyme loading afforded by PEG could allow a cost saving of approximately US$ 55 per tonne of pulp. In the context of a full-scale plant processing 200 tonnes per day this could lead to an annual cost saving of US$ 4 million.

5.3.4 Increasing enzyme activity with ascorbic acid

Objective: To determine whether the MSW pulp contained sufficient redox activity to allow maximal enzyme activity by the compound GH61/AA9.

Method Summary: To establish an appropriate concentration range an initial test was carried out in which Fisher brand filter paper was hydrolysed at a solids content of 4.5% TS. The water used for dilution contained varying concentrations of ascorbic acid (0.07, 0.13, 0.20 and 0.41 wt%). The MSW pulp was then tested at a solids concentration of 10.5% TS, where the pulp was diluted with pH 5 water containing 0.13 wt% ascorbic acid.

An enzyme concentration of 50 mg C-Tec3 g^{-1} pulp was used for both the filter paper and the MSW pulp and hydrolysis was conducted for 3 days.

Results: Addition of ascorbic acid at 0.13% in the liquid medium was the optimum concentration for increased filter paper conversion (see figure 5.3-4). This resulted

in a 13% increase in glucan conversion above that achieved for filter paper with no ascorbic acid.

The results in figure 5.3-5 indicate, however, that the presence of ascorbic acid has little or no effect on achievable hydrolysis of MSW pulp. Thus, following 3-days of hydrolysis 53% conversion was obtained both with and without the additive.

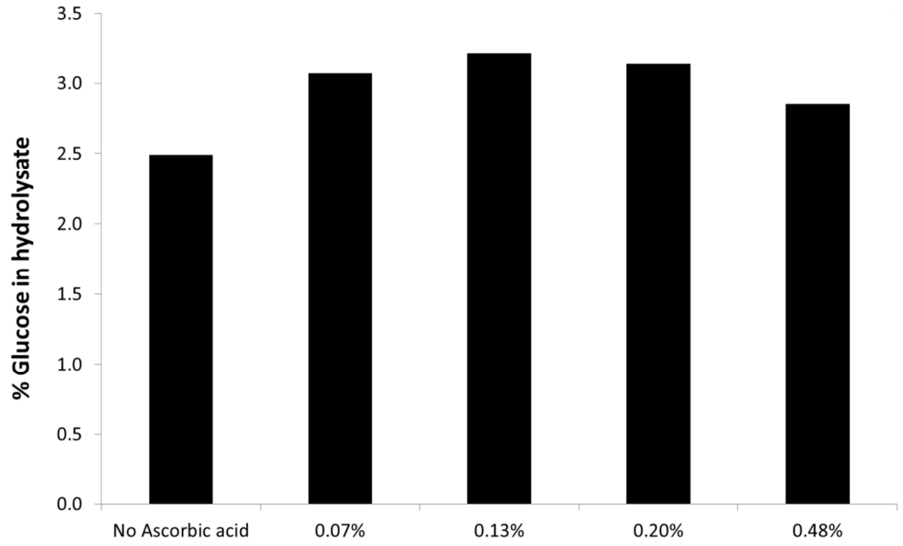


Figure 5.3-4. Glucose in hydrolysate. 3 day hydrolysis of Fisher brand filter paper either without or with ascorbic acid in the reaction medium at concentrations of 0.07, 0.13, 0.20 and 0.41 % Hydrolysis was carried out at 4.5% solids with 50 mg enzyme g^{-1} pulp.

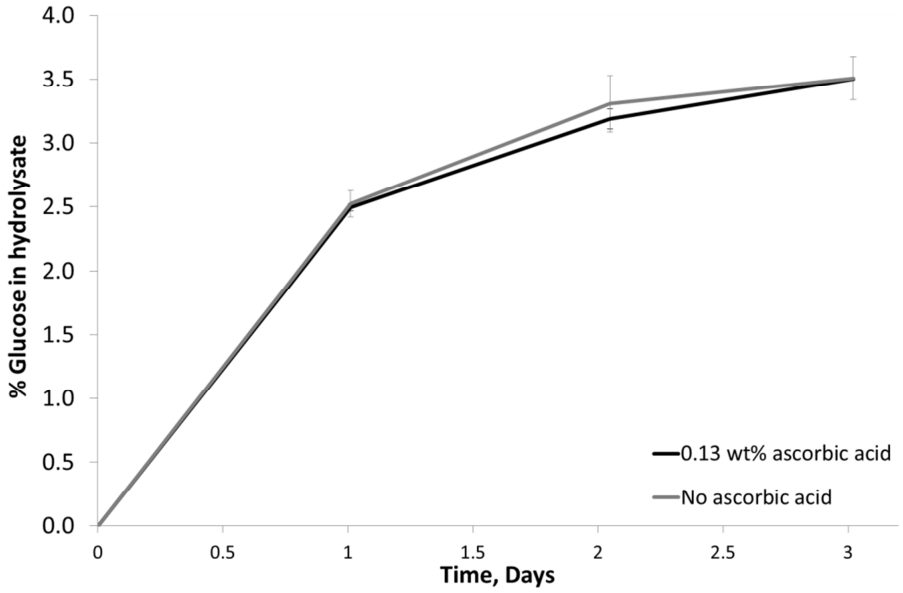


Figure 5.3-5. Glucose in hydrolysate. MSW pulp with and without 0.13% ascorbic acid in the reaction medium. The hydrolysis was carried out with 10.5% MSW pulp using 50 mg enzyme g^{-1} pulp over 3 days. Error bars represent range.

Discussion: An initial test was carried out with filter paper and varying concentrations of ascorbic acid in the liquid reaction medium as this substrate does not contain any lignin and therefore has no potential redox factor which can enhance the GH61/AA9 complex found in the enzyme preparation. This experiment on filter paper was carried out at low solids, 4.5% TS to mitigate interference from the solids effect. When ascorbic acid was applied to the MSW pulp the test was carried out at a higher total solids 10.5% TS content as this allowed for the solids effect which is prevalent at the high substrate concentrations used later in this research.

As C-Tec 3 is known to have a higher GH61/AA9 activity than earlier enzyme preparations it was important to check whether the maximum enzyme activity was being obtained on the MSW pulp. In order for the GH61/AA9 complex to act as a catalyst for the cellulase system it needs to interact with a redox factor, and it has been shown that lignin can act as this factor (Dimarogona et al., 2012). Ascorbic acid can be used in the absence of a natural redox factor (Horn et al., 2012a) and the results shown above in figure 5.3-4 indicate that ascorbic acid has an effect in increasing hydrolysis yield on filter paper which does not contain any redox reagent. As shown in figure 5.3-5, however, little or no difference was seen for the MSW pulp with or without ascorbic acid. This suggests that the lignin or pseudo lignin present in the MSW pulp has a sufficient redox factor to enable the GH61/AA9 complex in the enzyme mixture to achieve maximal enzymatic activity. This is in keeping with recent findings from (Hu et al., 2014).

It is interesting to note that in the filter paper experiment as the ascorbic acid concentration started to increase above 0.13% the glucan conversion started to decrease. This is most likely due to water constraint in the reaction medium and is a similar result to that seen for increasing glycine addition in section 5.3.1.

5.4 Substrate and enzyme feeding strategies to enhance conversion

Different substrate and enzyme feeding strategies can affect the conversion efficiency of lignocellulosic substrates. A number of feeding strategies were trialled to observe their effect on final hydrolysis yield and to determine the best conditions for high solids hydrolysis. The strategies trialled were fed batch, split batch and enzyme dilution and these were compared to a batch process where all the enzyme and substrate are added together at the beginning of the hydrolysis.

5.4.1 Fed batch substrate addition

Objective: To determine whether a fed-batch feeding strategy has a positive effect on glucan conversion and rate of enzyme hydrolysis. This strategy was used to maintain a low viscosity throughout the hydrolysis period.

Method summary: This experiment used dried MSW pulp. The total liquid (water adjusted to pH 5) for the experiment was added at the beginning of the experiment after which pulp was added in equal aliquots at hourly intervals for 8 hours, followed by a 17-hour break. Feeding at hourly intervals then resumed for a further 5 hours. Enzyme was added in equal aliquots with every second solid addition until a total loading of 50 mg C-Tec2 g^{-1} pulp was reached.

At the end of the substrate addition at 30 hours the reaction medium contained the equivalent of 20% TS if those solids had been added initially as a batch hydrolysis.

This fed-batch strategy was compared to a batch hydrolysis of 20% TS using 50 mg C-Tec2 g^{-1} pulp over a period of 5 days.

Results: After 5 hours of feeding on the second day the viscosity of the solution became too thick to allow adequate mixing between solid additions. It was therefore not worthwhile to add further solid. Although this strategy initially resulted in lower viscosity in the reaction medium, this was not maintained throughout the experiment. The final glucose concentration in the hydrolysate was

9.4% which equated to a glucan conversion of ~47%. These values were equivalent to the final result for a batch conversion of MSW pulp at 20% TS over a 5-day period. From Figure 5.4-1 it is clear, however, that the fed-batch experiment required 5 days to reach this conversion as compared to the batch experiment which achieved the same conversion in 2 days.

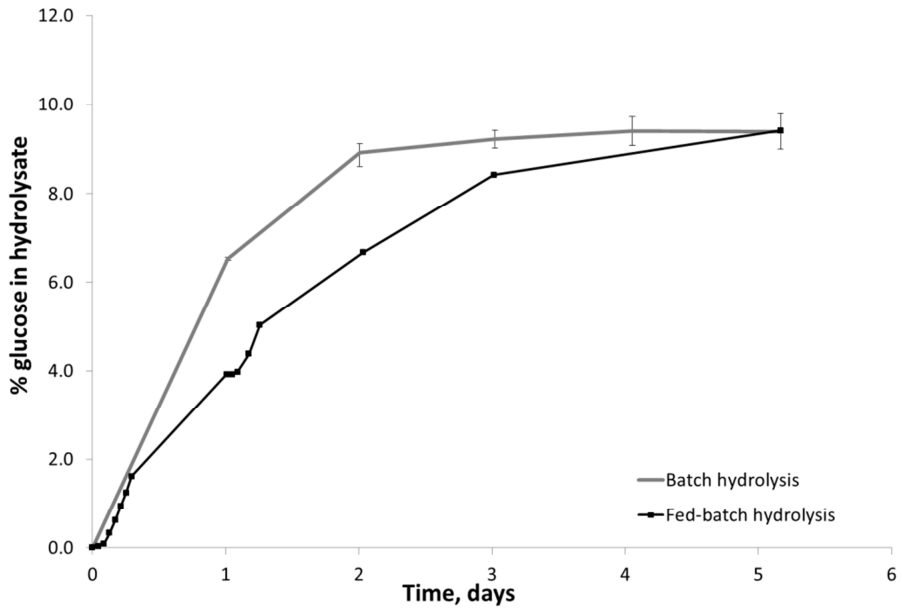


Figure 5.4-1. Glucose in hydrolysate. Fed batch and batch hydrolysis using 20% TS and 50 mg C-Tec2 g^{-1} dry pulp. Error bars represent range.

Discussion: This experiment was carried out to determine whether fed batch substrate addition could reduce the viscosity of the reaction medium to allow better initial mixing and therefore increased overall substrate conversion. As stated in the results the reaction medium became too thick on day 2 to allow adequate mixing and therefore solids addition had to be stopped. This could have been due to, or exacerbated by, an insufficient time interval between the feed additions.

Another possible reason for the reduced hydrolysis rate is that the build-up of sugars over day 1 could have inhibited the new enzymes added on day 2. The glucose content of the hydrolysate at the start of day 2 was already 3.9% and this has been shown to be a severely inhibitory concentration for the enzyme C-Tec 2 which was used in this experiment, as discussed later on in section 6.6.1.

This result is consistent with that of Rosgaard et al., 2007 who carried out fed-batch experiments on pre-treated barley straw. Their fed batch system involved adding substrate 3 times over 24 hours to reach a final dry matter content of 15% TS. Their findings showed that glucose production over a 72-hour period was greater per gram of substrate in a batch system than in the fed batch. They also trialled adding all the enzyme at the beginning versus adding it with each feed, and found that both methods resulted in similar final glucose yields. Initial addition of all of the enzyme gave higher initial glucose yields, however, possibly because more of the initially-added substrate was degraded than of the subsequently added batches. They found that glucose production suffered a lag phase every time substrate was added. This was attributed to a localised build-up of glucose which inhibited enzymes from adsorbing onto fresh substrate, until was it well mixed and localised glucose build-up was alleviated (Rosgaard et al., 2007).

5.4.2 Split-batch substrate and enzyme addition

Objective: To determine whether a split batch approach for substrate and enzyme addition is better than a batch hydrolysis in terms of overall glucan conversion and rate of hydrolysis.

Method Summary: Half of the wet MSW pulp at 40% TS and half of the total enzyme used and all of the pH 5 dilution water were added at the beginning of hydrolysis. The second half of the substrate and enzyme were added after 6 hours of hydrolysis. A total substrate concentration of 20% TS was reached after the addition of both lots of substrate and an enzyme loading of 50 mg C-Tec3 g^{-1} pulp was used. Hydrolysis was carried out for a total of 8 days.

This split-batch strategy was compared to a batch hydrolysis of 20% TS using 50 mg C-Tec3 g^{-1} pulp over a period of 8 days.

Results: Figure 5.4-2 shows the results for the split batch and batch processes over an 8-day period. As can be seen the batch hydrolysis gave a sugar solution with approximately 2% more glucose, equivalent to a 20% increase in conversion. The batch hydrolysis reached a glucose concentration of 9.4 ± 0.5 wt% in the

hydrolysate with a glucan conversion of 68% over 8 days, whereas the glucose concentration in the split batch experiment was only 7.7 ± 0.2 wt%, equating to 57% glucan conversion.

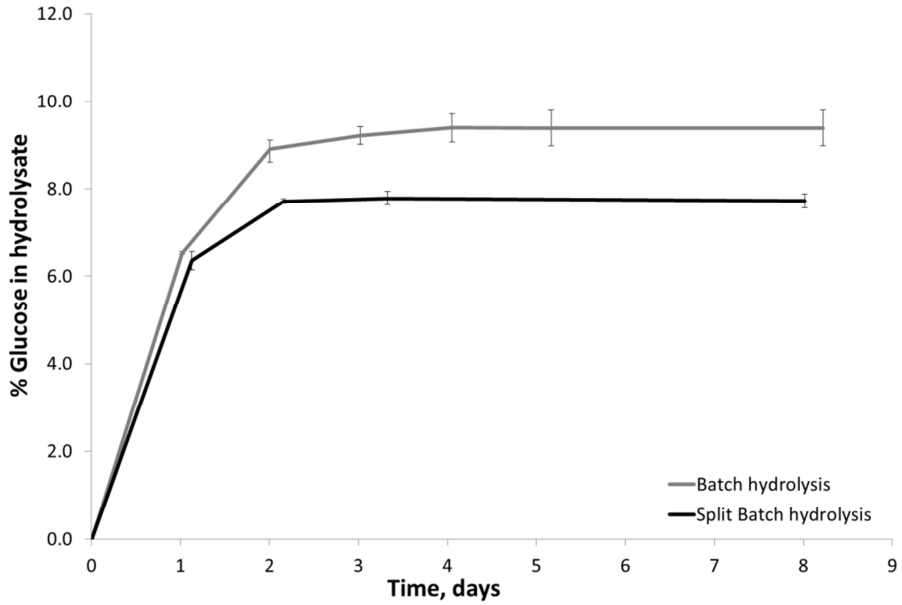


Figure 5.4-2.Glucose in hydrolysate. Split batch vs batch substrate addition. Error bars represent range.

Discussion: Split batch hydrolysis did not perform as well as batch hydrolysis, possibly for the same reason as in the fed batch system, where a rapid sugar build-up at the beginning of hydrolysis may subsequently prevent the new enzyme from properly adhering to the substrate. If no sugar was present when the second half of the enzyme was added to the substrate, however, then it is possible that more enzyme would adhere. A modified split batch experiment was therefore devised to test if this was the case.

5.4.2.1 **Modification of split-batch process**

Objective: To test whether enzyme-substrate binding for a split batch process is limited by the presence of sugars produced in the first 6 hours of hydrolysis.

Method summary: A split batch experiment was carried out in which enzymes were added to the second half of the substrate an hour prior to mixing this substrate with the initial hydrolysate started 6 hours earlier. Thus the enzymes were added to the second batch of substrate after 5 hours of hydrolysis, and this substrate and

enzyme mixture was added to the original hydrolysis after 6 hours of hydrolysis initiation. The same overall quantities of substrate and enzymes were used as in experiment described in section 5.4.2. A total respective substrate concentration and enzyme load of 20% TS and 50 mg C-Tec3 g^{-1} substrate were used for a hydrolysis period of 6 days.

Results: The glucose in the hydrolysate of this experiment reached 9.2 ± 0.5 wt% after 6 days compared to the batch control which contained 10.1 ± 0.2 wt% glucose. The total glucan conversion of the split batch and batch hydrolysis experiments were 73 ± 1% and 66 ± 3%, respectively (Figure 5.4-3).

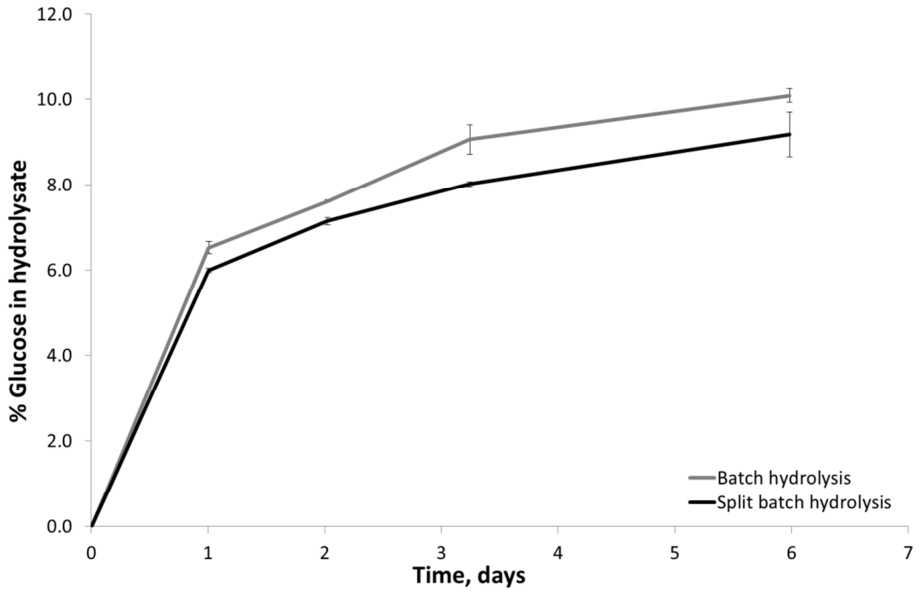


Figure 5.4-3. Glucose in hydrolysate. Split batch vs batch substrate addition.

Discussion: Using this approach it was found that a higher conversion was obtained than in the original split batch experiment (section 5.4.2). The difference in the glucan yields of batch and split batch in this section was 7% compared to section 5.4.2 where the difference was 11%. This result suggests that reduction in conversion in split batch could be partly due from enzymes not adhering to substrate in the presence of sugar.

It has been shown previously that during hydrolysis a proportion of the enzyme mix, between 35 – 65%, binds to substrate in the period between 20 minutes and 3

hours after addition and this proportion is primarily responsible for most of the hydrolysis (Yu et al., 2013). Thus in the current work an hour of adsorption time at 50 ^{o}C was allowed for the enzyme to bind without the presence of sugar. This period was chosen as it was not sufficient for the production of large amounts of sugar which could cause a decrease in enzyme activity; however it probably allowed most of the enzyme-substrate binding to occur.

In this experiment a loss in conversion is still seen compared to the batch experiment, and this could be due to the fact that the enzymes were not given enough time to adhere properly to the substrate. Another possible explanations is that some of the enzyme added to the second batch in this split batch experiment adhered to plastic reaction vessel (section 3.6), or to the very small amount of pulp (≤ 0.3 g of a total 10 g) that remained in the bottle after this second aliquot of substrate was added to the initial hydrolysate. It is possible that this small amount of pulp adhered to the bottle as it contained some of the enzyme preparation, which is adhesive due to its high sugar content, and this resulted in some enzyme not being transferred into the initial hydrolysate.

This second experiment was carried out with a different batch of substrate to that used in in the original experiment (section 3.4.2), and the degree of hydrolysis after 48 hours appeared to be higher for this second batch of pulp than for the pulp used in section 3.4.2 even after the 48 hours. This continued conversion could be due to a disc refining step which was added to the Fiberight pilot plant before the production of this batch of pulp. An increase in conversion following this type of substrate refining has been shown previously (Jones et al., 2013).

Conclusion: Split batch addition of substrate and enzyme did not result in increased glucan conversion over a batch hydrolysis process. It was noted, however, that if the enzymes are allowed to adhere to the second batch of substrate prior to its addition to the original reaction medium, then increased conversion is obtained in the split batch process as compared to adding the substrate and enzyme directly to the original reaction medium. When the substrate and enzymes are added directly to the initial hydrolysis it is thought that sugar build-up in the first 6 hours results in

inhibition which either does not allow the second batch of enzymes to adhere fully, or inhibits enzyme activity on the substrate. Glucose inhibition of enzymes is discussed in more detail in section 6.6.1.

5.5 Enzyme dilution vs no dilution

Objective: To determine whether addition of the enzyme preparation 'as is' or after dilution with water causes a difference in hydrolysis yield.

Method Summary: Hydrolysis was carried out for 48-hours using wet MSW pulp. The wet pulp was added at 40% TS and diluted to 15% TS with pH 5 dilution water. The enzyme was either added as is to the 15% solution or added as part of the dilution water (ie; it was mixed into the dilution water before being added to the wet pulp).

Results: The glucan conversion was equivalent in each case, at 67.3 ± 0.03% for direct addition and 66.8 ± 0.02% when diluted.

Discussion: This result confirms that mixing by the tumbling method adopted allows even distribution of the enzyme within the pulp, and the enzyme does not need to be diluted in the liquid medium beforehand.

5.6 Conclusions from chapter 5

This section of the research aimed to look at parameters which could limit substrate conversion. These included substrate properties, enzyme inhibition and limitations posed by the way that substrate and enzyme were added into the hydrolysis system.

The substrate properties investigated were (i) particle size, (ii) the presence of extractives, (iii) the presence of lignin and (iv) the presence of calcium carbonate. These were manipulated to determine what effect if any they had on glucan conversion.

It was found that reducing the particle size by milling to ≤0.5 mm did not enhance conversion, and in fact hindered the hydrolysis process, possibly due to the release of inhibitory compounds into the reaction matrix or through sugar build-up which restricted enzyme desorption/adsorption. Solvent extraction to remove extractives or pseudo lignin with acetone seemed to improve the substrate accessibility and led to an increase of up to 6% in conversion compared to un-extracted pulp. Mild alkali treatment had the opposite effect with a significant decrease in conversion, possibly due to deposition of compounds on the surface of the cellulose making it inaccessible. Lignin removal was undertaken with the use of sodium chlorite and acetic acid, as used successfully on a number of other substrates. This treatment did not seem to remove lignin and from SEM imaging of the material it appeared to lead to surface deposits or structural changes which reduced cellulose accessibility. It is possible that, due to complex nature of the substrate and the many processing steps it has been through prior to hydrolysis, the lignin is not is a form which is susceptible to removal. From these various extraction treatments it was concluded that any extractives or lignin present in the substrate do not significantly hinder substrate conversion. Further to this, the removal of calcium carbonate in the substrate had a neutral impact on the hydrolysis of the MSW pulp used in this research.

To enhance the enzyme activity the following strategies were trialled: (i) Nitrogen addition in the form of glycine to help reduce any unproductive binding of enzymes; (ii) pH control with buffer vs no buffer to determine any effect on conversion due to changes in substrate accessibility, enzyme performance or water availability; (iii) surfactant/polymer addition in the form of PEG 6000 to help reduce any unproductive binding of enzymes and increase enzyme activity; and (iv) addition of ascorbic acid to determine whether the substrate contained a sufficient redox factor to help catalyse hydrolysis.

It was found that glycine addition did have a positive effect on conversion; however it was not significant enough to warrant further investigation into this compound. The use of citric acid buffer as a reaction medium lowered the potential hydrolysis yield of the MSW pulp compared to when hydrolysis was carried out in water

adjusted to pH 5 with phosphoric acid. This may have been due to the phosphoric acid opening up pores in the substrate, or because the citric acid buffer reduced the water availability in the hydrolysis system. PEG 6000 was also trialled as an additive and was found to increase hydrolysis yield significantly. By using this compound a 40% decrease in enzyme requirement was observed. Lastly the MSW pulp contained sufficient redox potential to ensure maximal enzyme activity on this substrate.

In the last set of experiments various substrate and enzyme feeding strategies were trialled such as fed-batch, split batch and enzyme dilution prior to addition onto the substrate. It was found that a fed batch system resulted in the same sugar yields as a batch hydrolysis; however it required an extra 3 days of reaction to obtain this yield. A split batch system of substrate and enzyme addition also led to a reduced overall yield compared to batch hydrolysis. It is believed that this is largely due to sugar build-up during the first 6 hours of hydrolysis which then inhibits the fresh enzymes added at this point.

Tumbling mixing allowed even distribution of enzymes, and diluting the enzyme prior to substrate addition did not improve performance: it was thus confirmed that adding the enzyme preparation 'as is', is sufficient for good conversion.

Chapter 6: Results & Discussion – Optimisation of high solid hydrolysis process

6.1 Introduction

As was seen in figure 4.3-1, at low substrate concentrations most of the achievable substrate conversion occurs in the first 24 hours. As the amount of solids increases the hydrolysis rate falls, and a further 24-48 hours is required to obtain the same degree of conversion. The time taken depends on the substrate, its concentration and the enzyme dosage as well as other experimental conditions such as pH, temperature and the origin and condition of the substrate (wet or dry).

The aim of this work was to investigate and optimise the hydrolysis of MSW derived pulp under industrially relevant conditions. It was therefore necessary to consider parameters that would be of importance in a large-scale commercial context, such as relatively short process times, high conversion rates and yields and minimal enzyme dosing. As noted above, initial experiments at both high and low solids clearly indicated that the most of the achievable hydrolysis of the MSW pulp substrate occurred within the first 48 hours after enzyme addition, with insignificant conversion occurring thereafter. Thus most experiments in this part of the research focussed on conversion over a 48-hour period.

This process time of 48 hours compared very well with other high solids studies using either SHF or SSF where periods of 4-7 days were needed to attain an acceptable ethanol titre (Cannella & Jørgensen, 2013; Zhang et al., 2010; Zhang et al., 2012b)). A 48-hour hydrolysis followed by a 24-hour fermentation (as would be the case here) is a shorter overall process time than that proposed in other studies, and as such is more suitable for commercial application. The high yields achievable in the short process time with this substrate mean that significant value is drawn from the substrate, and the remaining solids can then either be hydrolysed further or processed to produce electrical energy and heat through biogas production or combustion of the residual solid (Dererie et al., 2011; Kemppainen et al., 2012).

These secondary process would help improve the overall energy balance as part of the bio-refinery concept.

Most of the studies in the earlier chapters which investigated substrate limitations and feeding strategies were conducted at low solids so as to avoid any interference from the 'solids effect'. Commercially, however, it is considered preferable that the hydrolysis processes should run at high solids concentration (≥ 18% TS) to reduce water consumption, capital investment and energy costs (Koppram et al., 2014; Larsson & Zacchi, 1996; Modenbach & Nokes, 2013). Thus the work in this chapter focused on examining a number of interdependent factors that control enzyme efficiency, and can be manipulated to improve overall performance and product quality at high solids concentrations. The overall goal was to maximise the efficiency of enzyme usage to obtain a concentrated sugar solution from a waste feedstock, without compromising yields or prolonging process times.

Unless otherwise stated, it should be noted that all hydrolysis process data presented in this and the next chapters (chapters 6 and 7) used wet pulp supplied from the Fiberight pilot plant. This pulp was provided at a solids content of between 30 – 50% TS, and was then diluted to the appropriate concentration required for each process strategy. As noted previously, there was slight batch to batch variation of the pulps due to changes in the pilot plant over the course of this work; however this was accounted for when calculating conversion yields, as conversion was based on actual glucan content in each batch.

6.2 Maximum conversion capability

Objectives: To quantify the maximum obtainable glucan conversion at high solids concentrations.

Method summary: Hydrolysis was carried out using 50 mg C-Tec2 g^{-1} pulp for 4 days with dried MSW pulp diluted to 25% TS with pH 5 water. After 4 days the hydrolysate was centrifuged for 10 minutes, the sugar solution supernatant was removed, and the residual solid was washed twice with deionised water. The residual solid was then re-suspended in pH 5 water to make up a suspension with

15% TS. Extra enzyme was added, in the same quantity as that used initially, and the mixture was allowed to hydrolyse for another 4 days. This procedure was then repeated once more.

Results & Discussion: It was found that 90 ± 5% of the glucan in the substrate was hydrolysed (figure 6.2-1). As this experiment was carried out with the enzyme C-Tec2 it is possible that more complete conversion could be achieved with C-Tec3 and with wet pulp. The result suggests, however, that the substrate is not 'non-fibre material' limiting at high solids and thus high glucan conversions and high glucose yields are achievable.

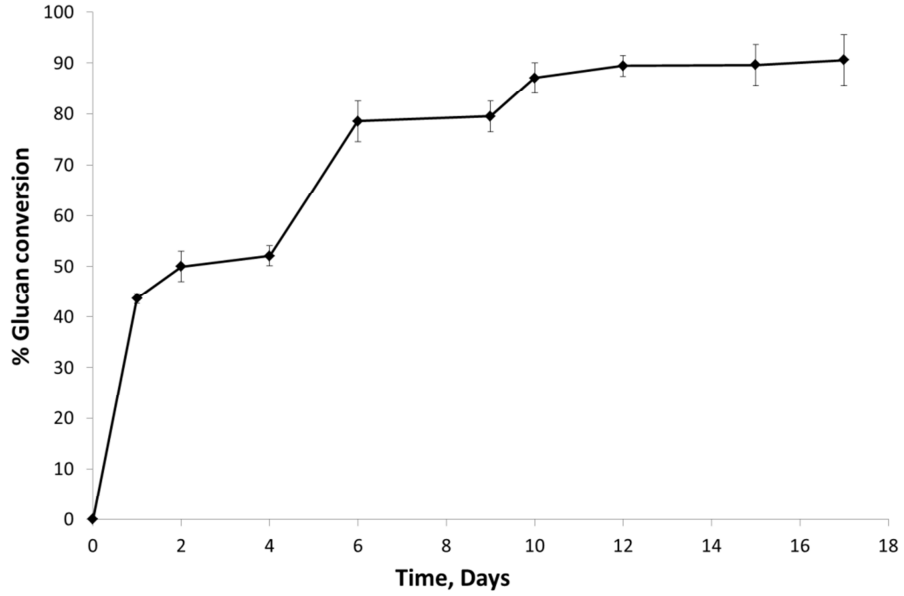
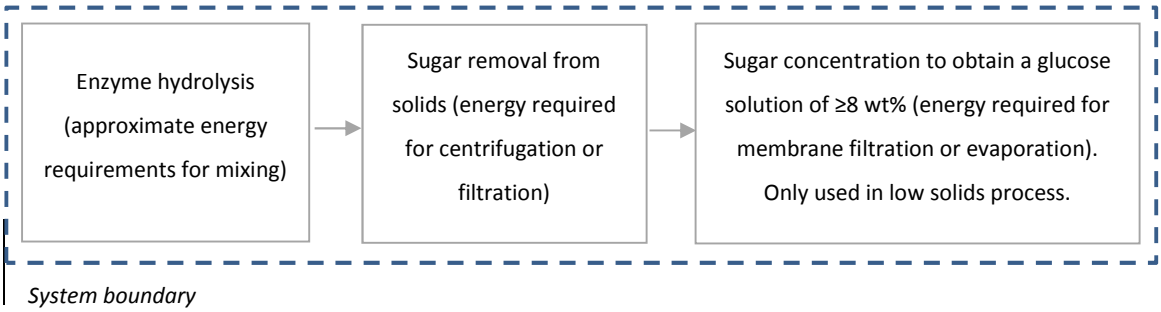


Figure 6.2-1. % Glucan conversion. Evaluation of maximum conversion capability of MSW pulp.

6.3 Low solids vs high solids hydrolysis – energy balance

Objective: To develop an outline an energy balance for an enzyme hydrolysis process at both low and high initial solids concentrations in order to determine the relative energy efficiency of the two processes.

Method summary: Energy usage in an enzyme hydrolysis process was estimated using literature data for the steps within the system boundary shown:



Overall it is difficult to obtain an absolute value for the difference in energy requirement for a low vs. high solids hydrolysis process. This is because at low solids the tank volume, water use and heating requirement are higher than that for a high solids process. On the other hand, the high initial viscosity for high solids concentrations would require an increased power input for mixing. Here an attempt is made to outline a very approximate comparative energy balance between the two process configurations.

The calculations were based on a starting feedstock of 1000 kg of dried MSW with a total carbohydrate content of 75%. These parameters, the conversion capability and water requirement of the theoretical process are given in Table 6.3-1.

Table 6.3-1. Parameters used to calculate energy balance for low and high solids hydrolysis process

Solid content description	Total $solid_i$ (kg)	Total $water_i$ (kg)	Total $Solids_i$ (%)	$Carb_i$ solid (%)	Carb conversion (48 h) (%)	Total $liquid_f$ (kg)	Total $solids_f$ (kg)
Low	1000	19,000	5	75	85	19,638	362
High	1000	4000	20	75	70	4,525	475

Nomenclature; Carb – Carbohydrate, i – initial, f - final

Using the conversion bases stated in table 6.3-1 a hydrolysis period of 2-days would yield respective total sugar concentrations of 3.4 and 10.3 wt% for the low and high solids hydrolysis processes. This means that the low solids process would require a sugar concentration step prior to a fermentation and distillation if it is to be used for bio-ethanol (Larsson & Zacchi, 1996).

The optimum temperature for the hydrolysis process is 50 ^{o}C and therefore the water coming into the process needs to be heated. Assuming that the heating takes place over 2 hours and based on the heat capacity of water of 4.18 kJ kg^{-1} and a

temperature change of 35°C (from 15°C to 50°C), the power requirement can be calculated as 146 MJ tonne^{-1} water which is equivalent to 40.6 kWh tonne^{-1}. For the conditions outlined in table 6.3-1 this implies that the low and high solids processes would respectively require 19x and 4x this amount of energy. If it is assumed that the heat capacity of the pulp slurries is equivalent to that of water then total heating requirement for the low solids material is 406 kW and for the high solids process is 102 kW.

The heating requirement for water as calculated above is not included in the following sections as it is most likely that in practice the waste heat from other sections of the bio-refinery process would be used in heating the water for hydrolysis, and thus electricity would not be required. It is important to note, however, that this part of the process would require a significant amount of heat, especially under low solids conditions.

6.3.1 Energy requirement - Mixing in hydrolysis

The first section of the energy balance considers the mixing requirement for hydrolysis. The experimental work in this thesis uses gravity mixing and it is envisioned that if the process was to be carried out at full scale a rotational drum reactor would be used with baffles to promote gravity mixing in addition to the rotational action (see figure 6.3-1). This design is similar to that used in the Inbicon pilot plant in Denmark (Larsen et al., 2012; Larsen et al., 2008).

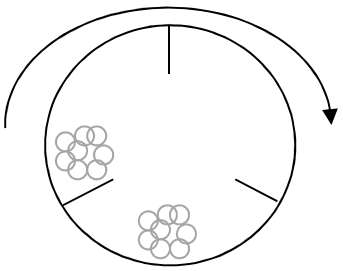


Figure 6.3-1. Cross-sectional diagram of a rotational reactor with internal gravity mixing.

Although this type of reactor has been used in the hydrolysis of lactose (Honda et al., 1991) and of wheat straw (Inbicon), and in drying applications (Shaffer, 1997),

little or no data is available on its energy requirements during operation. It has however been noted previously in the hydrolysis of wheat straw that liquefaction in a horizontal rotary reactor is as efficient at 3.5 RPM as it is at 11 RPM (Larsen et al., 2012), which suggests a potentially low mixing energy requirement in a rotational reactor.

The energy requirement for an impeller stirred device, on the other hand, can be calculated because a large amount of data and theory exists for this process. At low solids concentration the behaviour of the solution is Newtonian and the viscosity therefore does not vary with shear rate. Previous studies which have looked at the mixing of similar suspensions have quoted values of between 0.5-8 W $m^{-3,}$ usually with a default value of 5 W m^{-3} being selected (Christodoulides, 2001; Milledge, 2013).

Thus, on the assumption that a 5% pulp solution has a similar density to water, the power requirement for mixing a volume of 20 m^3 would be approximately 100 W or 0.1 kW. Over a 24 hour hydrolysis period this converts to an energy consumption of 2.4 kWh.

The energy requirement for high solids (20% TS) process is not estimated here as in this case the substrate used would behave as a wet solid and would not be able to be mixed in a stirred tank unless a fed-batch system was employed. It should be noted that the higher energy requirement for mixing high solids in a batch reactor would only apply in the first 12-24 hours of hydrolysis, after which the hydrolysate is sufficiently liquefied for the mixing power requirement to be reduced.

6.3.2 Energy requirement - Separation of concentrated sugar solution post hydrolysis

After hydrolysis, if the sugar solution is to be used for fermentation then either the whole slurry can be fermented or a concentrated sugar solution can be removed from the hydrolysate and fermented. The advantage of whole slurry fermentation is that all the sugar in the hydrolysate can be used for downstream processes. The disadvantages of this strategy are threefold: (i) the high temperatures used for

distillation would not allow enzyme recovery post distillation (Skovgaard & Jorgensen, 2013); (ii) at low solids the ethanol content of the fermentation broth is too dilute for economically feasible distillation, and the hydrolysate would require concentration prior to fermentation; and (iii) whole slurry fermentation can be affected by mass transfer limitations which increase the time required to produce the ethanol. Accordingly, for the scenario used here it is assumed that both processes (low and high solids) go through a sugar separation step. Even if ethanol fermentation is not the final goal of the process, and the sugar solution is to be used as a basis for other bio-refinery products, then it is still preferable to have a concentrated solid-free solution to feed into other bacterial processes or into a chemical reactor.

The sugar solution can be removed from the pulp using either centrifugation or filter pressing. As energy data for large-scale processing and separation of hydrolysate are not readily available, data from dewatering processes used in primary sludge treatment has been used. As the hydrolysate is relatively easy to dewater it is assumed that it behaves similarly to primary sludge dosed with polymer. In sludge dewatering centrifugal concentration can be carried out to increase the solids content from 3-5% to ~20% TS, which is similar to the processing that would be required in the low solids hydrolysis. After hydrolysis the solids content of the high solids process is around 9-10% insoluble solids. Although this is higher than in the low solids process, it was not possible to find data for the power requirement for centrifugation at this solids concentration. It is therefore assumed that the centrifugal power requirement would be similar for both the high and low solids process, given the low viscosity of the material.

The primary sludge data used here were obtained from a CDM Smith webinar entitled 'Trends in Dewatering' and accessed through the water environment association of Texas (Moss, 2012). The centrifuge used in this webinar was a horizontal centrifuge and the press was a horizontal screw press. The power requirements for centrifugation and filter pressing of primary sludge with a high polymer concentration were 53 kWh per tonne and 6 kWh per tonne respectively. These values are within the range given in a wastewater dewatering design manual

published by the United States Environmental Protection Agency, which states that low speed centrifuge energy requirements range from 30-60 kWh per tonne and that a belt filter press uses between 10-25 kWh per tonne, for an incoming feed with 3% solids (U.S.EPA, 1982).

Table 6.3-2 shows the relative amounts of solids and liquid recovered from the two selected separation steps. The total solids concentration following centrifugation is the result obtained on a laboratory centrifuge while the total solids achieved through filter pressing is based on the total solids content achieved with the screw press in the Fiberight pilot plant, Lawrenceville Virginia.

Table 6.3-2. Parameters required for sugar solution separation with either low solids or high solids hydrolysis processes.

Solids Content	TS% of pulp after centrifuge	TS% of pulp after filter press	Energy required centrifuge kWh tonne^{-1}	Energy required filter press kWh tonne^{-1}	Total liquid recovered in centrifuge kg & (%)	Total liquid recovered in filter press kg & (%)
Low	40	50	53	6	19094 (97)	19275 (98)
High	35	50	53	6	3643 (81)	4050 (90)

Table 6.3-3. Energy calculations for sugar solution separation with either low solids or high solids hydrolysis processes.

Solids Content	Total energy requirement for centrifuging kWh	Total energy requirement for filter pressing kWh
Low	1060	120
High	265	30

6.3.3 Energy requirement - Concentration of low solids sugar solution

A concentration step is required for the sugar solution produced from the low solids hydrolysis process to achieve a sugar solution with greater than 8 wt% glucose. This can be done in a number of different ways of which two are considered here, namely (i) membrane filtration and (ii) climbing film evaporation of the type used for fruit juice concentration.

Data from Metcalf and Eddy (Tchobanoglous et al., 2003) was used in order to assess the energy requirement of the membrane process (table 6.3-4). The two process steps required to concentrate the sugar solution with membrane filtration

are ultrafiltration followed by nanofiltration, with the retentate from the nanofiltration process containing the concentrated sugar solution. The permeate resulting from this process is an almost pure water which could be re-used in the hydrolysis process.

Table 6.3-4. Operating pressure and energy consumption data for membrane filtration

Membrane Process	Operating Pressure (kPa)	Energy consumption (kWh m^{-3})	Product recovery (%)
Ultrafiltration	525	3	70-80
Nanofiltration	875	5.3	80-85

(Tchobanoglous et al., 2003)

Table 6.3-5 below shows the amount of liquid that can be recovered with the membrane filtration set-up, following filter pressing (using the lower limits of recovery), and the energy cost for this. The results are calculated on the basis that the dilute sugar solutions have a density equal to water at 1000 kg m^{-3}.

Table 6.3-5. Liquid recovery through membrane filtration and the associated energy cost with this process.

	Liquid (kg or m^3)	After Ultrafiltration permeate (kg or m^3, maximum)	After Nanofiltration (retentate) (kg or m^3, maximum)	Total energy required after separation by filtration (kWh)
Liquid recovered by filter press	19275	134953	2699	(3 x 19.3) + (5.3 x 13.5) = 130

The amount of liquid recovered was based on the lower limits from table 6.3-4.

Glucose concentrations of up to 17 wt% can be obtained after nanofiltration.

The second concentration option is to use a climbing film evaporator. In this case it may be possible to concentrate the sugar solution directly from the hydrolysate without the separation step.

As this is an evaporation process the energy requirement can be calculated using a simple heat balance as shown in equation 6.3-1.

$$Q = m_{out} \times C_p \times (T_{out} - T_{in}) + m_{vap} \times C_p \times (T_{out} - T_{in}) + m_{vap} \times H_{vap} \qquad \textbf{Equation 6.3-1}$$

where

Q - Heat requirement (kJ)

m - Mass of liquid (kg)

C_p - Heat capacity of water (4.18 kJ kg^{-1})

T – Temperature (T_{out} = 100 ^oC and T_{in} = 50 ^oC)

H – Latent heat of vaporisation (2260 kJ kg^{-1})

If the whole slurry is evaporated then the starting liquid content is 19638 kg as taken from table 6.3-1 and of this 12961 kg is evaporated to produce a final solution with 10% sugar. Thus, it follows that

$$Q = 6677 \times 4.18 \times (100-50) + 12961 \times 4.18 \times (100-50) + 12961 \times 2260$$

$$= 3.3 \times 10^7 \, kJ$$

Assuming a processing time of 5 hours implies an energy requirement of 1.9MW or 9.5 x 10^3 kWh.

The calculated heat requirement is a rough estimate and most likely an underestimation. As time progresses the sugar in solution increases and a higher temperature is therefore required for evaporation, thereby increasing the heat requirement. In an industrial application, however, it is likely that multi-effect evaporation would be used which reduces the heat requirement.

6.3.4 Conclusion: Assessment from mixing energy

In this section the energy requirements for a hydrolysis process at low (5% TS) and high (20% TS) solids were compared in order to support the assumption that carrying out the process at low solids is not energetically efficient.

Energy calculations were made on the basis of 1000 kg dry MSW pulp solids. Three different process steps were considered (i) mixing, (ii) separation of sugar solution from solids and (iii) concentration of sugars from the hydrolysate of the low solids hydrolysis process.

Although the current research used free-fall mixing, it was beyond the scope of the study to calculate the energy requirement of this type of mixing. Instead calculations were based on a stirred tank model, although this is only applicable to the low solids process where the slurry behaves as a Newtonian fluid. The energy

cost for mixing in the low solids process was found to 2.4kWh for the 20,000 kg (solid + liquid) of material used over a 24 hour period. In a high solids process the viscosity of the pulp suspension is too high to mix in a batch process, and a fed batch process or different reactor configuration would be needed. It is likely that the mixing energy for the high solids process would be higher than that required at low solids for the first 12-24 hours until the mixture has liquefied, after which the suspension behaves as a Newtonian fluid. In addition to mixing the other energy consideration in hydrolysis is the energy required to heat the water up to 50 ^{o}C, which can be almost 5 times higher for the low solids process than for high solids due to the larger amount of water needed. If the water was heated over a 2-hour period then the energy consumption for this process would be 40.6 kWh tonne^{-1}.

The second step in the process is carried out to separate the sugar solution from the residual solid after hydrolysis and this can be achieved with either centrifugation or filter pressing. It was found that the energy requirement of a filter pressing step (6 kWh tonne^{-1}) was much lower than that for centrifugation (53 kWh tonne^{-1}). Thus when using the value for filter pressing for separation it was found that 120 kWh would be required for the low solids process and 30 kWh for the high solids processes.

Although the energy required for separation was calculated for both high and low solids, in practice the high solids process may not necessarily require a separation step. This is because the whole high solids slurry could be sent directly to fermentation and then distillation. The disadvantages of this, however, are the potential for mass transfer limitations during fermentation and the fact that enzyme cannot be recovered from the residual solid. Also it limits the end product use of the sugar.

The dilute sugar solution obtained from a hydrolysis process with low solids requires concentration before fermentation. It is more efficient to use membrane filtration to perform the concentration as the energy requirement is significantly lower than using evaporation. The total energy requirement for membrane

filtration following filter pressing is 130 kWh as opposed to direct evaporation of the hydrolysate which would require 1.9MW for the total mass considered.

In summary, for a low solids process the total power requirement for the three process steps noted above are approximately 2 kWh (mixing) + 120kWh (separation) + 130 kWh (membrane filtration) totalling 252 kWh. For a high solids process the power requirement is (unknown) kWh (mixing) + 30 kWh (separation) totalling (x + 30) kWh. Therefore in order for the two processes to have an equivalent energy balance it is possible to spend up to 250 kWh of energy on mixing in the high solids process. This is equivalent to 125 times the amount of energy required for mixing at low solids. Thus at high solids any mixing energy requirement below 250 kWh would result in a lower energy cost as compared to the low solids process.

The one shortcoming of the high solids process is the lower conversion yields obtained. Thus the research reported in following sections in this chapter aimed to increase these yields using various process manipulations. As these manipulations can add to the energy cost of the process, efforts were also made to minimise this, for example by trying to avoid the need for a sugar concentration step following hydrolysis.

6.4 Enzyme dosing at high solids

Objective: to establish appropriate working dosages of C-Tec2 and 3 for high solids hydrolysis so that a glucose concentration of 8 wt% can be achieved in a hydrolysis period of 2 days.

Method: Hydrolysis was carried out on dried MSW pulp from a single batch at 20 and/or 25% TS with 50 mg C-Tec2 and C-Tec3 g^{-1} pulp, using the hydrolysis procedure outlined in section 3.6.1 and 3.6.2. Wet pulp from the Fiberight pilot plant was also hydrolysed with 50 mg C-Tec3 g^{-1} pulp. Hydrolysis was carried out for a total of 4 days.

Results & Discussion: To achieve 8 wt% glucose in the hydrolysate using 25% TS and 50 mg C-Tec2 g^{-1} dried pulp a hydrolysis period of 3 days was required (figure 6.4-1). It was not possible to achieve this concentration of glucose at 20% solids using the same enzyme loading. The relative glucan conversions at ~4 days were on average 43% for the 20% TS and 39% for the 25% TS experiments.

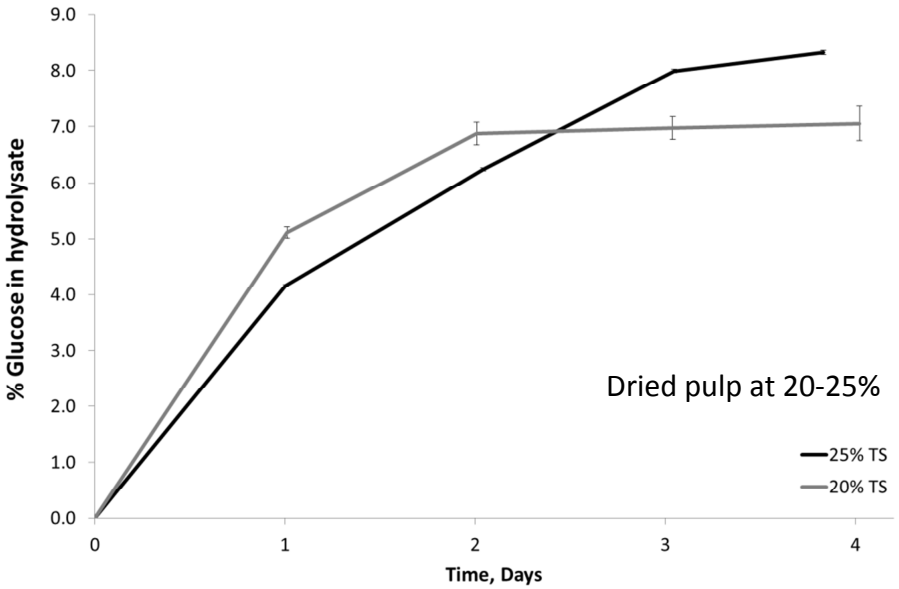


Figure 6.4-1. Glucose in hydrolysate from hydrolysis of dried MSW pulp with 50 mg C-Tec2 g^{-1} pulp at 20 and 25% TS. Error bars represent range.

When C-Tec3 became available the experiment was repeated on dried pulp at 25% TS using 50 mg C-Tec3 g^{-1} pulp. As can be seen in figure 6.4-2 it was possible to reach a glucose concentration of 8 wt% in 2 days rather than 3 using this newer enzyme preparation.

Furthermore, when using C-Tec3 and wet pulp from the Fiberight pilot plant it was possible to achieve the desired glucose concentration in a 2-day period using only 20% TS (figure 6.4-3). The average glucan conversion at 4 days for the dried pulp at 25% TS was 49% and for the wet pulp at 20% TS it was 73%. This result reinforces the finding that an increased hydrolysis yield can be achieved when using wet processed pulp as opposed to pre-dried pulp. This improved result for 20% TS may also be due in part to the reduction in viscosity within the reaction medium.

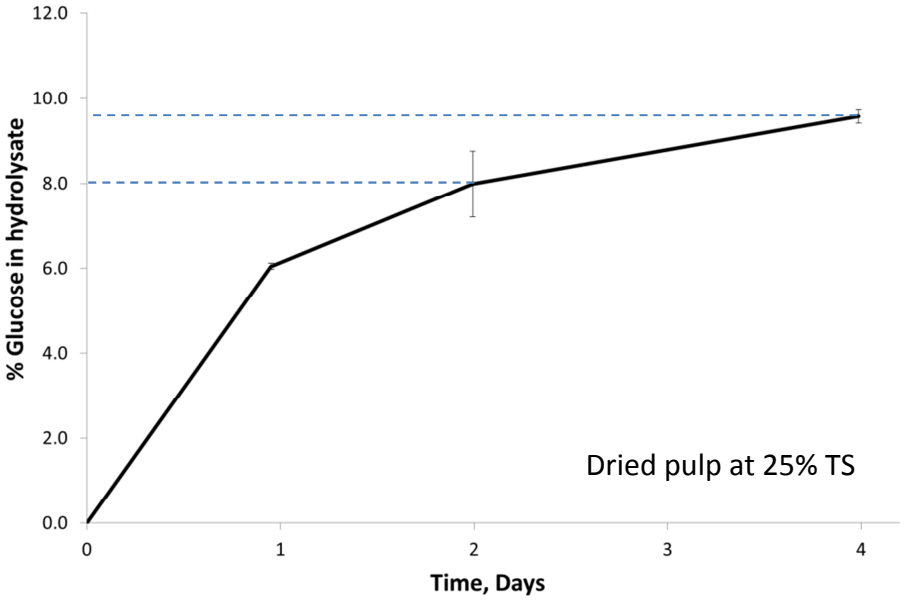


Figure 6.4-2. Percentage glucose in hydrolysate from hydrolysis of dried MSW pulp with 50 mg C-Tec3 g^{-1} dried pulp at 25% TS. Error bars represent range.

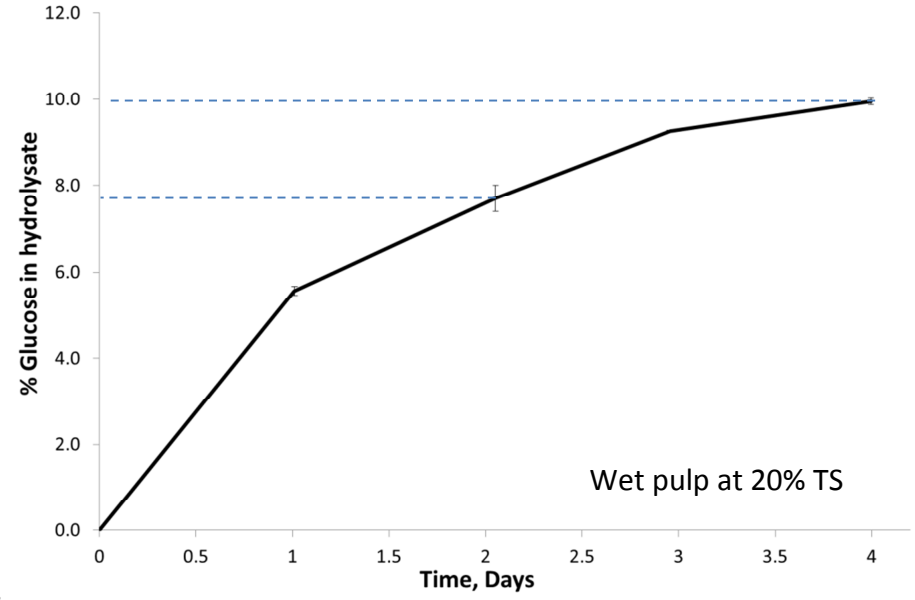


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Figure 6.4-3. Percentage glucose in hydrolysate from hydrolysis of wet MSW pulp with 50 mg C-Tec3 g^{-1} wet pulp at 20% TS. Error bars represent range.

Conclusion: Using C-Tec3 it was possible to obtain the desired glucose concentration in 2 days with 25% TS dried pulp or 20% TS wet pulp. This result formed the basis for choosing the enzyme dosing used in the following experiments in this chapter.

6.5 Two-stage hydrolysis with intermediate fermentation

Objective: To determine whether hydrolysis yields could be improved by using a two-stage hydrolysis with intermediate fermentation strategy.

Method Summary: A 24 or 48-hour hydrolysis was performed at 18.5% TS or 20% TS with 50 mg C-Tec2 or C-Tec3 g^{-1} pulp at 50 ^{o}C. Following hydrolysis the hydrolysate was cooled to 30 ^{o}C and yeast was added for a 24-hour fermentation. After this the temperature was readjusted to 50 ^{o}C for a second round of hydrolysis.

Results: Figure 6.5-1 shows the results for a two-stage hydrolysis with intermediate fermentation using C-Tec2. A sugar concentration of 6.2 ± 0.2% was reached after 2 days of hydrolysis and this was subsequently almost completely converted to ethanol after 24 hours of fermentation between day 2 and 3. The ethanol content at day 3 was found to be ~2.8 wt%. The enzymes continued to hydrolyse following the temperature increase at day 3, and a further 0.7 ± 0.1% of glucose was produced in the hydrolysate. A second fermentation performed on day 5 resulted in a final ethanol yield of ~3.5 wt%. It should be noted that the experiment was performed on dried MSW pulp and with the enzyme C-Tec2, and this could be the reason for the relatively low sugar yield obtained in the first-stage of hydrolysis.

When C-Tec3 became available the experiment was repeated using either 24 or 48 hours of initial hydrolysis followed by fermentation. The results in figure 6.5-2 show that with an initial hydrolysis of 48 hours the glucose concentration in the hydrolysate was 8.8 ± 0.2% wt. This was then fermented for 24 hours, yielding an ethanol concentration of 2.9 ± 0.6% wt. The yeast was not removed and reaction conditions were then optimised for a secondary hydrolysis (50 ^{o}C, mixing by tumbling). After a total of 5 days an additional 1.8 ± 0.2% wt of glucose was found in the reaction mixture. This was then fermented once more (not shown on graph) and gave a final ethanol yield over the two cycles of 5.8 ± 0.3 wt%.

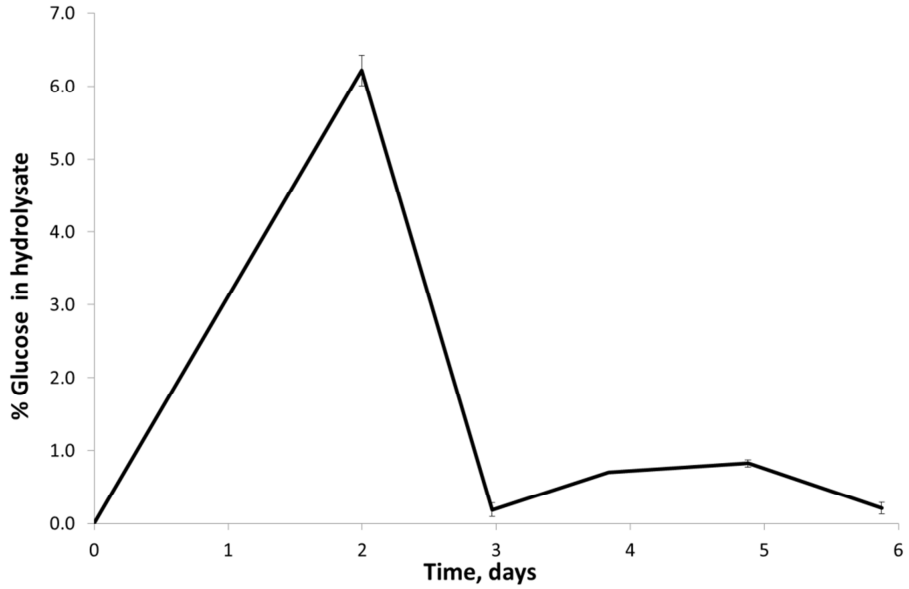


Figure 6.5-1. Glucose in hydrolysate. Evaluation of the effect of an intermediate fermentation step using MSW pulp and C-Tec2 The MSW was hydrolysed for 2 days at 21% TS with 50 mg C-Tec2 g^{-1} pulp at 50 ^{o}C, this was followed by 24 hours of fermentation at 30 ^{o}C and then a further hydrolysis at 50 ^{o}C for another 2 days. This second hydrolysis was then followed by a final 24 hour fermentation period. Error bars represent range.

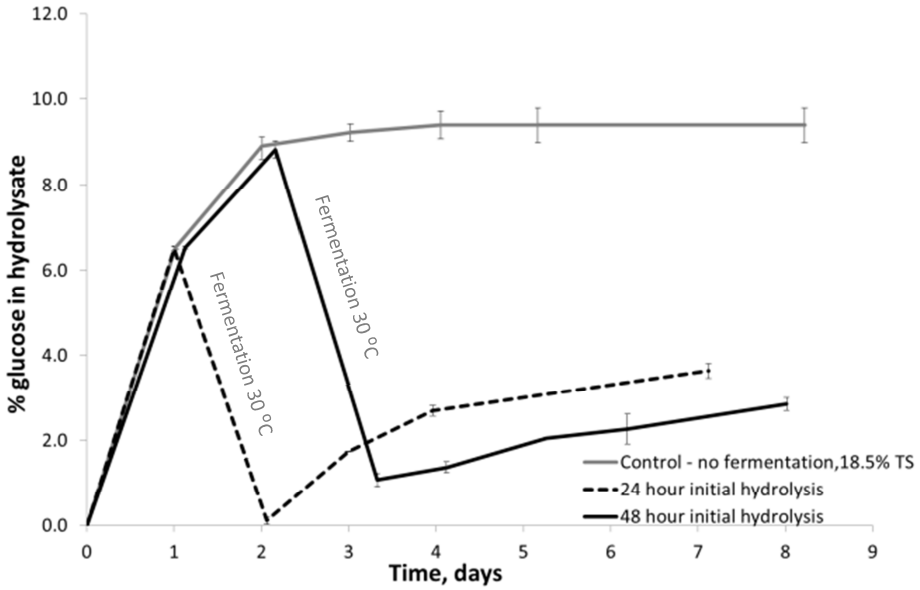


Figure 6.5-2. Glucose in hydrolysate. Evaluation of the effect of an intermediate fermentation step using MSW pulp and C-Tec3 at 18.5% TS with 50 mg C-Tec3 g^{-1} pulp: overall substrate conversion. For (i) no fermentation (–) (ii) fermentation following an initial 24 hour hydrolysis (– –) and (iii) fermentation following an initial 48 hour hydrolysis (–).

In a second strategy the initial hydrolysis cycle was reduced to 24 hours. This gave a lower glucose concentration of around 6.5% wt in the first hydrolysis, which was converted to 1.8 ± 0.3% wt of ethanol following fermentation. This was followed by

a second hydrolysis stage, which was much faster than the second-stage in the previous strategy with a 48-hour first- stage hydrolysis and yielded around 3% wt additional glucose within 48 hours, rising to ~4% wt over the 6-day hydrolysis period, giving a further 3.7 ± 0.3% wt of ethanol. The total ethanol concentration for the combined two-stage process was 4.2 ± 0.3% wt in a 4-day hydrolysis period and 5.5 ± 0.3% over the total 6-day hydrolysis period. Both two-stage systems gave a higher percentage glucan conversion than was achieved in the 18.5% TS control, as can be seen in Figure 6.5-3. Both the 24 and the 48-hour first stage hydrolysis procedure gave similar final yields, corresponding to total glucan conversions of 76.5 ± 0.5% and 73.1 ± 1.7% respectively (Figure 6.5-3), compared to 68 ± 0.5% in the single-stage hydrolysis control over the same time period.

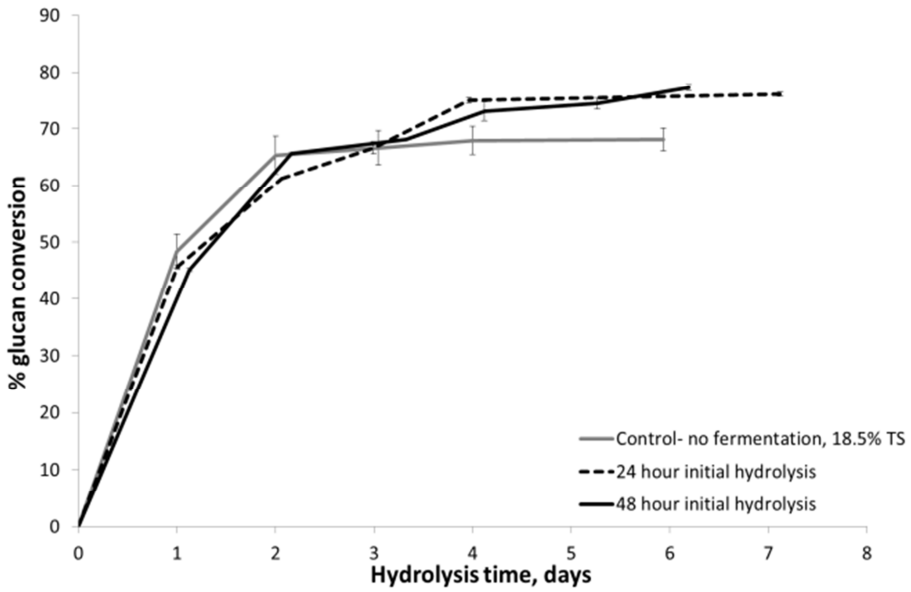


Figure 6.5-3. Glucan conversion. Evaluation of the effect of an intermediate fermentation step using MSW pulp at 18.5% TS with 50 mg C-Tec3 g^{-1} pulp: percentage of glucan conversion. For (i) no fermentation (–) (ii) fermentation following an initial 24 hour hydrolysis (– –) and (iii) fermentation following an initial 48 hour hydrolysis (–).

Discussion: As noted in the literature review, product inhibition is one of the major limitations in realising the full potential of enzymatic hydrolysis. It has been shown that cellulase-containing enzyme preparations are inhibited by their products glucose and cellobiose (Andric et al., 2010; Xiao et al., 2004). For this reason continuous removal of glucose through simultaneous saccharification and fermentation (SSF) was once considered to be the best way of obtaining reasonable titres of ethanol within relatively short process times. Many researchers today

continue to believe that this is the best option (Kemppainen et al., 2012; Olofsson et al., 2008; Watanabe et al., 2012). As the efficacy of commercial enzymes continues to improve, however, it must be questioned whether it is necessary to forgo optimal hydrolysis conditions, as is invariably the case when SSF is used (Viikari et al., 2012). Furthermore, it has recently been shown that SHF is better than SSF at achieving good ethanol titres under very high solids loadings (20-30% TS) when using the newer enzyme preparations (Cannella & Jørgensen, 2013). Although SSF can overcome the problem of glucose inhibition it has a further slight drawback, as ethanol can also inhibit the enzyme activity. This has been clearly shown for those cellulases taken from cultures of *Trichoderma Resei* used at 30°C (Holtzapple et al., 1990; Wu & Lee, 1997).

From the results in figures 6.5-2 and 6.5-3 it can be seen that the two-stage system is beneficial in achieving higher glucan conversion than a batch hydrolysis. In the experiment with C-Tec3 although the greatest improvement of 5% in glucan conversion was achieved with 48-hour initial hydrolysis, the 24-hour initial hydrolysis showed a slightly smaller increase of 3.5%. There may, however, be a slight advantage in using the 24-hour initial hydrolysis as early production of ethanol may help to maintain sterility within the system. The rate of reaction in the second cycle following 24-hour hydrolysis was faster than after the 48-hour initial hydrolysis. This is consistent with the observation made by Pribowo et al., 2012, who suggested that if enzyme recycling is to be undertaken then it should be carried out within 24 hours (Pribowo et al., 2012).

All the ethanol yields given here were based only on conversion of the glucose: in all the experiments the hydrolysate also contained up to 1.8 wt% xylose, which could potentially be fermented to give additional ethanol if a C5/C6 fermenting strain were used.

The strategy trialled in this experiment proved effective at alleviating product inhibition from glucose. It does not, however, negate the influence of the non-metabolised sugars and other soluble compounds in the hydrolysate / fermentation broth on water availability and product inhibition, which can also have negative

effects on hydrolysis (Selig et al., 2012; Xiao et al., 2004). Additionally ethanol is known to have a negative effect on hydrolysis, and further experiments were therefore conducted with this alcohol to quantify its effect on both C-Tec2 and C-Tec3. These experiments are discussed in the following section.

6.5.1 Ethanol inhibition of C-Tec2 and C-Tec3

Objective: To determine whether and to what extent C-Tec2 and C-Tec3 are inhibited by ethanol.

Method Summary: Filter paper was added to pH 5 water containing 4% ethanol to give a solids content of 20% TS. The mixture was hydrolysed with either C-Tec2 or C-Tec3 at an enzyme loading of 50 mg g^{-1} filter paper.

Results: Figure 6.5-4 and 6.5-5 show the results for hydrolysis carried out in 4% ethanol with C-Tec2 and C-Tec3 respectively. It is clear that ethanol does inhibit enzyme activity in both cases. For C-Tec2 glucan conversions of 42 ± 1% and 49% were achieved for reaction media with and without ethanol, respectively; for C-Tec3 the glucan conversions were 48 ± 1% and 53% with and without ethanol respectively.

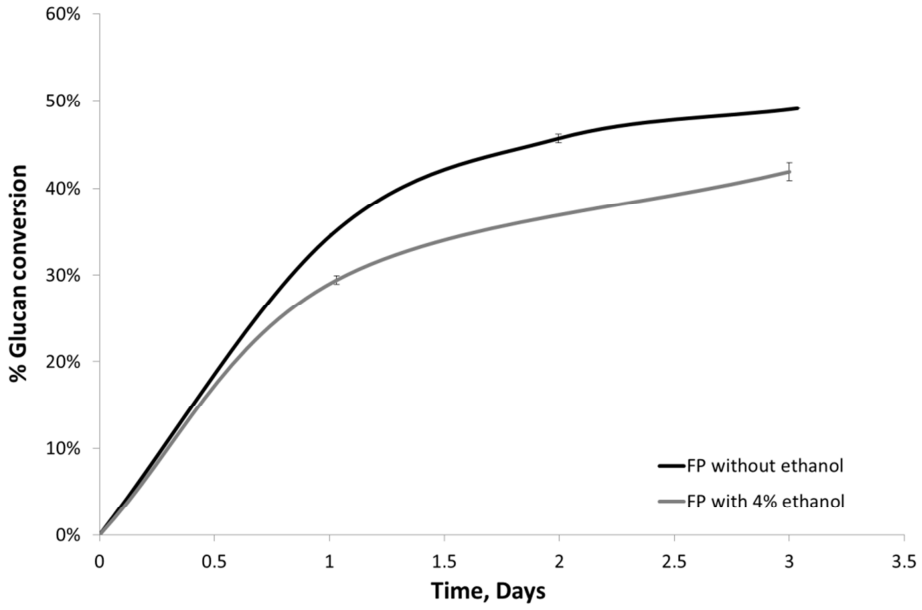


Figure 6.5-4. Ethanol inhibition of C-Tec2. Hydrolysis of filter paper was carried out at 20%TS and 50 mg C-Tec2 g^{-1} pulp. The hydrolysis was carried out either with 4% ethanol in the liquid medium (grey line) or without ethanol (black line).

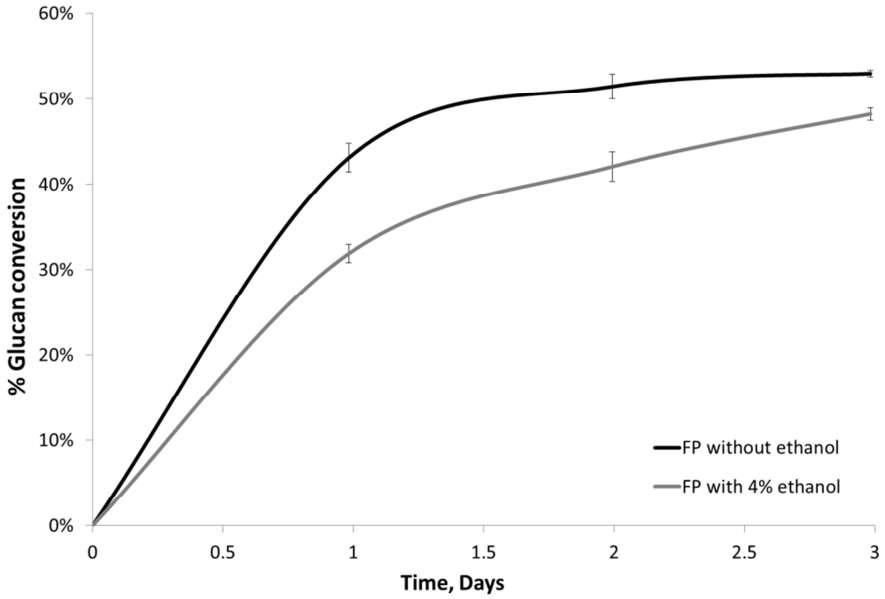


Figure 6.5-5. Ethanol inhibition of C-Tec3. Hydrolysis of filter paper at 20%TS and 50 mg C-Tec3 g^{-1} pulp. The hydrolysis was carried out either with 4% ethanol in the liquid medium (grey line) or without ethanol (black line).

Discussion: Both enzyme preparations were affected by the presence of ethanol but the tolerance of C-Tec3 was marginally better, with an average reduction in glucan conversion of 5% compared to 7% for C-Tec2.

The sensitivity of glucan conversion to the presence of ethanol suggests that the two-stage hydrolysis with intermediate fermentation may not be the optimum way in which to obtain high glucose yields, as the enzyme activity is likely to be affected by ethanol in the second stage of hydrolysis. Alternative means of removing product sugar whilst also increasing the water availability in the system are explored in the next section. Further to this, by using this system in this section it requires that the final product is ethanol which is limiting if this process is to be part of a sugar-platform bio-refinery.

6.6 Two-stage hydrolysis with intermediate product removal

Objective: To determine the effect on hydrolysis yield by removing a portion of the liquid in the hydrolysate, which contains product sugars, and replacing this with water.

Method Summary: A preliminary experiment was carried out in which hydrolysate that had undergone 48 hours of hydrolysis was centrifuged at 1000 – 10000 g and the residual solids content was determined in order to identify the optimum centrifuge speed for maximising product recovery but minimising shear stress to the enzymes in the system.

Hydrolysis was carried out at 25% TS with 50 mg C-Tec2 or C-Tec3 g^{-1} pulp for 48 hours. The resulting hydrolysate was centrifuged and the concentrated sugar solution was removed and replaced with water adjusted to pH 5 with phosphoric acid without any additional enzyme. The hydrolysis was then allowed to continue for a further 2-4 days either with or without another product removal step.

Results: As can be seen in figure 6.6-1 similar solids contents were obtained for centrifuge speeds of 5000 g and 10,000 g. Due to the shear sensitive nature of enzymes 5000 g was therefore chosen as an acceptable centrifuge speed. This speed is consistent with that used in other studies (Ouyang et al., 2013; Rodrigues et al., 2014) as well as in other biological applications (Kimura & Okabe, 2013; Richter et al., 2012).

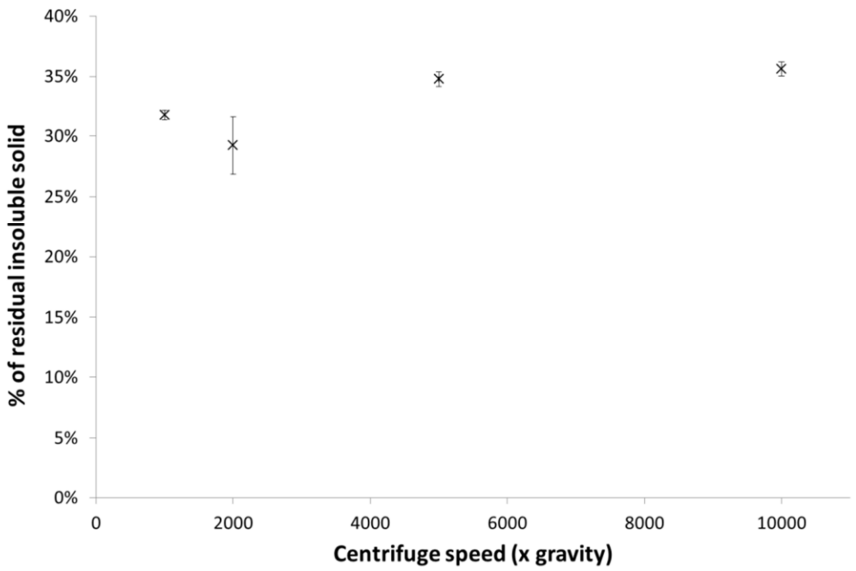


Figure 6.6-1. Percent of residual solids vs centrifuge speed following 48 hours of MSW pulp hydrolysis at 20% TS with 50 mg enzyme g^{-1} pulp.

Figure 6.6-2 shows glucan conversion in the experiment carried out with C-Tec2. As can be seen, glucan conversion in the first 48 hours was approximately 29%. The sugar solution was then removed from the mixture and replaced with pH 5 water. The following 2 days of hydrolysis produced an overall 4-day conversion of 48%, which is 9% higher than the control experiment with no sugar removal. An image of the centrifuged hydrolysate is shown in figure 6.6-3.

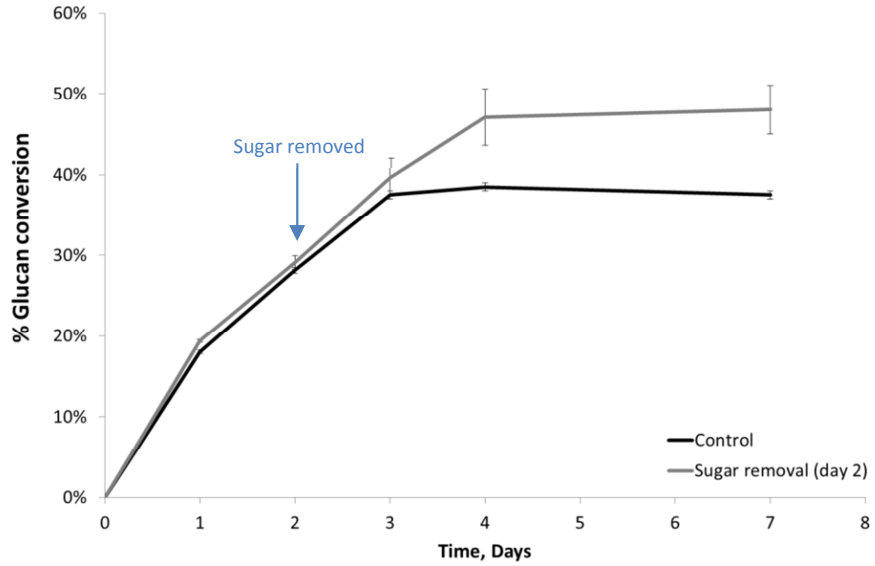


Figure 6.6-2. Impact of intermediate product removal with C-Tec2. In the hydrolysis of 25% TS MSW pulp with 50 mg C-Tec2 g^{-1} pulp. Sugar removal at day 2 (grey) and no sugar removal (black).

Figure 6.6-3. Image of centrifuged hydrolysate showing the concentrated sugar solution separated from the residual solid after centrifugation.

The experiment was repeated with C-Tec3 and the concentrated sugar solution was removed at day 2 and day 4. In this experiment both the control and the product-removal experiment (PRE) had an average 2-day conversion of 36%. After 4 days of hydrolysis the conversion of the control and the PRE were ~43% and ~47% respectively. By the end of the experiment, at 7 days, both the control and the PRE had achieved a similar conversion (49 ± 3% and 51 ± 3% respectively) (figure6.6-4).

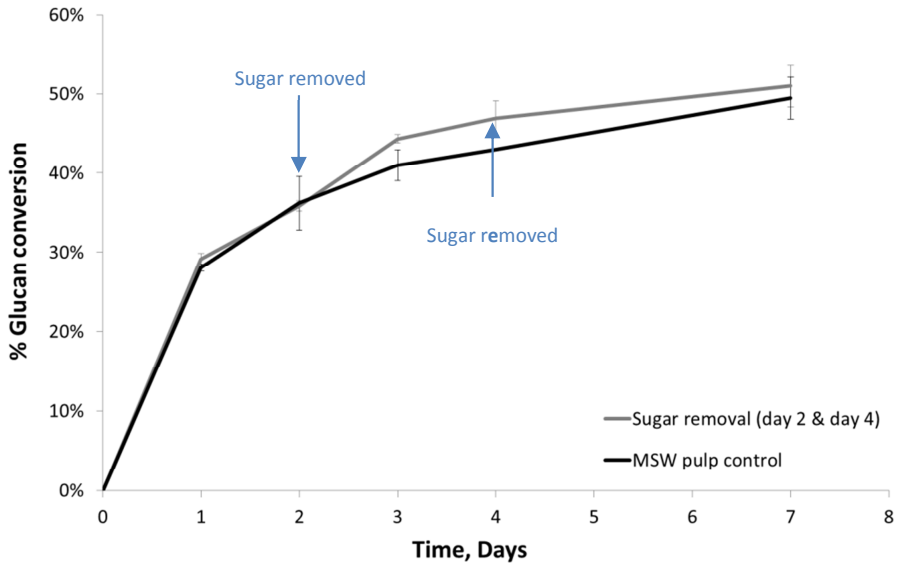


Figure 6.6-4. Impact of intermediate product removal with C-Tec3 in the hydrolysis of 25% TS MSW pulp with 50 mg C-Tec3 g^{-1} pulp. Sugar removal at day 2 and 4 (grey) and no sugar removal (black).

Discussion: As can be seen from the result, aside from removing sugars through fermentation, as was done in the section above, a portion of the concentrated sugar solution can also be removed by centrifugation.

Enzymes are a biological product and are therefore shear sensitive (Ganesh et al., 2000). As a result low centrifuge speeds are preferred in order to maximise water removal from the hydrolysate with minimal stress to the system hence 5000 g was chosen as an acceptable centrifuge speed.

For the trial with C-Tec2, centrifugation removed 43% of the liquid, which contained on average 6.3% glucose. The liquid was then replaced with pH 5 water to the same volume that was removed, reducing the soluble glucose content to 2.6 wt%. The insoluble solid content of the concentrated sugar solution was measured and was found to be 0.3 wt% of the liquid. This negligible loss indicated that most of the unreacted solids remained in the residual solid material. The system was then left to undergo a secondary hydrolysis without the addition of extra enzyme. The results from this experiment are shown Figure 6.6-2 and clearly indicate that C-Tec2 was severely inhibited, probably by the product sugars and/or the reduction in water availability. Removing this product and replacing it with fresh water resulted in an increase in glucan conversion of 9%. This procedure therefore seemed a promising approach for optimisation of the hydrolysis system.

Soon after this initial experiment was performed the newer generation of the enzyme C-Tec3 became available, and the same experiment was therefore repeated with this new enzyme. As before, the hydrolysis was carried out at 25% TS with an initial enzyme loading of 50 mg g^{-1} pulp; the sugar solution was removed at day 2 and day 4 and replaced with pH 5 water. After the first hydrolysis the glucose concentration in solution had reached 7.7 wt%, and this was reduced to 3.3% by removal of the concentrated sugar solution and water replacement. In the second hydrolysis the sugar concentration increased again to 5.8 wt% and was reduced to 2.6% after the second concentrated sugar removal. Although there was a small improvement in glucan conversion with product removal, it was not as pronounced as when using C-Tec2. This suggests that the C-Tec3 enzyme preparation is much

more tolerant to its product sugar and/or to the reduction in water availability, and is therefore not affected by the removal of concentrated sugar to the same extent as C-Tec2. This result was further tested by conducting a glucose loading analysis to determine the product tolerance of the two enzyme preparations: the results of this are presented in the next section.

The results of this experiment also indicate that much of the enzyme activity remains adhered to the solid and any loss of activity into the sugar solution is insignificant. This finding is in agreement with Yu et al., 2013 who studied the adsorption behaviour of enzymes. They found that the enzymes that bind to the substrate within 3 hours perform majority of the hydrolysis and, once bound, there is little desorption into solution during hydrolysis (Yu et al., 2013). In all the literature to date, where experiments have involved sugar removal to increase conversion, extra enzyme has been added to compensate for the loss in enzyme. As proved here and in the work by Yu et al., (2013), it is not necessary to include this enzyme compensation to increase conversion.

6.6.1 Glucose inhibition analysis

Objective: To determine the tolerance C-Tec2 and C-Tec3 to an initial glucose loading and to determine whether an initial loading of product sugar is inhibitory to conversion.

Method Summary: Hydrolysis was carried out at 6.5 % TS (to limit the solids effect) at enzyme dosings between 10 and 120 mg g^{-1} pulp over a time period of 48 hours. Glucose was added to the reaction medium before the enzyme, at concentrations between 3 to 6 wt%. The test was carried out against controls of MSW pulp and Fisher brand filter paper without the addition of glucose.

Results: Results from this experiment are shown in figures 6.6-5 and 6.6-6. It can be seen that C-Tec2 was severely inhibited by a starting glucose concentration of 4 wt% (figure 6.6-5). C-Tec3 however was not subject to the same degree of inhibition and had a comparatively high tolerance for a glucose solution of up to 4.7 wt% (figure 6.6-6).

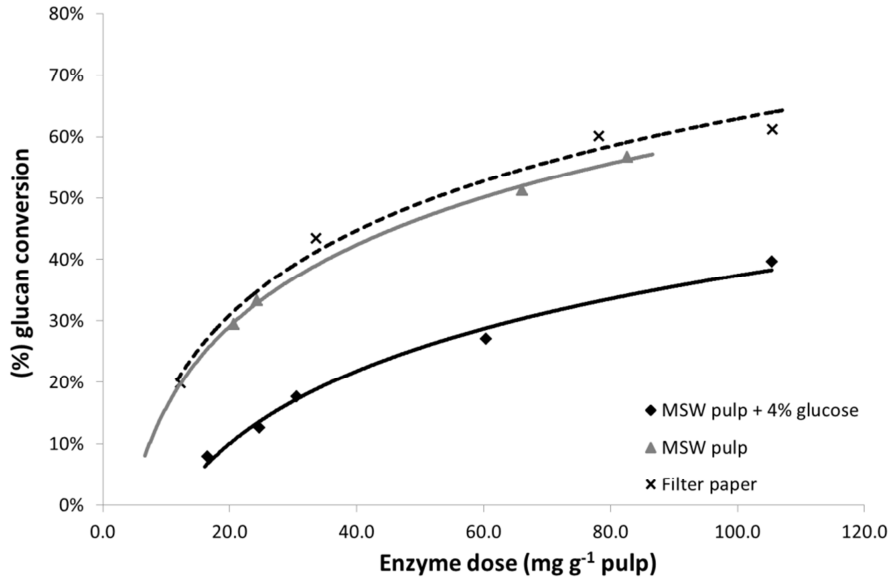


Figure 6.6-5. Effect of initial glucose concentration on final glucan conversion at different enzyme doses. Hydrolysis was carried out over 48 hours using C-Tec2.

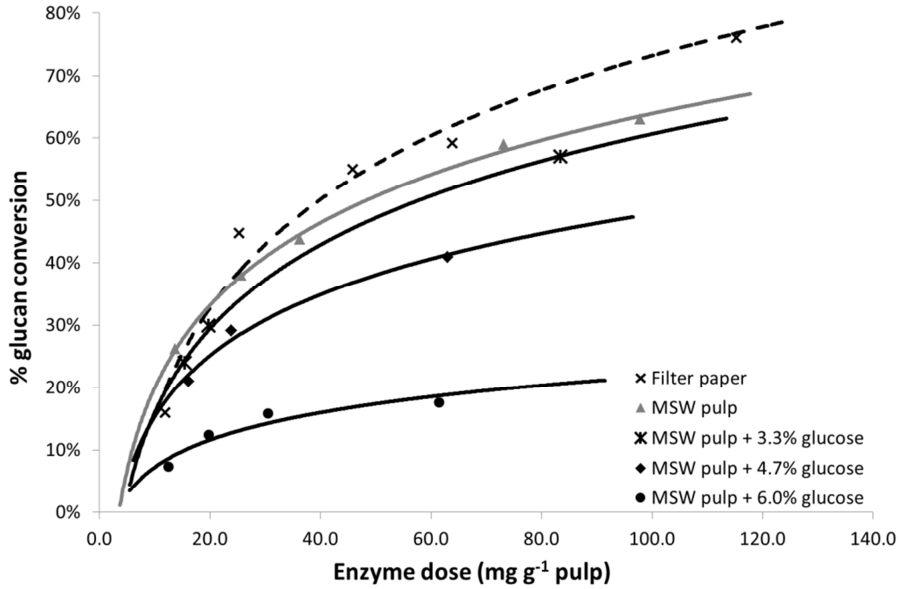


Figure 6.6-6. Effect of initial glucose concentration on final glucan conversion at different enzyme doses. Hydrolysis was carried out over 48 hours using C-Tec3.

Discussion: Glucose tolerance and the inhibitory sugar concentration in the reaction medium are important parameters in cellulose hydrolysis. Based on the literature review, glucose inhibition can occur for one of two reasons: (i) a direct inhibitory effect on cellulase activity; or (ii) accumulated glucose may obstruct the transfer of enzymes between glucan fibres. If the latter is the cause of decreasing enzyme

activity as hydrolysis proceeds, then reducing the glucose concentration during a reaction should have a positive effect on hydrolysis and be able to increase the overall glucan conversion.

As can be seen in figure 6.6-6 at low enzyme loadings (< 30 mg enzyme g^{-1} pulp) C-Tec3 shows no inhibition in a medium containing up to 4.7 wt% glucose. C-Tec2 however is significantly inhibited at these low enzyme loadings. Thus it can be concluded that product tolerance has advanced significantly in the new generation of enzymes, especially at low enzyme concentrations. Furthermore it was found that the hydrolysis rate plateaus at a later stage when using C-Tec3 than with C-Tec2.

Figure 6.6-6 also shows that glucose concentrations up to 3 wt% are well tolerated by C-Tec3 over the range of enzyme loadings tested. It should be noted, however, that during hydrolysis of lignocellulosic substrates other sugars and soluble compounds such as calcium are also released, and these can act to reduce the water availability of the system and cause enzyme inhibition. Although the 3% glucose concentration used in this trial did not show a great impact, a 3% glucose solution with other soluble compounds might hinder hydrolysis. To allow for this possibility, it may be that glucose concentrations should be limited to an appropriate range (e.g. of 1-2 wt%) in a second or third stage of hydrolysis of an MSW pulp to minimise inhibition of C-Tec3 activity. This theory was later verified by experimentation in section 6.7-4.

The use of C-Tec3 improves the efficiency of the system in terms of hydrolysis rate and glucose produced per unit of enzyme; but the pulp still does not hydrolyse completely and the hydrolysis curve follows the same pattern as when using C-Tec2. This suggests that further optimisation of hydrolysis with the newer and improved enzyme is still desirable and possible, in order to decrease enzyme usage and cost.

One other interesting point to note from this experiment was that the overall conversion on filter paper and MSW pulp without glucose addition was similar. This result may indicate that the same proportion of enzyme bind to the substrate and then produce glucose in the same way. The amount of glucose produced from filter

paper is of course greater than from MSW due to the higher glucan content but the proportion of enzymes that bind, be productively or unproductively, is most likely the same.

6.7 Two-stage hydrolysis with intermediate product removal and wash step

When working at high solids concentrations there is significant retention of liquid within the residual solids (Ioelovich & Morag, 2012). After a 48-hour hydrolysis at 20% TS, the residual solids from a centrifuged hydrolysate typically contain 35% solids, inclusive of sugar. This means that on average 43-45% of the hydrolysate liquid is extractable whilst 55-57% remained with the MSW pulp. The liquid remaining with the solid therefore represents a considerable amount of unrecovered sugar. The next sets of experiments were thus designed to recover as much of this residual sugar as possible for use in production of ethanol or other value-added products whilst also preventing product inhibition and improving water availability.

From the experiments in section 6.6 it was clear that the sugar concentration inhibition threshold for C-Tec3 was much higher than for C-Tec2, but that removal by centrifugation alone was not sufficient to reduce the product sugar concentration to a level where it does not inhibit the enzymes. The experiment also showed that much of the enzyme activity remains adhered to the substrate following removal of the concentrated sugar solution. It was therefore decided to add a wash step after the concentrated sugar removal, in an attempt to increase the enzyme activity of C-Tec3 by increasing the water availability in the system for a second stage hydrolysis. It was decided to perform the wash step after the sugar solution reached a concentration of > 8 wt% glucose so that an acceptable ethanol titre could be attained and/or to minimise the requirement for further concentration steps for other bio-refinery products.

Objective: To determine whether enzyme activity could be increased by reducing the concentration of product sugars associated with the residual solid in the hydrolysate to less than 2 wt% glucose.

Method Summary: Hydrolysis was carried out for 48 hours at 18.5% TS using 55 mg C-Tec3 g^{-1} pulp, after which the hydrolysate was centrifuged and the concentrated sugar solution removed. The residual solid was washed with pH 5 water for one hour. The washwater was then removed by centrifugation and fresh pH 5 water was added to make up the volume of hydrolysate to the original volume before removal of the concentrated sugar solution. No extra enzyme was added in this process.

Results: The results presented in figure 6.7-1 show that when a pulp of 18.5% TS was hydrolysed for 48 hours with 55 mg CTec3 g^{-1} pulp and washed at pH 5, the sugar concentration in the residual solids reduced from ~9.5 wt% to ~1 wt%. The second stage hydrolysis of the re-suspended solids resulted in a further 14% of glucan conversion, taking the total conversion efficiency from 67.2 ± 1.3% to 81.2 ± 0.7% (figure 6.7-2). This result can be compared with that for a control which did not undergo product removal and a wash step, and had an overall conversion efficiency of 68.0 ± 5.0%. The results suggest that the increase in total conversion efficiency is due to an improvement in water availability brought about by removal of the residual sugars.

Analysis of the hydrolysate from the secondary hydrolysis showed a slight increase (1.1 fold) in cellobiose concentration as compared to the initial hydrolysis, indicating a possible loss of β-glucosidase from the original hydrolysate. This adds support to previous evidence that this enzyme does not bind to the substrate (Varnai et al., 2011).

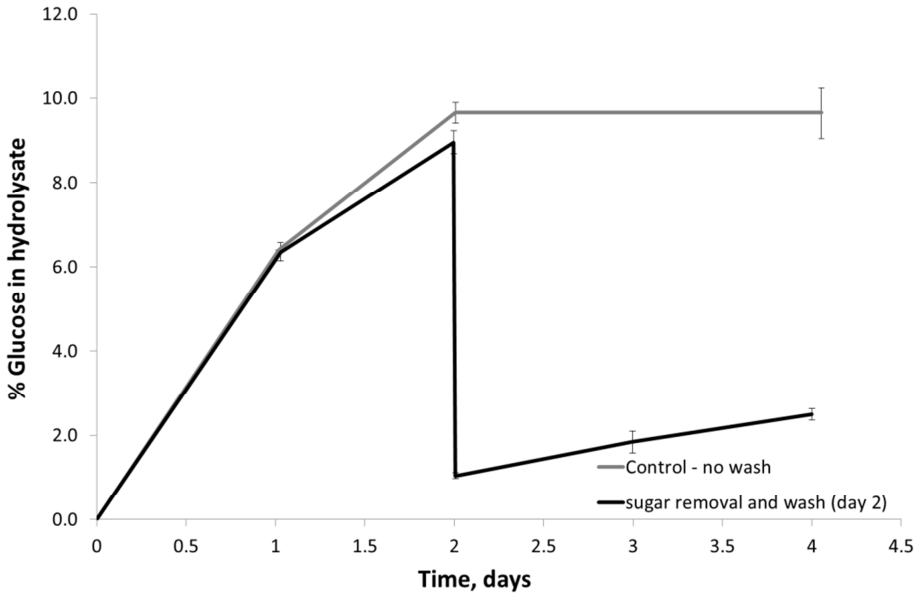


Figure 6.7-1. Effect of intermediate washing on MSW pulp (18.5% TS) hydrolysis: Glucose in hydrolysate. Unwashed control (grey line), washed at day 2 (black line)

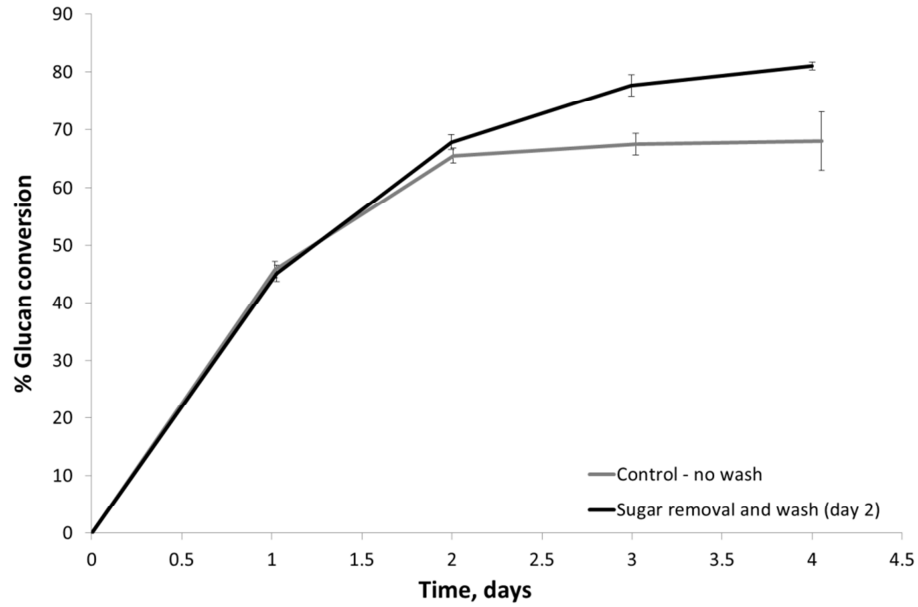


Figure 6.7-2. Effect of intermediate washing and β-glucosidase enzyme addition on MSW pulp (18.5% TS) hydrolysis: Overall substrate conversion. Unwashed control (grey line), washed at day 2 (black line)

Discussion: Adding an intermediate wash step is an effective means of increasing product conversion. While the percentage conversion to sugar is a prime consideration, however, there is also a requirement to produce a high sugar concentration in the hydrolysate (Larsson & Zacchi, 1996). There must therefore be a trade-off between conversion efficiency, conversion rate, and product concentration. Yang et al., 2010 & 2011 showed that it is possible to obtain a very

high substrate conversion (85%) in a period of 30 hours using a 3-stage hydrolysis system with intermediate washing steps (Yang et al., 2011; Yang et al., 2010). The sugar stream arising from each stage contained a maximum sugar content of ~5.5% or 55.5 g L^{-1}, however, which is well below the ideal value for further fermentation. Once fermented, a hydrolysate of this strength would contain less than 4 wt% ethanol, unless the sugar stream was first concentrated by methods such as multiple-effect evaporation or nanofiltration (Qi et al., 2012) that would reduce the net energy yield.

As is evident from the results, although the wash step enhances the enzyme activity, the glucose concentration in the hydrolysate from the secondary hydrolysis was only 2.5 wt%. Such a dilute solution would certainly require concentration for use in ethanol production, and probably most other purposes. It should be noted, however, that the amount of liquid requiring concentration is less than that using the system of Yang et al., 2011 or from performing hydrolysis at 5% TS. Although a low sugar yield was obtained during the second stage the process was not optimised, and the dilute sugar solution is still a good source of energy. This latter aspect suggests that use of this procedure may be more appropriate in an integrated bio-gas/bio-ethanol process as it could potentially help to improve the overall energy balance.

The commercial enzyme product C-Tec3 contains a mixture of enzymes and their associated binding domains, to provide a strong affinity with the substrate and its sub-components (Ramos et al., 1993; Van Dyk & Pletschke, 2012). Cellulases tend to bind strongly to the substrate, and after hydrolysis remain associated with the solid fraction: the results in the present tests gave further evidence in support of this. Weiss et al., 2013 showed that by recycling 85% of the insoluble residual solid with its bound enzymes, plus fresh substrate at 15% TS, the subsequent enzyme requirement could be reduced by 30% (Weiss et al., 2013). A commercial cellulase enzyme preparation will also contain β-glucosidase which cuts cellobiose and cellotriose into glucose monomers. It is thought that this enzyme does not form a strong bond with the substrate, and will most likely partition into the sugar solution after hydrolysis. Further to this, cellulases have been used for up to four rounds of

hydrolysis (Tu et al., 2007b) whereas β-glucosidase is known to be less stable over prolonged or multi-stage reaction periods (Varnai et al., 2011); so in addition to potential loss of this enzyme in the concentrated sugar solution or washwater the enzyme may also be denatured during hydrolysis. It has recently been shown, however, that β-glucosidases from newer enzyme preparations such as C-Tec2 contain a proportion of β-glucosidase that tends to remain adhered to the solid and can retain a large proportion of its catalytic activity (Haven & Jorgensen, 2013).

The two-stage hydrolysis with intermediate wash step experiment suggested that a proportion of the β-glucosidase was removed in the washing process, as indicated by an increasing cellobiose concentration measured in the second stage. A complete loss of β-glucosidase is unlikely, as glucose was still produced during the secondary hydrolysis. This enzyme is important as it is required to convert cellobiose to glucose and its action may possibly release cellobiose from the active sites of cellobiohydrolases, thus further facilitating the processive action of the enzymes (Bu et al., 2012). As it is an essential component in reactivation of the enzyme system, it was thought that an even higher glucan conversion might be achieved if the depletion observed as a result of washing could be replenished (section 6.7.1).

6.7.1 Two stage hydrolysis with intermediate wash step and β-glucosidase addition in the second-stage of hydrolysis

Objective: To determine the performance of a two-stage hydrolysis process with an intermediate wash step when β-glucosidase is added in the second stage of hydrolysis.

Method Summary: The experimental procedure used was the same as in section 6.7, with addition of β-glucosidase at a concentration of 12.5 and 25 mg enzyme g^{-1} original pulp at the beginning of the second-stage of hydrolysis.

Results: No significant effect was seen when β-glucosidase was added at 12.5 mg g^{-1} of original pulp. As can be seen in figure 6.7-3, however, there was a marked increase in glucose concentration for β-glucosidase addition at 25 mg g^{-1} of original

pulp, resulting in a hydrolysate with >5 wt% glucose. The final glucan average conversion following this addition was 88% (figure 6.7-3 B). This conversion was verified by measuring the weight-loss of the substrate, which was found to be 87.8%. These results provide support for the premise that either β-glucosidase is denatured/loses activity during hydrolysis; or a proportion is lost during washing and that replacing this can result in an increase in glucan conversion. Mixing of the concentrated sugar stream (~10 wt%) from the first-stage hydrolysis with the sugar stream from the second stage hydrolysis (~5 wt%) would give a glucose concentration of ~7.5% wt for the final product of the two-stage hydrolysis process.

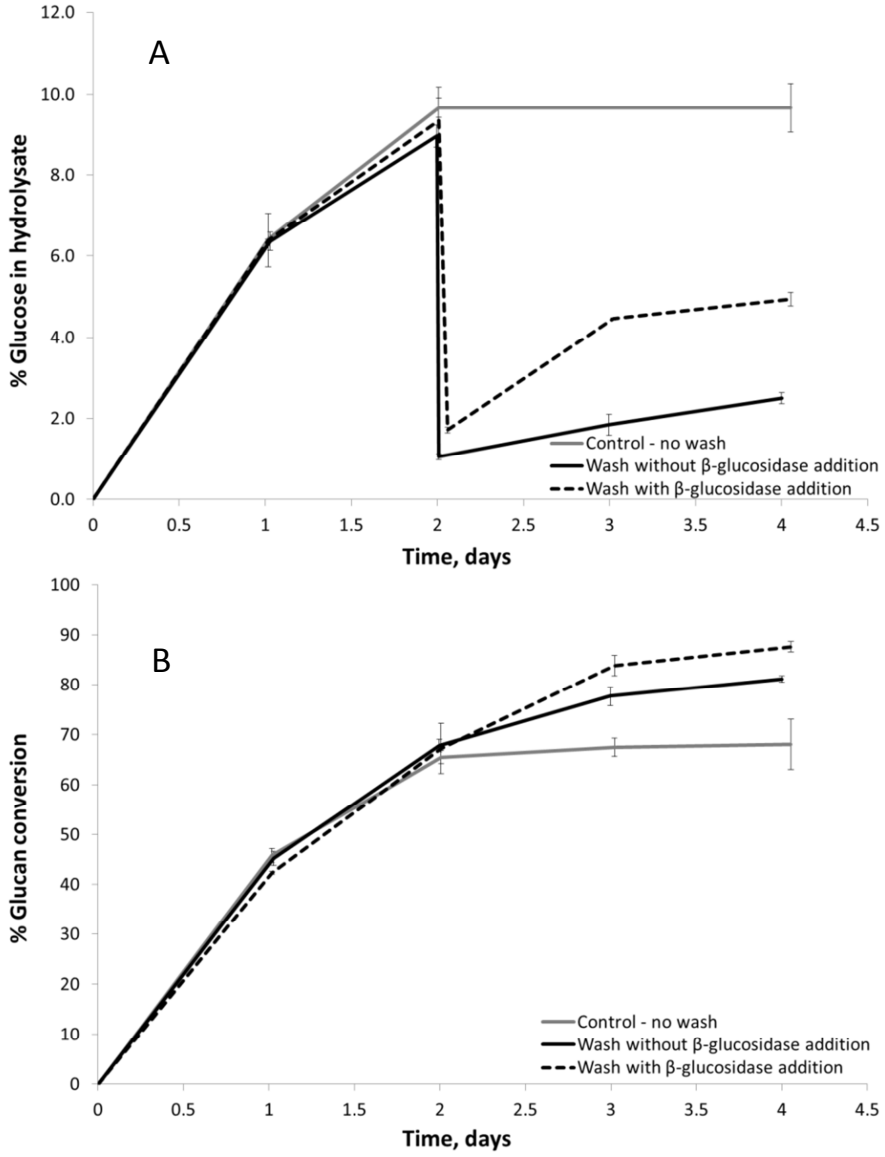


Figure 6.7-3. Effect of intermediate washing and β-glucosidase enzyme addition on MSW pulp (18.5% TS) hydrolysis: A) percentage of glucose in hydrolysate, B) overall substrate conversion. Unwashed control (–), washed without β-glucosidase addition (–); washed with β-glucosidase addition (– –).

The experiment was repeated with a second batch of pulp at a slightly higher initial TS concentration of 20% and a lower initial enzyme dose of 50 mg CTec3 g^{-1} pulp. The enzyme dose was lower due to the minor variations in substrate characteristics occurring between and within batches. The same response to β-glucosidase addition was noted, although the final glucose concentrations were lower at 8.0 wt% and 4.1 wt% in the first and second stage respectively: this was possibly due to increased solids effects coupled with the impact of the slightly reduced enzyme dose. The final glucose concentration of 6 wt% from mixing the primary and secondary hydrolysates was also lower than the ideal 8 wt%. This variability clearly shows the need both for optimisation and for careful control of process conditions.

The focus in the research thus far was on glucose yields as the actual quantities of the other sugars were minimal and followed the same hydrolysis trend as glucose. In this experiment, however, an interesting result was seen with respect to xylose. In addition to the positive effect glucan conversion, the results also suggested that the β-glucosidase addition may have had some effect on xylan which is surprising as strictly β-glucosidase should not affect xylan conversion. As seen in Figure 6.7-4 when β-glucosidase was not added or when it was added at a low concentration of 12.5 mg g^{-1} original pulp, the xylose in solution did not increase; whereas when β-glucosidase was added at 25 mg g^{-1} original pulp there was an increase in xylose within the solution, which coincided with the increased glucan conversion. This could be due to a number of reasons, including: (i) the presence of xylanase in the β-glucosidase preparation; or (ii) the release of extra cellulose/cellobiose as a result of β-glucosidase addition which then allowed increased access for hemicellulases to hydrolyse unconverted xylan.

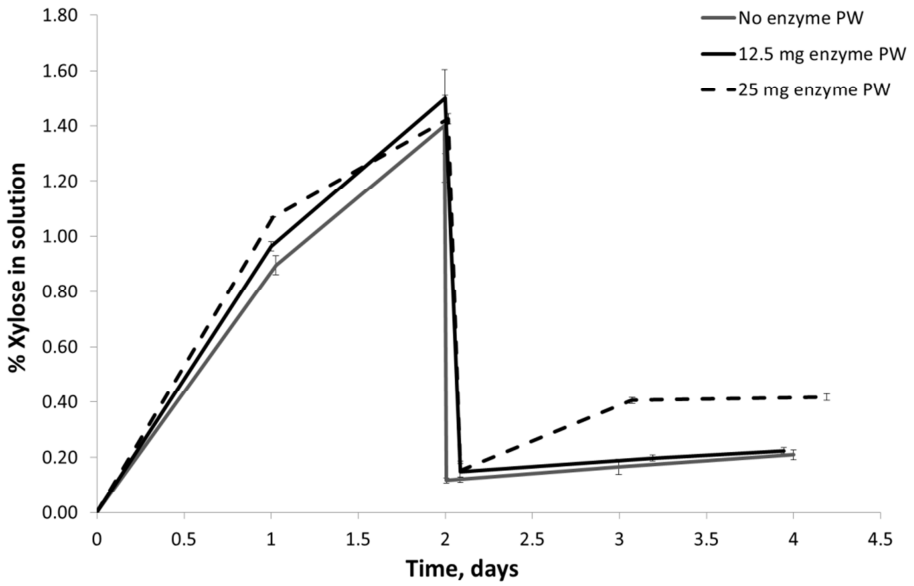


Figure 6.7-4 Effect of different β-glucosidase additions on hydrolysate xylose concentration. No enzyme post wash [PW] (–), 12.5 mg β-glucosidase g^{-1} original pulp (–),25 mg β-glucosidase g^{-1} original pulp (– –).

Discussion: The final glucose concentration expected from mixing of the sugar solutions from the first and second stage was ~7.5 wt%. Although this is just below the preferred starting point for fermentation, there is clearly considerable potential for optimisation of the system. Furthermore, this result can be achieved in a 4-day period compared to the 7 days required to obtain a similar yield using the two-stage hydrolysis with fermentation approach.

Varnai et al., 2010 showed that even a very small amount of xylan, 0.34% on a TS basis, can limit the hydrolysability of a lignocellulosic substrate and that adding xylanases to their system gave a 12% increase in cellulose hydrolysis (Várnai et al., 2010). Other researchers have also shown the detrimental effect of xyloligomers on cellulose hydrolysis and have demonstrated that synergistic effects can occur when xylanases are added to a cellulase enzyme mix to help increase cellulose conversion (Hu et al., 2011; Qing et al., 2010; Zhang et al., 2012a). These studies point to the importance of the xylan distribution and its role in limiting enzyme hydrolysis. It is therefore of interest to determine the mechanism of xylose release and this is considered in the next section.

6.7.1.1 Investigating the mechanism of β-glucosidase in relation to increased glucan and xylan conversion

There are a number of possible reasons why the addition of β-glucosidase to the second stage hydrolysis may have led to an increase in both glucose and xylose production, the three most likely are: (i) The β-glucosidase preparation used may have contained significant xylanase activity; (ii) the β-glucosidase may have contained or released cellulases which converted a proportion of cellulose that was obstructing access to hemicellulose. Once this hemicellulose was accessible it could then be degraded by the residual hemicellulases in the system; or (iii) a synergistic effect existed between cellulose and hemicellulose conversion.

Objective: To determine why the addition of β-glucosidase leads to a simultaneous release of glucose and xylose in second-stage of hydrolysis (section 6.7.1).

Method Summary: The following experiments were set-up to try and determine the mechanism of action for the β-glucosidase:

(1) The cellulase and xylanase activities of the β-glucosidase preparation were determined and compared to the activity of C-Tec3. Activity measurement was carried out using the modified FPU test described in section 3.10 on both Whatman number 1 and Fisher brand filter paper (Cat no. FB59035). The latter substrate was included to ensure that any xylanase activity could be seen, as it contains both cellulose and hemicellulose, whereas the Whatman paper does not.

(2) Two-stage hydrolysis experiments with an intermediate wash step were performed and various enzyme preparations (C-Tec3, H-Tec3, β-glucosidase or a combination of these) were added to the second stage of hydrolysis. These extra enzyme preparations were added at a concentration of 12.5 mg g^{-1} original substrate, except for the combination of β-glucosidase and H-Tec3 in which both enzyme preparations were added at a concentration of 6.3 mg g^{-1} original substrate.

The first-stage hydrolysis was carried out at 20% TS using 50 mg C-Tec3 g^{-1} pulp for 48 hours. This pulp was centrifuged, and the concentrated sugar solution was removed, then the pulp was washed and re-suspended in pH 5 water with enzyme additions at the concentrations noted above.

(3) Based on the result from step (2), a β-glucosidase activity test (section 3.10) was performed with β-glucosidase and C-Tec3 enzyme preparations to clarify possible mechanisms for any differences in performance.

Results:

(1) Figure 6.7-5 shows the relative glucose release in mg from the two enzyme preparations. While figure 6.7-6 shows the relative xylose release from the two enzyme preparations.

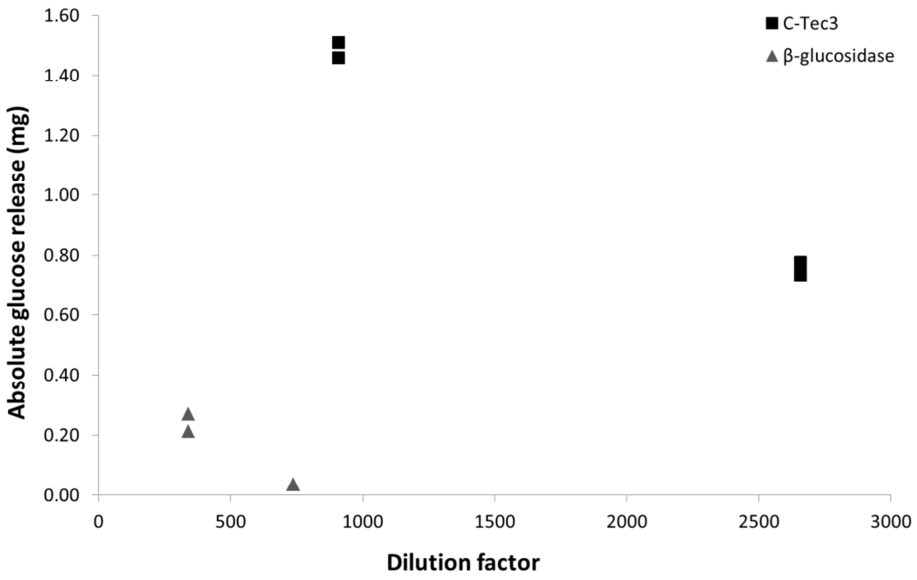


Figure 6.7-5. Comparative glucose release from Whatman no. 1 filter paper of both C-Tec3 and β-glucosidase enzyme preparations at various dilutions.

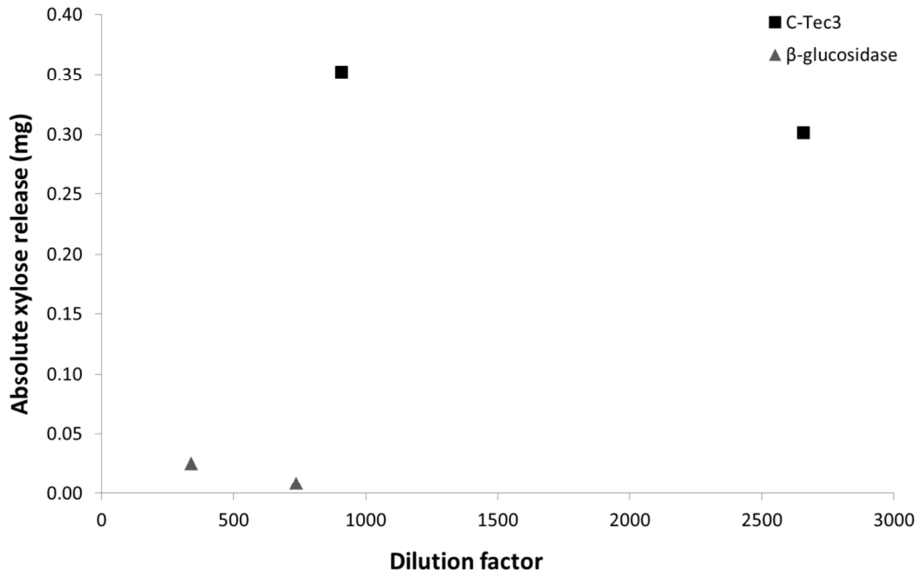


Figure 6.7-6. Comparative xylose release from fisherbrand filter paper of both C-Tec3 and β-glucosidase enzyme preparations at various dilutions.

The dilution factor for β-glucosidase in the preceding experiment (figure 6.7-3) was 160, giving a higher concentration than the dilutions used here which were between 300 and 2500. As can be seen from figure 6.7-5 the β-glucosidase preparation did contain a low level of cellulase activity and this combined with the β-glucosidase activity could account for the increase in glucose yield. In contrast figure 6.7-6 shows that there is very little xylanase activity in the β-glucosidase compared to C-Tec3. It is therefore unlikely that the β-glucosidase addition led to the release of hemicellulose, and the increase in xylose (Figure 6.7-4) was probably due to cellulose hindering hemicellulose access.

(2) The reason for doing this experiment was to observe whether xylan conversion always related to the increase in glucose yield. If hemicellulose removal was the reason for cellulose release in the second stage of hydrolysis (section 6.7-1) then a secondary hydrolysis with the addition of H-tec3 or C-Tec3 would in theory produce better results than the use of β-glucosidase in the second-stage of hydrolysis.

It was found that all of the enzyme preparations tested gave an increase in glucan conversion over the control which did not undergo an intermediate product removal and a wash step (figure 6.7-7).

An overall 4-day glucan conversion of 65 ± 0.1% was achieved for the control hydrolysis, as can be seen in figure 6.7-7. In ascending order the average overall glucan conversion achieved over both rounds of hydrolysis was 74 % with C-Tec3, 77% with H-Tec3, 79% with β-glucosidase, and 80% with β-glucosidase and H-Tec3.

Xylan conversion was also measured and the results are shown in figure 6.7-8. The average 4-day xylose conversion in each experiment was as follows: control 78%, H-Tec3 91%, C-Tec3 93%, β-glucosidase & H-tec3 99% and β-glucosidase alone 100%.

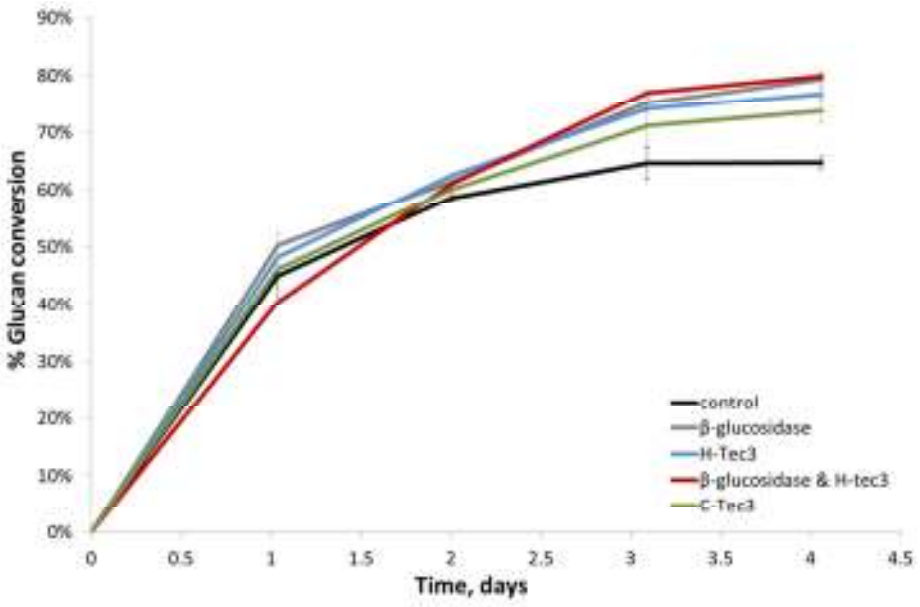


Figure 6.7-7. Effect on glucan conversion of different enzyme preparations used during second-stage hydrolysis. Control, no wash or additional enzyme (black), β-glucosidase addition (grey), H-Tec3 addition, (blue), a combination of B-glucosidase and H-tec3 addition (red) and C-Tec3 addition (green).

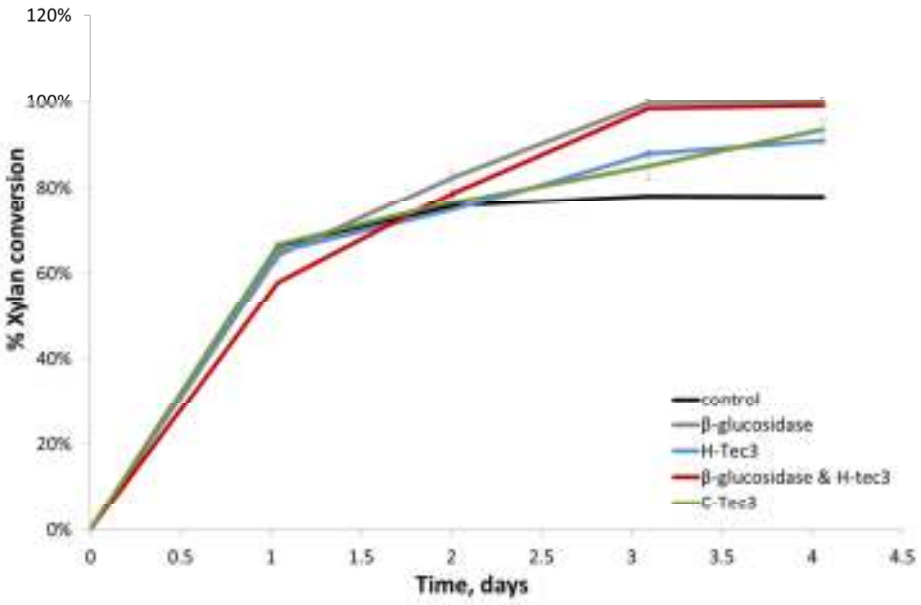


Figure 6.7-8. Effect on xylan conversion of different enzyme preparations used during secondary hydrolysis. Control, no wash or additional enzyme (black), β-glucosidase addition (grey), H-Tec3 addition, (blue), a combination of B-glucosidase and H-tec3 addition (red) and C-Tec3 addition (green).

(3) The result from experiment 3 is shown in figure 6.7-9 and it can be seen that the β-glucosidase activity of H-Tec3 was much lower than C-Tec3 and β-glucosidase. The latter two preparations hydrolysed all of the cellobiose present in test when used at the lower dilution ranges.

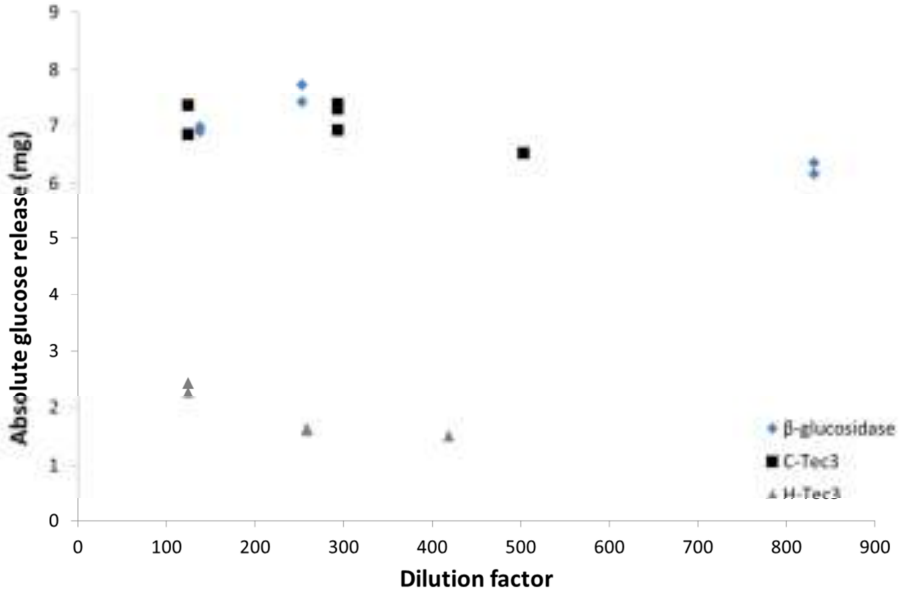


Figure 6.7-9. Comparative glucose release from cellobiose using β-glucosidase enzyme preparation (blue), C-Tec3 (black) and H-Tec3 (grey) at various enzyme dilutions.

The low β-glucosidase activity of H-Tec3 compared to the other two enzyme preparations could explain its relatively poor performance in secondary hydrolysis.

Discussion: From the first results shown in figure 6.7-5 and 6.7-6 it was clear that the cellulase and xylanase activity of the β-glucosidase preparation was very low in comparison to the C-Tec3 enzyme preparation. This suggests that cellulase activity alone was not the reason for the increase in glucose yield initiated by β-glucosidase addition, and instead a combination of cellulase and β-glucosidase activity was needed. Figure 6.7-9 shows that β-glucosidase activities of both C-Tec3 and β-glucosidase were quite high and were similar at the dosing used in the experiment. It is therefore of interest to note from Figure 6.7-7 that the secondary hydrolysis with C-Tec3 addition showed the poorest performance of all the enzyme preparations, even though this preparation contains the whole mixture of enzymes required to increase hydrolysis.

The results shown in Figure 6.7-7 indicated that higher yields occurred in the second-stage of hydrolysis in the presence of enzyme mixtures containing the β-glucosidase enzyme preparation. This suggests that the mechanism of cellulose release is specific to this enzyme. It almost seems as though the addition of the β-glucosidase preparation alters the conversion equilibrium and allows further reactivity of the enzymes present. It should be noted, however, that even though the various enzyme preparations performed differently in second stage of hydrolysis the difference was not significant enough be conclusive.

It is also interesting to observe from figure 6.7-8 that the preparations with β-glucosidase appeared to release more xylose than the C-Tec 3 preparation which has significant xylanase activity within its mix. This result once more points towards some form of interaction between β-glucosidase and xylose release. It may be that a cyclical effect is occurring where β-glucosidase releases some cellobiose which in turn releases trapped xylan that is crucial for further cellulose hydrolysis. This still does not explain why the C-Tec3 addition results in lower xylose release than β-glucosidase addition. It is possible, however, that the remaining cellulases bound to the residual solid after the initial hydrolysis prevent the adsorption of new

cellulases or cause enzyme jamming on the remaining cellulose fibrils and thus prevent new cellulases from productively binding to the substrate, thereby limiting the effectiveness of adding C-Tec3.

The current experiment suggests that the β-glucosidase helps to release a proportion of cellulose / cellobiose which contains the tightly bound xylan / xylose and this allows increased xylose release. This observation is based on the results presented in figure 6.7-6 which shows that the β-glucosidase preparation has little xylanase activity. This finding is in agreement with observations by Penttilä et al., 2013 that there are two xylan fractions; one which is loosely bound and one more tightly bound with cellulose.

It should be noted that this experiment was carried out with a different batch of pulp than that used in the original two–stage hydrolysis with intermediate wash step. The slightly lower initial hydrolysis yields found in the current test could be attributed to the use of 20% TS rather than 18.5% TS due to the solids effect; a similar result was also seen in section 6.7.1.

Further to this, the experiment presented in this section showed an increased glucan and xylan conversion in the second stage of hydrolysis when using a low dose of β-glucosidase (12.5 mg g^{-1} original pulp), which was not the case in the experiment in section 6.7.1. Two possible reasons for this result are: (i) the lower hydrolysis yields obtained in the first stage of hydrolysis figure 6.7-7 compared to the experiment in figure 6.7-2, section 6.7.1 meant that a proportion of readily degradable or easily accessed cellulose was still present at the start of the second-stage of hydrolysis which allowed residual substrate to be converted by a lower enzyme dose; or (ii) the pulp used in the later experiment may have had increased substrate accessibility as it was disc refined in the Fiberight pilot plant, which the original pulp was not.

Conclusion: The results from these experiments indicate that the mechanism by which β-glucosidase enhances glucan and xylan conversion in the second stage of hydrolysis is connected with β-glucosidase activity, as this enzyme preparation does not have significant xylanase activity. It is possible that the β-glucosidase releases

trapped cellobiose molecules which allows for the 'reactivation' of cellulases. This release of cellobiose may also release trapped or tightly bound xylan which is associated with cellulose.

6.7.1.2 Two-stage hydrolysis with intermediate wash step & PEG 6000 addition

Objective: To determine whether; (i) the addition of PEG 6000 could further enhance hydrolysis in the two-stage hydrolysis strategy with an intermediate wash step and (ii) the reduction in the number of bound enzymes after the initial hydrolysis affects the second stage hydrolysis.

Method Summary: In this experiment a 1% PEG solution was added to a substrate at 47% TS to dilute the solids to 20% TS, resulting in a final PEG concentration of 0.6 wt%. Once diluted, the substrate was dosed with 50 mg C-Tec3 g^{-1} pulp and the mixture was left to hydrolyse for 48 hours. Following this the concentrated sugar solution was removed by centrifugation, and the residual hydrolysate was washed with a 1% PEG solution for 1 hour at pH 5. After washing the pulp was centrifuged again and the washwater was removed and replaced with a 1% PEG solution to the volume of the hydrolysate (concentrated sugar solution) removed originally. Two experiments were carried out: (i) with no enzyme addition post wash and (ii) with β-glucosidase at 25 mg g^{-1} initial pulp added post wash.

Results: The average glucose concentration after 48 hours with the addition of PEG and 50 mg C-Tec3 g^{-1} pulp was 7.7 ± 0.1%, which was equivalent to a 57 ± 2% glucan conversion. This yield is less than that obtained without PEG, as shown in section 6.7.1. After the washing, the second stage of hydrolysis began with an average of 1.2% glucose in solution; after 24 hours this rose to ~3.4 wt% both with and without β-glucosidase addition. After 48 hours, however, the hydrolysates without and with β-glucosidase had respective glucose contents of 3.6 ± 0.1 wt% and 4.0 wt%. It seems that this procedure with the addition of PEG is not as effective as without the additive.

Two additional interesting results were seen in this experiment: (i) The volumes of liquid removed by centrifugation after the initial 48 hours of hydrolysis in this experiment and the equivalent one in section 6.7.1 were 52% and 45% with and without PEG respectively; and (ii) pH 5 water was used for dilution in this experiment, but after 48 hours of hydrolysis the pH had adjusted to 5.3. The sugar solution was removed; the pulp was washed and re-suspended in pH 5 water. The pH was measured after 12 hours of the second hydrolysis and found to have risen to pH 5.5. This was then re-adjusted to pH 4.7 using phosphoric acid for the hydrolysate without β-glucosidase and 4.8 for the hydrolysate with β-glucosidase. The significance of these findings is discussed below.

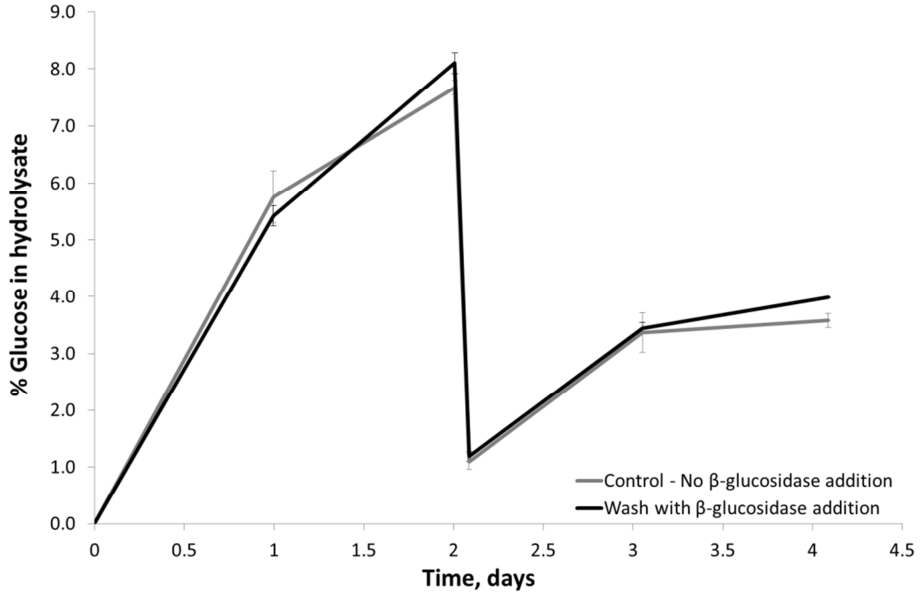


Figure 6.7-10. Effect of intermediate washing and β-glucosidase enzyme addition on MSW pulp (20% TS) hydrolysis with PEG addition. Control with no β-glucosidase (grey), β-glucosidase added at the start of secondary hydrolysis (black)

Discussion: As noted in section 5.3.3 PEG appears to prevent unproductive binding and unnecessary binding of cellulases which can lead to enzyme jamming. These mechanisms led to an overall increase in glucan conversion of 15% when using 30 mg enzyme g^{-1} pulp. Thus, it was of interest to see what effect addition of PEG would have on the two-stage hydrolysis with intermediate wash step strategy as described in section 6.7.1. The purpose of these experiments was to see how the addition of PEG affected the two-stage wash system and whether the decrease in

enzyme adhered to the substrate as a result of PEG addition led to a decreased yield in the second stage.

Comparing the present result (figure 6.7.10) to that in figure 6.7-3A (section 6.7.1) it can be seen that, contrary to expectation based on the results in section 5.3.3, the primary hydrolysis in this experiment with PEG gave a decreased glucose yield compared to that in the same experiment without PEG (section 6.7.1). The reason for this may be related to the mechanism by which PEG acts, whereby the compound adheres to a cellulose fibre preventing unproductive binding of cellulases which in turn can cause jamming or early termination of glucan hydrolysis through hydrophobic interactions. Thus, increasing the enzyme loading from 30 to 50 mg g^{-1} pulp could have resulted in an increase in hydrophobic interactions with greater enzyme binding and jamming in certain areas of the cellulose fibril. This action of PEG which prevents enzymes binding at low enzyme loadings may be a hindrance at higher loadings where the hydrophobic synergy may be prevented by the increased number of interactions occurring.

This experiment was also designed to test whether unbound enzymes resulting from the addition of PEG (Yu et al., 2013) caused a loss in conversion during secondary hydrolysis. The overall conversion of the substrate in the study with PEG was 73 ± 3%, which was a 17% increase over the first-stage hydrolysis yield. For comparison the previous experiment without PEG (section 6.7.1) only gave a 14 % increase in the second stage. This suggests that PEG does not prevent the binding of crucial enzymes, which is in agreement with (Yu et al., 2013).

The experiment with β-glucosidase addition gave a 19% increase in conversion in the second stage of hydrolysis, similar to the 20% increase achieved without PEG (section 6.7.1). Thus it is possible that when PEG is used the amount of β-glucosidase retained in the residual solid is increased, and the β-glucosidase addition therefore has little effect on increasing conversion further. The PEG could also help to stabilise the enzymes and stop them from denaturing, as has been shown with other surfactants (Kaar & Holtzapple, 1998). This stabilising effect would be most significant for β-glucosidase, which is believed to lose its activity

faster than the cellulases (Varnai et al., 2011). The result here confirms that the addition of PEG does not impede secondary hydrolysis, and all the enzymes required for continued hydrolysis are still present in the residual substrate after the initial hydrolysis. The overall glucan conversion, however, was 10% lower than in the experiment without PEG (section 6.7.1).

The increase in the amount of liquid removed by centrifugation after the first-stage hydrolysis with PEG compared to that without PEG suggests that PEG may affect endoglucanase activity in the enzyme preparation. This enhancement, however, was not linked to a corresponding increase in cellobiohydrolase activity. Endoglucanases have been shown to be primarily responsible for substrate liquefaction (Szijarto et al., 2011), with a corresponding decrease in viscosity; but this does not directly lead to an increase in sugar yield. Once a certain degree of liquefaction is achieved a full complement of enzymes is required to decrease the fibre length further (Skovgaard et al., 2014). Endoglucanases release bound water associated with cellulose fibrils, and this also reduces the viscosity of the reaction medium (Felby et al., 2008). The increase in water removal by centrifugation when using PEG could thus be related to PEG enhancing endoglucanase activity. This theory is in accordance with previous reports that the surfactant Tween 20 can enhance hydrolysis by inhibiting the non-productive binding of endoglucanases to the cellulose surface (Mizutani et al., 2002; Ooshima et al., 1986): PEG may allow the same mechanism to occur. Alternatively it is possible that as a polymer PEG binds water and thus allows increased water removal during centrifugation. PEG is not commonly used in dewatering of sludges, however, and thus its dewatering capabilities are not believed to be high (Tchobanoglous et al., 2003).

The argument for the increase in endoglucanase activity is further strengthened by the rise in pH seen in both the first and second stage hydrolyses. It is possible that an increased endoglucanase enzyme activity increases the release of calcium into the solution. It has also been shown that PEG can help to solubilise calcium in solution rather letting it precipitate out (Cölfen & Qi, 2001; Polowczyk et al., 2013). An increase in dissolved calcium could cause an increase in pH, which has a negative effect on hydrolysis. An increase in calcium within the liquid medium could also

cause unproductive binding of enzymes to the calcium itself, making them unavailable for hydrolysis (Chen et al., 2012). This behaviour could also help to explain the lower yield obtained in the primary hydrolysis, and is discussed further in section 7.2.

6.7.2 Enzyme activity in washwater and sugar solution

Objective: To determine how much enzyme activity was lost in the concentrated sugar solution and washwater in the original (without PEG) two-stage hydrolysis with intermediate wash step and whether an increase in activity could be obtained by performing the wash a pH 9 rather than pH 5.

Method Summary: The concentrated sugar solution obtained after 48 hours of hydrolysis (containing 8+ wt% glucose) and the washwater (obtained by washing the residual solid after the initial hydrolysis, as described in section 6.7.1) were combined and processed using ultrafiltration to remove the product sugars (see section 3.11). A relative enzyme activity test was then carried out in which the activities of these ultrafiltered solutions were compared to that of a fresh enzyme solution at the dilution used in the experiment (1:80).

To observe whether enzymes as well as sugars were recovered, the washwater and concentrated sugar solution was tested for enzyme activity using the filter paper and β-glucosidase activity tests. The tests were carried out with washwaters at pH 5 and 9. The activity was tested in 0.5 ml of ultrafiltered solution. To obtain the total recovered activity in the sugar solution and washwater, the result was multiplied by [the total volume of recovered solution (ml) / 0.5 (ml)].

Results: 15% of the original β-glucosidase activity from the C-Tec3 preparation was recovered in the pH 5 wash, whereas 30% was recovered in the pH 9 wash. These values do not include any recovered but denatured enzyme, however, which could account for a significant portion of the β-glucosidase initially added (Varnai et al., 2011).

The cellulase (or FPA) activity in the sugar solution with pH 9 washwater was twice as high as with pH 5 washwater, while the β-glucosidase activity was 3.5 times higher. These results are in accordance with those of other researchers, who have shown higher enzyme recovery in alkaline conditions (Rodrigues et al., 2012; Wang et al., 2012b). It was noted, however, that recovery at the higher pH still only accounted for ≤ 10% of the original FPA enzyme activity.

Discussion: These results support those in in section 6.7, by suggesting that most of the cellulase enzyme remains with the pulp and enzyme recovery by this method is not feasible. This indicates that it might be more beneficial to recycle the pulp with its bound enzymes rather than trying to recover enzymes in the washwaters only, as has been the focus in other research (Tu et al., 2007a; Xue et al., 2012a).

The advantages of an alkaline wash are discussed in section 2.2.6.4 . The result here shows that the pH 9 wash was more effective at recovering enzymes in the high solids conditions used. This is an important result as previous studies on alkali elution of enzymes have only been carried out under low solids conditions (Rodrigues et al., 2012; Wang et al., 2012b).

6.7.3 Reusing the washwater as a means of reclaiming sugars

Objective: To determine the effect on hydrolysis performance and enzyme efficiency, of using the washwater from an intermediate pH 5 wash as dilution water in a subsequent round of hydrolysis.

Method summary: The washwater from the pH 5 wash experiment described in section 6.7.1 was used as part of the dilution medium for a subsequent round of hydrolysis. The washwater was added to new substrate with an initial solids content of 35% TS to give a final solids content of 20% TS. The enzyme was added simultaneously with the washwater at a loading of 50 mg C-Tec3 g^{-1} pulp.

Results: The initial glucose concentration in the new round of hydrolysis was 1.05 wt%. It was found that the conversion efficiency over a 48-hour period was the

same as with no initial sugar, at 54 ± 0.5%; but the final sugar yield was higher due to the augmentation of the initial glucose content by the washwater (Figure 6.7-11).

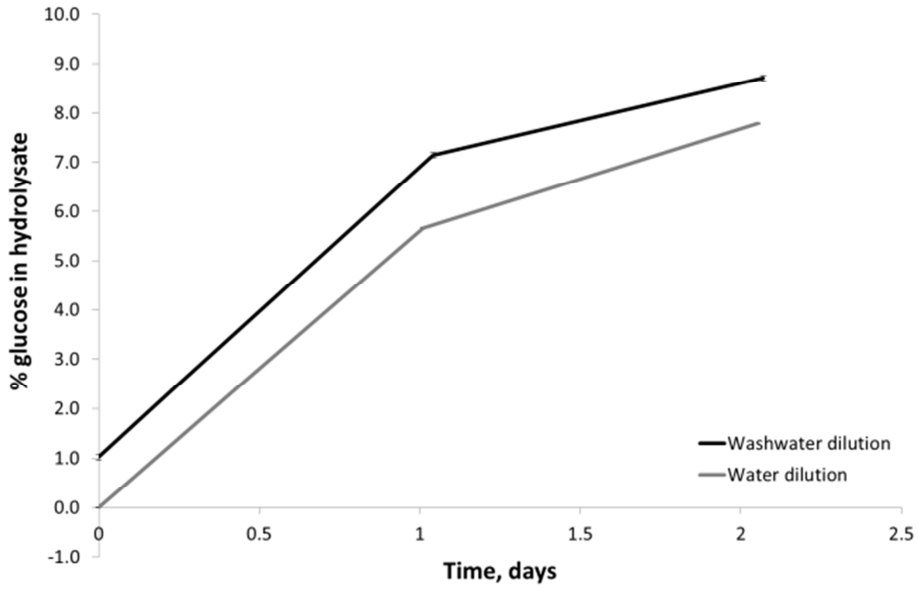


Figure 6.7-11. Effect of the re-use of washwater as dilution water for a new batch of hydrolysis. Control with dilution by pH 5 water (grey), dilution with pH 5 washwater containing residual 1.05% wt glucose (black).

Discussion: The washwater that is discarded in the process described in section 6.7.1 is a rich source of carbohydrate; however it is dilute in strength and therefore is more suitable for use as a feed stream for biogas production than for further sugar extraction. This experiment explored an alternative strategy of using the washwater from the previous experiment, corresponding to a preceding hydrolysis cycle, as dilution water for a subsequent round of hydrolysis with the idea that this could help in reclaiming sugars that remain bound to the substrate post primary hydrolysis. This strategy also ensures re-use of any enzyme activity recovered during the wash process. The success of the strategy was mainly due to recovery of the dilute sugar from the washwater as conversion efficiencies were not affected, indicating that little or no enzyme activity was carried over from the first round.

6.7.4 Washwater and enzyme addition – method development

Objective: To determine the optimum order of addition of enzyme and washwater for maximum sugar conversion.

Method: Two experiments were carried out to determine the best sequence of washwater and enzyme addition.

(1) Hydrolysis was carried out with dried MSW pulp at 5% TS using washwater from a previous 20% TS hydrolysis as part of the dilution medium. In experiment 1A washwater was added either simultaneously with the enzyme or two hours after enzyme addition (table 6.7-1). In experiment 1B and 1C washwater or water was added simultaneously with the enzyme.

Table 6.7-1. Experimental set-up for method 1

Expt.	Pulp concentration (%)	Glucose in washwater (wt%)	Washwater in dilution medium (%)	pH 5 water in dilution medium (%)	Glucose concentration at start of experiment (wt%)
1A	5	4.1	50	50	2.0
1B	5	4.1	25	75	1.0
1C	5	4.1	0	100	0

(2) A hydrolysis of filter paper was carried out at 10% TS in which glucose was added to the reaction medium either simultaneously to the enzyme or 1 hour after enzyme addition. The amount of glucose added gave a glucose concentration of 4 wt% in the liquid portion of the reaction medium.

In both experiments the enzyme loading used was 50 mg C-Tec3 g^{-1} pulp.

Results: The results from experiment 1A (figure 6.7-12) show that with 5% TS pulp and a washwater containing 2.0 wt% glucose addition of the washwater simultaneously with or two hours after enzyme addition made no difference, as the same overall glucose yield was achieved. A total glucose concentration of 3.5 ± 0.2 wt% was obtained in the hydrolysate of both experiments, inclusive of the initial 2.0 wt% glucose in the dilution water. The total glucan conversion of both experiments was 42.5 ± 0.5%: the conversion was low as dried MSW pulp was used.

Experiment 1B used less washwater in the dilution medium than 1A, and the washwater was added simultaneously with the enzyme. In this experiment an overall conversion of 50% was achieved which was equivalent to the conversion when no washwater was used (expt 1C) (figure 6.7.13 & 14). A possible explanation is that 2 wt% glucose together with the other soluble compounds present in the system may be inhibitory to hydrolysis; if so the optimum concentration of glucose in the washwater is likely to be slightly below the value used here (see section 6.6.1)

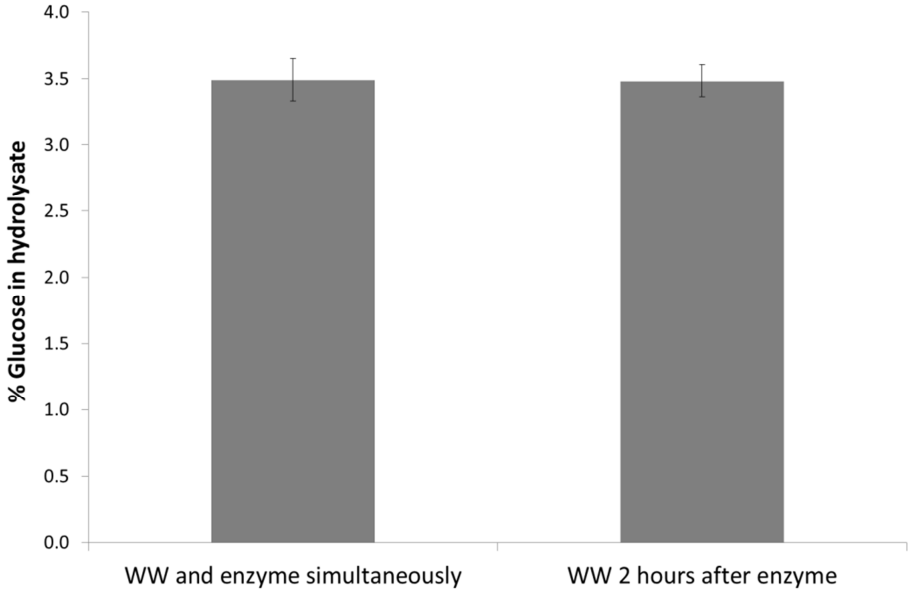


Figure 6.7-12. Experiment 1A. Comparison of 3-day hydrolysis yield of dried MSW pulp at 5% solids using washwater containing 2.0 wt% glucose as dilution water. The washwater was added either simultaneously to the enzyme or 2 hours after enzyme addition.

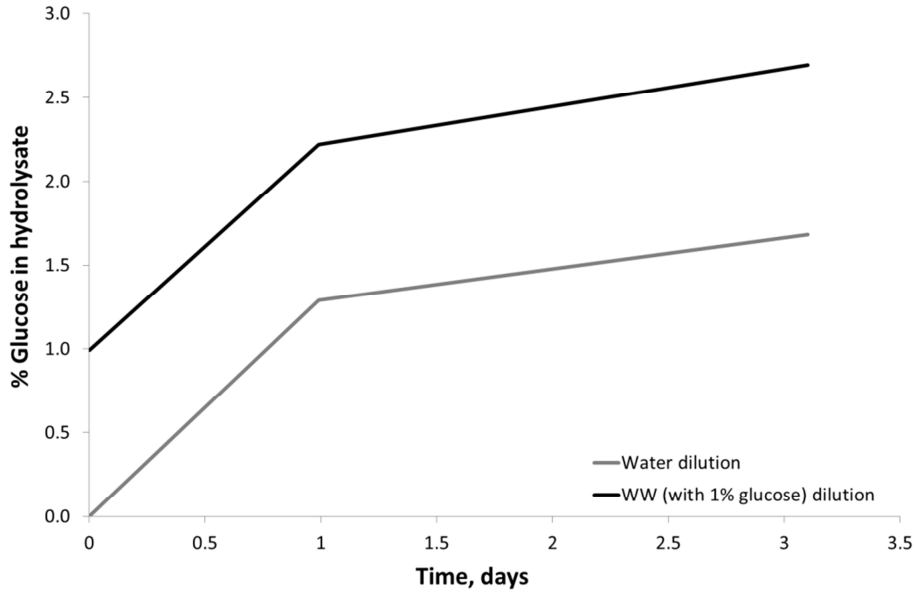


Figure 6.7-13. Comparison of 3-day hydrolysis yield of dried MSW pulp at 5% solids using either water for dilution or washwater containing 1 wt% glucose as dilution water. The washwater was added simultaneously to the enzyme addition (Experiments 1B & 1C).

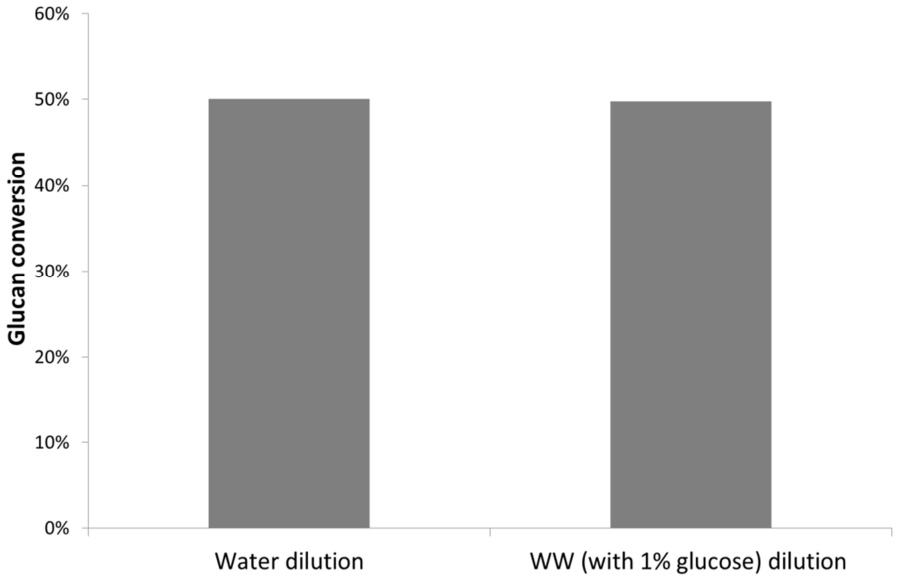


Figure 6.7-14. Comparison of the overall glucan conversion of a 3-day hydrolysis of dried MSW pulp at 5% solids using either water for dilution or washwater containing 1 wt% glucose as dilution water. The washwater was added simultaneously to the enzyme addition (Experiments 1B and 1C).

In the experiment with filter paper (method 2) it was seen that addition of glucose after the enzyme addition gave better results. The final increase in glucose concentration (i.e. from 4 wt%) in the experiments with simultaneous enzyme and glucose addition was 2.9 ± 0.2 wt% whereas in subsequent glucose addition the increase in concentration was 3.6 ± 0.2 wt% (Figure 6.7-15). This was equivalent to conversions of 29 ± 2 wt% and 35 ± 2 wt%, respectively.

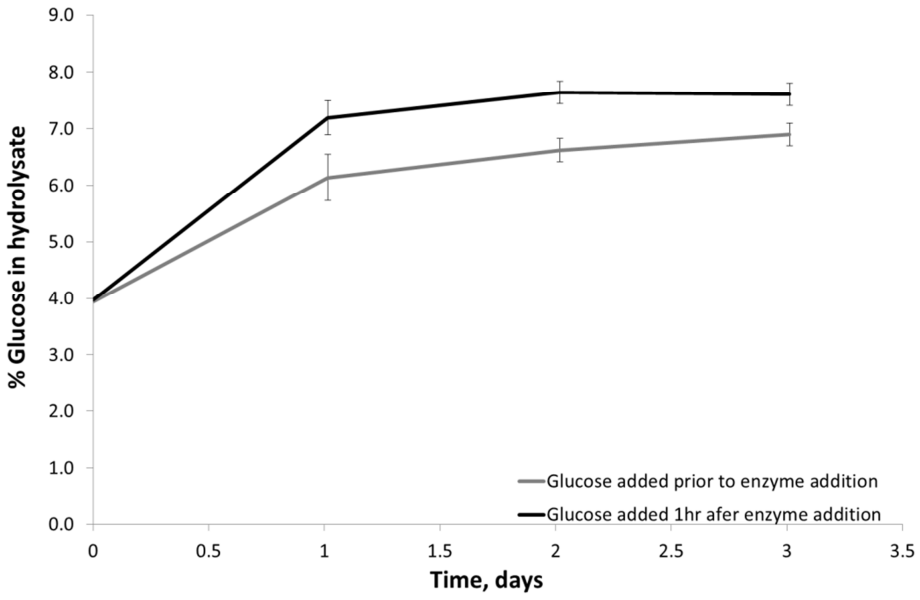


Figure 6.7-15. Comparison of 3-day hydrolysis yield of filter paper with 4% glucose in solution. Glucose was added either simultaneously with the enzyme or added 1 hour subsequent to enzyme addition. Error bars represent range.

Discussion: The results of the experiments indicated that the order in which enzyme and washwater containing a relatively low concentration of sugar are added does not impact on the comparative overall conversion.

It was found however when this experiment was compared to dried pulp in water or a washwater with only 1% glucose in solution an overall conversion of 50%, as opposed to the 42.5% conversion achieved with the higher initial glucose loading (figure 6.7-13 & 14). It was thought that perhaps a 2 wt% glucose plus the other soluble compounds present in the system may be inhibitive to hydrolysis and as such the washwater should contain a slightly lower content of sugar as was suggested in section 6.6.1.

The use of washwater with a 2% wt glucose concentration gave a lower conversion than at 1 wt% glucose. This finding has been very recently confirmed by Hsieh et al., 2014 who showed that hydrolysis in 10 g L^{-1} glucose (equivalent to 1 wt%) actually enhanced glucan conversion, but an inhibitory effect can be seen at concentrations a higher than this (Hsieh et al., 2014).

The second set of experiments was performed on filter paper to reduce or prevent interference from other inhibitory compounds such as other sugars, lignin, liberated ions and ash in the pulp. The initial 4% glucose concentration was chosen as this is above the 'safe level' of 3% which did not severely inhibit substrate conversion when added prior to the enzyme C-Tec3 (section 6.6.1). The low level of conversion seen in this experiment is due to the inhibitory effect of sugar.

The results show that a higher substrate conversion was achieved under the conditions where glucose was added post enzyme. A possible explanation for this is that that glucose inhibits some enzymes from binding to the substrate, leading to a reduction in overall conversion, rather than only inhibiting the enzymes once they are bound.

It should be noted that the results from figure 6.7-11 and 6.7-13 show the same outcome regardless of whether the pulp is hydrolysed at 5% TS or 20% TS.

6.8 Conclusions from chapter 6

Results from the set of experiments described in this chapter showed that 90 ± 5 % conversion could be obtained with the MSW pulp at a high solids concentrations (>20% TS), and indicated that hydrolysis was not limited by the build-up of lignin and other non-sugar containing compounds. An outline assessment of the energy requirement for low solids vs high solids hydrolysis indicated that unless the energy required for mixing of the high solids process is at least 125-fold higher than that at low solids, a low solids process would be much more energy intensive. This is due to the increased water heating requirement, larger throughput quantity in the separation process, larger tank sizes and the need for a sugar concentration step following hydrolysis.

The main difficulty arising from the operation of a high solids hydrolysis process is the prevalence of the solids effect which results in a decrease in percentage sugar conversion as the concentration of solids increases. The reasons for this include but are not limited to: insufficient mixing; product inhibition as a result of increasing sugar concentration; decreased water availability; irreversible binding of adsorbed enzyme to the substrate, including non-productive binding to lignin; inhibition of enzyme adsorption; and enzyme denaturation.

To overcome some of these limitations a number of experiments were devised to alleviate product inhibition and increase water availability in the hydrolysis system. These parameters were manipulated so that it was possible to obtain higher overall sugar yields, ethanol titres and substrate conversions compared to those in a normal batch hydrolysis where the enzyme activity diminishes significantly after 48 hours. The specific conversion yields for each experiment are given in table 6.8-1.

The first set of experiments aimed to alleviate product inhibition by using an intermediate fermentation step at 30 ^{o}C between two hydrolysis stages at 50 ^{o}C. Using this method it was possible to reactivate the enzyme system which otherwise

stagnated after 48 hours of hydrolysis. The effectiveness of this strategy was however limited due to the unfermented soluble sugars released during hydrolysis and the decreased water availability caused by their presence in the system. Further to this the ethanol present after fermentation was shown to decrease enzyme activity.

The second set of experiments tested a strategy to relieve product inhibition and increase water availability by removing part of the concentrated sugar solution after 48 hours of hydrolysis. The residual solid was then washed and the sugar solution removed was replaced with an equal volume of pH 5 water. This procedure also reactivated the enzyme system and led to increased conversion compared to controls which did not undergo a wash step. The strategy was further enhanced by the addition of β-glucosidase in the second stage, indicating that this enzyme may have been lost or denatured during the initial steps of the hydrolysis. The addition of β-glucosidase in the second-stage hydrolysis led to the simultaneous release of glucose and xylose when the enzyme system was reactivated. A possible explanation for this is related to the release of a certain portion of cellobiose which is tightly bound to xylan: thus by the freeing the cellobiose the adjoined xylan is free to be converted by the hemicellulases in the system.

The strategy of a two-stage hydrolysis with intermediate wash step was also carried out in the presence of PEG 6000 using 50 mg C-Tec3 g^{-1} pulp. Overall a lower yield was obtained with this additive than without it, possibly due to unproductive hydrophobic interactions between the PEG and enzymes. In section 5.3.3 it was noted that the use of PEG resulted in enhanced conversion when using 30 mg C-Tec3 g^{-1} pulp . The fact that this synergy occurs at a low enzyme loading but not at higher solids loadings may be due to the increased concentration of hydrophobic species, although this hypothesis was not verified here. A positive result from the two stage experiment with PEG was that increased glucan conversion was seen in the second hydrolysis stage compared to when no PEG was used. This may have been due to the enzyme stabilising properties of PEG allowing increased β-glucosidase activity in the second round. Furthermore it was found that the use of PEG increased the proportion of liquid that could be removed from the hydrolysate

by centrifugation: this is thought to be due to an increase in endoglucanase activity afforded by PEG, or alternatively could also be caused by the polymer's water binding capacity.

Table 6.8-1 compares the 4-day single stage results for glucan conversion with those obtained from both the intermediate fermentation and intermediate wash strategies. Two-stage hydrolysis with intermediate fermentation improved glucan yield, but this was more successful with a 24-hour than 48-hour initial hydrolysis, indicating that product inhibition was less likely to occur when the glucan conversion was more evenly distributed between the two stages. The use of an intermediate wash step that removed residual sugars from the substrate produced an even higher glucan conversion, confirming that water availability may have been a key factor in reducing the activity of enzymes retained on the solid fraction.

Table 6.8-1. Comparison of all two stage hydrolysis systems in chapter 6 after 96 hours of hydrolysis

Experimental condition	% Conversion in primary hydrolysis	% Total conversion after secondary hydrolysis	% Conversion improvement compared to control (no fermentation or wash step)
A. Single stage hydrolysis (96 hours)	68.0 ± 5	-	-
B. Two-stage hydrolysis with intermediate fermentation (24-hour initial hydrolysis)	46.5 ± 0.36	76.5 ± 0.5	8.5 ± 0.5
C. Two-stage hydrolysis with intermediate fermentation (48-hour initial hydrolysis)	65.8 ± 0.3	73.1 ± 1.7	5 ± 1.7
D. Two stage hydrolysis with intermediate wash step	67. 2 ± 1.3	81.2 ± 0.7	13.2 ± 0.7
E. Two stage hydrolysis with intermediate wash step and β-glucosidase addition	67.2 ± 0.8	87.6 ± 0.4	19.6 ± 0.4
F. Two stage hydrolysis with intermediate wash step and PEG	55.0 ± 0.1	71.8 ± 1.0	-
G. Two stage hydrolysis with intermediate wash step, PEG and β-glucosidase	55.0 ± 2.0	76.6 ± 2.0	-

The most significant improvement was obtained when β-glucosidase was replenished after the first stage hydrolysis, as this apparently helped to reactivate cellulases that were bound to the substrate. Using this strategy gave a higher glucan conversion at a lower enzyme dose than in many other high solids hydrolysis studies

(Xue et al., 2012b; Zhang et al., 2009; Zhang et al., 2012b). The importance of obtaining a concentrated sugar solution from this high conversion yield should also be noted. The liquid hydrolysate stream from an intermediate wash strategy may not require a concentration step prior to fermentation, saving on energy costs and allowing higher net yields per tonne of substrate. Moving away from SSF to a separate hydrolysis process also opens up opportunities for use of the concentrated sugar stream for other bio-refinery applications beside ethanol production (Connor & Liao, 2009).

A further strategy was trialled in which the washwater from the intermediate wash step was used as the dilution water in a subsequent batch hydrolysis. This showed no detrimental effects on glucan conversion, while the glucose concentration in the hydrolysate was increased due to augmentation by the 1 wt% glucose in the washwater. Enzyme recovery in the washwater was increased if the residual solid was washed at pH 9 rather than 5.

The results from this chapter are utilised in the next section which looks at employing a continuous hydrolysis process where washwater from one hydrolysis was used as the dilution water in a subsequent hydrolysis over 3-6 rounds. This was undertaken in order to maximise the yield of sugars and to determine whether any carryover of enzyme activity could be seen.

Chapter 7: Washwater recycling in two-stage hydrolysis

7.1 Introduction

The research in this chapter attempted to bring together a number of discoveries made during the previous experimental studies. The aim was to develop a continuous steady state process where washwater from one hydrolysis was used as the dilution water in a subsequent hydrolysis. One of the main reasons for this process strategy was to determine whether enzyme activity as well as sugar could be carried over in the washwater in a steady state system, and/or whether a build-up of inhibitory compounds would occur. Another minor aim was to integrate the two stage hydrolysis with intermediate wash step into this steady state cycle.

A series of process sequences were designed where the washwater was recycled and used as dilution water for a subsequent batch of substrate over 3-6 rounds of hydrolysis of 48 hours each. The timeframe of 48 hours was selected as a recent study by Lindedam et al., 2013 recommended that if enzyme recycling is to be undertaken it should be done within 48 hours for a hydrolysis process at 50 ^{o}C or 96 hours for a hydrolysis process at 40 ^{o}C to obtain similar yields in each case.

In this chapter a lower enzyme dose of 30 mg CTec3 g^{-1} pulp was used at the beginning of each round of hydrolysis to make it easier to identify whether enzyme activity was being carried over from the previous round of hydrolysis (see general process sequence figure 3.9-1 section 3.9.4). The use of a higher enzyme dose may have resulted in a saturation effect, especially when the substrate conversion was near the theoretical maximum, making it difficult observe any enzyme carryover or whether the wash procedure had a negative effect on hydrolysis. The recycle scheme in this chapter was investigated using washwaters at pH 5, pH 7, and pH 9; and at pH 5 and pH 7 with the addition of PEG 6000 in the washwater. The main sequence of unit processes is described in the methods in section 3.9.4. Modifications of this main method are described in the following sections.

Although experiments similar to those described in this chapter have been carried out by other researchers on different types of lignocellulosic substrates, the conditions used in these other studies were generally not suitable for scale-up to an industrial process. Flaws in the previous studies include: (i) the enzyme activity of the hydrolysate following hydrolysis was measured on filter paper rather than on a real substrate (Lindedam et al., 2013); (ii) insufficiently high solids concentrations were used, and sugar concentrations in solution were not given (Xue et al., 2012a); (iii) Experiments which used alkaline pH desorption are carried out either on model substrates and/or at 5% TS where there were many more free enzymes in solution post hydrolysis; and pH control was achieved with buffers, which is not realistic for an industrial application (Rodrigues et al., 2014; Rodrigues et al., 2012). Thus, the current work used a real substrate at high solids concentrations to obtain an adequate sugar concentration in the hydrolysate. Enzyme recovery was carried out over a short time period without buffers, and the process was performed using industrially relevant timescales.

7.2 pH 5 washwater recycle

Objective: To assess the performance of a steady state washwater recycle process where pH 5 washwater from the previous round of residual solid washing is used as part of the dilution water for the subsequent round of hydrolysis.

Method summary: Hydrolysis was carried out by charging a pulp at 40% TS with 30 mg C-Tec3 g^{-1} pulp. After 1.5 hours the pulp was diluted to 20% with either pH 5 water (first round), or pH-adjusted washwater from the previous round of hydrolysis. The substrate was then left to convert for a further 47 hours. Following this the concentrated sugar solution was removed by centrifugation from the hydrolysate and the residual pulp was washed with water adjusted with phosphoric acid to a pH between 3 and 5. This washwater was then collected and adjusted to pH 5 with phosphoric acid and used as dilution water for a subsequent round of hydrolysis. This process was carried out for a total of 6 rounds of hydrolysis. For a more detailed description of the standard method used see section 6.9.5.

Results & Discussion: Figure 7.2-1 shows the glucose concentration in the first-stage hydrolysate over 6 rounds of recycling. It can be seen that using this strategy it was possible to obtain a glucose concentration of more than 7 wt% even at the lower enzyme dose used (30 mg C-Tec3 g^{-1} pulp). Table 7.2-1 and figure 7.2-2 summarise the glucose yields and glucan conversions as well as showing the initial and final washwater pH in each round of the process sequence: the reasons for the change in the initial adjusted pH value in each round are discussed below.

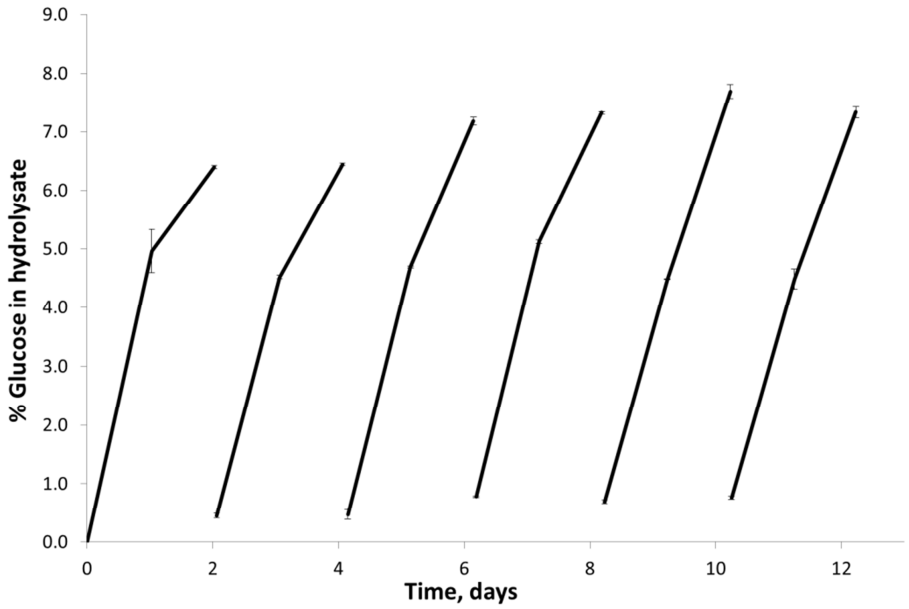
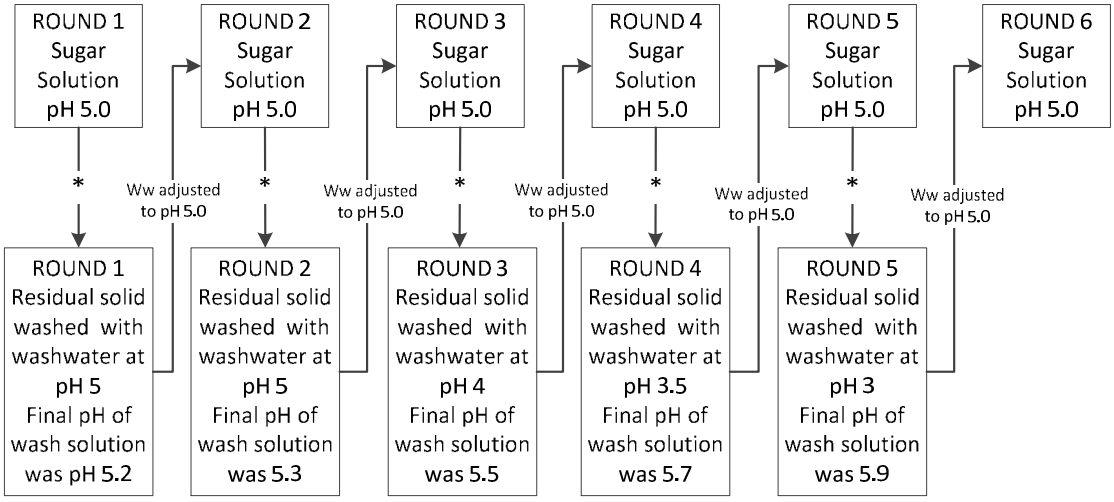


Figure 7.2-1. Glucose concentration in hydrolysate from 6 consecutive hydrolysis rounds with MSW pulp, using pH-adjusted washwater recycled as dilution water for the subsequent round of hydrolysis.

Table 7.2-1. Glucose concentration, glucan conversion and pH values from the pH 5 washwater recycling strategy.

	R 1	R 2	R 3	R 4	R 5	R 6
Glucose concentration after 48 hours hydrolysis	6.4 ± 0	6.4 ± 0	7.2 ± 0.1	7.3 ± 0	7.7 ± 0.1	7.3 ± 0.1
Glucose concentration at start of hydrolysis	0.0	0.5 ± 0	0.5 ± 0.1	0.8 ± 0	0.7 ± 0	0.8 ± 0
% Glucan conversion after 48 hours	46 ± 0	41 ± 0	47 ± 1	48 ± 0	49 ± 1	46 ± 1
Initial pH of washwater used	5.0	5.0	4.0	3.5	3.0	--------
pH of the washwater after 1 hour of washing	5.2	5.3	5.5	5.7	5.9	--------

Nomenclature; R - Round



* Centrifugation of hydrolysate and removal of concentrate sugar solution. Residual solid then washed.

Figure 7.2-2. Schematic diagram of pH 5 washwater recycle process Nomenclature: Ww - washwater

In the round 1 first-stage hydrolysis sugar production was low, reaching an average glucose concentration of 6.4 wt% in the hydrolysate which was equivalent to an average glucan conversion of 46%. The round 2 conversion was also low, but this was most likely due to a fault in the mixer which meant that the sample was not mixed for the final 12 of 48 hours, which would have led to lower glucose production. Following this there was a slight increase in sugar concentration, mostly due to augmentation by the sugar from the washwater of the new sugar produced during hydrolysis in round 3. A slight increase in conversion was seen in rounds 3, 4, 5 and 6 where the conversions were 47, 48, 49 and 47% respectively. Problems with the mixer occurred again in round 6, and this could be the reason for the slightly decreased conversion seen in that round. In round 5 the glucose concentration in solution reached 7.7 wt% which is just short of the desired 8 wt%. This result was achieved with a 40% decrease in enzyme loading compared to the previous high solids processes described in section 6.7.1. If the mixing had not failed, it is possible that enough enzyme was present in the final round to increase the glucose concentration to above 8 wt%. Comparison of the 5th and 1st rounds seems to indicate that a small amount of enzyme carryover occurred which resulted in a maximum 4% increase in glucan conversion. The result here also confirmed that the sugar in the washwater could be used to augment the sugar produced during hydrolysis without a detrimental build-up of soluble compounds.

Interestingly, in round 2 it was noted that the pH of the washwater increased to 5.3 and in round 3 it increased to 5.5 after an hour of washing. These washwaters were then adjusted to pH 5 for use as dilution water in the next round of hydrolysis. After round 3 of hydrolysis, it was decided to try reducing the pH of the washwater to below pH 5 to see if this would reduce or prevent the pH increase in the subsequent wash step. The amount of phosphoric acid added to the washwater was therefore increased to give a target pH value 0.5-1 units lower in each round successive wash round. As the acid addition to the washwater progressively increased through rounds 3 - 5, however, somewhat counterintuitively the final pH in the subsequent washwaters continued to increase. This may have been due to the presence of calcium carbonate or other ions which leached out of the substrate during the acidic wash. As more acid was added, this may have increased the amount of calcium carbonate in solution which drove up the pH. This explanation is in keeping with observations by Wang et al., 2011 who showed that the addition of calcium carbonate at a dosage of as little as 0.02 g g^{-1} TS increased the final pH of the hydrolysate from 5 to 6.6. This calcium carbonate content is similar to that found in the MSW pulp used in this research. It was therefore considered useful to analyse the calcium content in the washwater following the wash process, as reported in the following section.

It is also noteworthy that no problems with contamination were observed in this washwater recycle process, even though the water was not sterilised and no agents/compounds were used to control microbial contamination.

7.2.1 Calcium and magnesium build up in pH 5 wash water

Objectives: To determine whether a decrease in washwater pH leads to an increase in the cation content when the washwater is used for washing. A further aim was to determine the effect on hydrolysis yield when using a washwater at pH 7 rather than pH 5.

Method: Three pieces of experimental work are reported in this section;

(1) pH measurements were taken of deionised water at a conductivity of 1MΩ and of a 0.1 wt% solution of calcium carbonate made up in this deionised water.

(2) Three hydrolyses were carried out at 20% TS for 48 hours using 30 mg CTec3 g^{-1} pulp. The concentrated sugar solution from each hydrolysate was then removed by centrifugation and the residual solid was washed with water at a pH of 5, 3.73 or 3.33. The washwaters were collected and the calcium and magnesium contents were analysed.

(3) Hydrolysis was carried out by charging a pulp at 40% TS with 30 mg C-Tec3 g^{-1} pulp. After 1.5 hours the pulp was diluted to 20% TS with either pH 5 water (round 1), or washwater adjusted to pH 5 from the previous round of hydrolysis. The substrate was then left to convert for a further 47 hours. Following this the concentrated sugar solution was removed by centrifugation from the hydrolysate and the residual pulp was washed with pH 7 water. This washwater was then adjusted to pH 5 with phosphoric acid before being used as dilution water for a subsequent round of hydrolysis. This process was carried out for a total of 3 rounds of hydrolysis. A detailed general method can be found in section 3.9.5.

Results: The pH of deionised water at a conductivity of 1MΩ was 6.4 and the pH of a 0.1 wt% (equivalent to 0.004 g g^{-1}) solution of calcium carbonate in this deionised water was 8.5. This result confirmed that even a very small amount of calcium carbonate can causes a dramatic increase in pH.

The calcium and magnesium content of the washwater from the strategy described in method (2) above was measured and as expected the concentration of these ions increased as the pH of the washwater decreased (Figure 7.2-3).

As the ion concentration increased when the pH of the washwater decreased, it was decided to conduct a washwater recycle process sequence with a pH 7 washwater in order to decrease the amount of calcium and magnesium leaching out of the pulp during the wash step, and thus the amount of cations going into the subsequent round of hydrolysis.

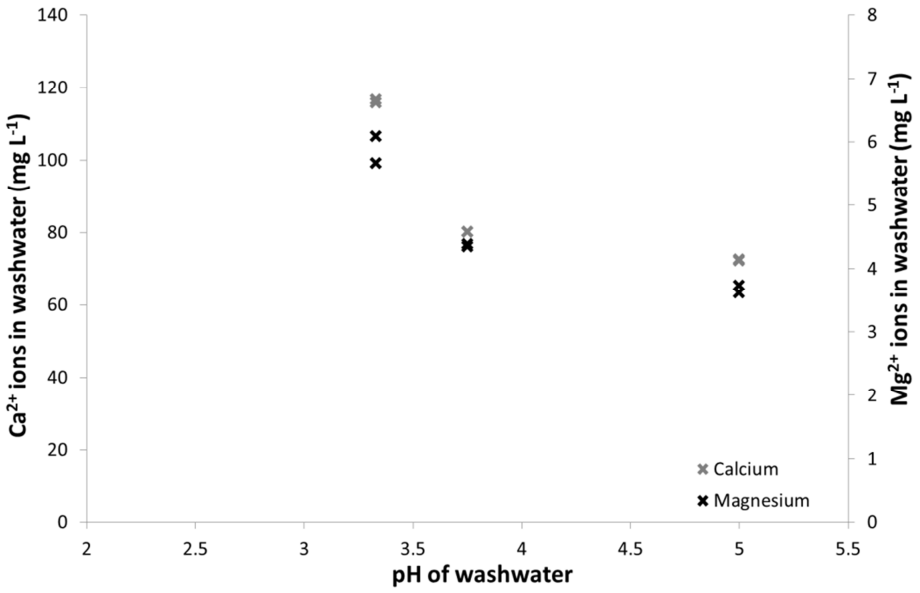


Figure 7.2-3. Cation content (calcium and magnesium) in washwater adjusted to pH between 3.3 and 5 before washing of residual solids after a 48 hours hydrolysis of MSW pulp at 20% TS.

Figure 7.2-4 shows the results for the three consecutive hydrolysis cycles using pH 7 washwater. It was observed that conversion through all three rounds was consistent at 44 ± 1%; however the absolute glucose yield increased over the three rounds due to sugar augmentation. The average glucose concentrations in the hydrolysate in rounds 1, 2 and 3 were 6.3, 7.3 ± 0.1 and 7.3 ± 0.1 wt%.

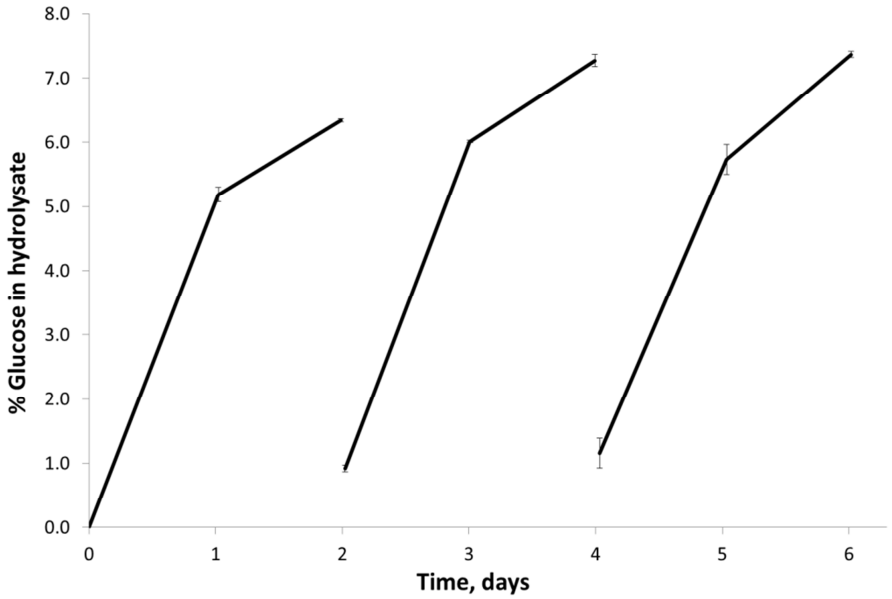


Figure 7.2-4. Glucose concentration in hydrolysate for 3 consecutive rounds of hydrolysis with MSW pulp, using pH 7 washwater recycled as dilution water for the subsequent round of hydrolysis.

Discussion: The results shown in figure 7.2-3 confirm the hypothesis that the calcium content in the washwater increases with a decrease in initial washwater pH, leading to an overall increase in the pH of the hydrolysate.

If the results of the initial strategy in figure 7.2-1 are compared to those in Figure 7.2-4 it can be seen that the enzyme performance was enhanced when using the acidic washwater, which was not the case when using the pH 7 wash.

It has been shown previously by Liu et al., 2010 that calcium and magnesium at concentrations of 10mM (400 mg L^{-1} or 240 mg L^{-1} respectively) can cause increased enzyme activity by the formation of a lignin-metal complex which prevents unproductive binding of the cellulases.

Thus although a high calcium content can be detrimental to hydrolysis (see section 2.2.7.1), the low concentration in this case seems to have been beneficial, perhaps by increasing the enzyme activity. The calcium content in the hydrolysate from method (2) (i.e., before washing at a pH between 3 and 5) in this section was measured and found to be 308 ± 8 mg L^{-1}. If the calcium content in round 1 of the process in section 07.2 above was similar, then calcium concentrations in rounds 2 – 6 may have been successively higher due to the acidic washes used. If the calcium content approached the 400 mg L^{-1} reported as beneficial by Liu et al., 2010, this may also account for the increased conversion as hydrolysis proceeded through the rounds.

It was noted that even a small amount of calcium carbonate drives up the pH of the hydrolysate dramatically, and it may therefore be important to maintain proper pH control throughout the hydrolysis process when using substrates of this type.

As the benefits of calcium and magnesium are believed to be due to prevention of unproductive enzyme binding, it is possible that a greater increase in conversion could be achieved if the enzyme and washwater were added simultaneously rather than enzyme first and then washwater 1.5 hours after.

7.2.2 Increasing glucose yields in secondary hydrolysis

Objectives: To determine the maximum glucan conversion achievable in a second-stage of hydrolysis during the washwater recycle scheme (see section 3.9.4.1), and to evaluate the effect on conversion of limiting the amount of pH 5 water added in the second-stage hydrolysis.

Methods: This process sequence follows on from that in section 7.2. Hydrolysis was carried in accordance with the method in section 7.2 where a total of 6 rounds of hydrolysis were conducted with washwater recycle.

In the recycle rounds strategy the washwater was removed by centrifugation, as was the concentrated sugar solution which left behind a residual solid (see section 3.9.2 & 3.9.2.1 for detailed method set-up). It was found that the residual solid retained a slightly higher amount of water after washing than it did after the sugar solution was removed. Thus, after washing 10 ± 3 ml of the washwater remained in the residual pulp from rounds 1 and 4 giving a residual solids content of 21 and 22% TS respectively.

In the current strategy, these residual washed solids from rounds 1 and 4 were left to undergo a second-stage hydrolysis at 50 ^{o}C for 3-days without addition of extra pH 5 water or enzyme (see section 3.9.4.1).

Results: The glucose concentration in the hydrolysate after 3 days of second-stage hydrolysis of the round 1 was 7.1 ± 0.3% and the total glucan conversion over the two stages of hydrolysis was 74 ± 2%. This meant that the second-stage of hydrolysis from round 1 yielded an extra 27.5% glucan conversion over the 3 days. In the round 4 sample the second-stage glucose concentration and glucan conversion were similar to those found for round 1, at 7.1 ± 0.1 wt% and 71 ± 1% respectively (figure 7.2-5). It should be noted that this experiment was planned as an initial trial only, and hence samples were not taken every 24 hours in round 1.

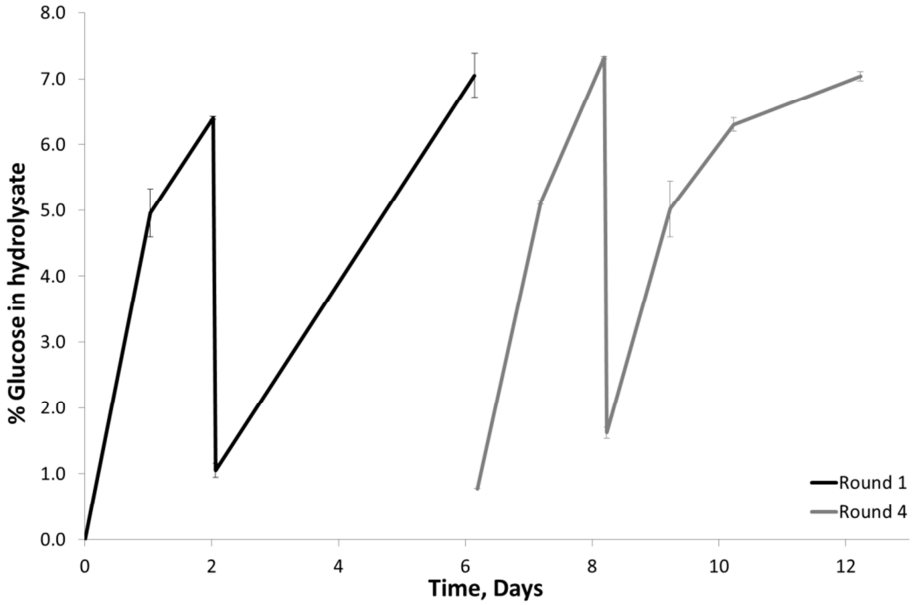


Figure 7.2-5. Glucose concentration in first and second stage hydrolysis of MSW pulps used in rounds 1 and 4 of the pH 5 washwater recycle scheme in section 7.2.

Discussion: In this adjunct to the recycle rounds process strategy (section 7.2), it was found that glucan conversion did increase significantly without additional dilution water in the second-stage hydrolysis. The glucose concentration produced in this second hydrolysis was high, and would require minimal concentration for use in an ethanol producing bio-refinery.

A total conversion of 74 ± 2% was achieved using this two stage hydrolysis scheme. The result obtained here is just short of the 81% conversion achieved in section 6.7.1, but at only 40% of the enzyme loading used in the previous strategy. The one shortcoming in this process is the high viscosity of the pulp in the second-stage hydrolysis. Methods and effects of reducing viscosity are explored in section 7.5.1.1.

7.3 Alkaline washwater recycle

Objective: To assess the performance of a steady state process configuration where alkaline washwater from the previous round is used as part of the dilution water for the subsequent round of hydrolysis.

Method summary: Hydrolysis was carried out at 20% TS with 30 mg C-Tec3 g^{-1} pulp for 48-hours, after which the concentrated sugar solution was removed from the

hydrolysate by centrifugation and the residual pulp was washed with water adjusted to pH 9+ with sodium hydroxide. After washing, the washwater from the first-stage of hydrolysis was removed and its pH was readjusted to 5 with phosphoric acid. This water was then used as dilution water for a subsequent round of hydrolysis where the pulp was diluted to 20% TS from the initial 40% TS. This process was carried out for 6 rounds of hydrolysis.

Note: In this process strategy the sugar concentration results were obtained after the conclusion of the work, and therefore decisions on pH adjustment were made based on the pH after washing rather than the sugar concentration after each 48-hour first-stage hydrolysis.

Results & Discussion: Three attempts were made at carrying out this strategy. In the first two it was found that, although the pH of the residual solid and washwater was adjusted to between 9-10 at the beginning of washing, an hour later at the end of the wash process the pH had buffered between 7 and 8. When this happened no positive effect was seen from reusing this washwater. When this behaviour recurred, it was decided a higher pH should be used to counteract this buffering effect.

Table 7.3-1 summarises the glucose yields and glucan conversions as well as the initial and final pH of the washwaters from this process strategy. Figure 7.3-2 shows the glucose concentration in the first-stage of hydrolysis through rounds 1-6. Figure 7.3-3 is a schematic diagram of the pH adjustment in the strategy.

Table 7.3-1. Glucan conversion, glucose concentration and pH results from the alkaline washwater recycle strategy.

	R 1	R 2	R 3	R 4	R 5	R 6
Glucose concentration after 48 hours hydrolysis	6.3 ± 0	7.5 ± 0.2	8.2 ± 0.1	7.8 ± 0.3	6.6 ± 0.3	7.0 ± 0
Glucose concentraton at start of hydrolysis	0	0.7 ± 0.1	1.0 ± 0.1	0.8 ± 0	1.1 ± 0.2	1.0 ± 0
% Glucan conversion after 48 hours	45 ± 0	49 ± 0	50 ± 1	50 ± 2	39 ± 3	43 ± 2
pH of residual solid & ww after 1 hour of washing	11.1	9.9	11.0	8.6	9.1	------
Adjusted pH of washwater (dilution water)	5.0	5.0	4.9	5.0	4.9	------

Nomenclature; R- Round, ww - washwater

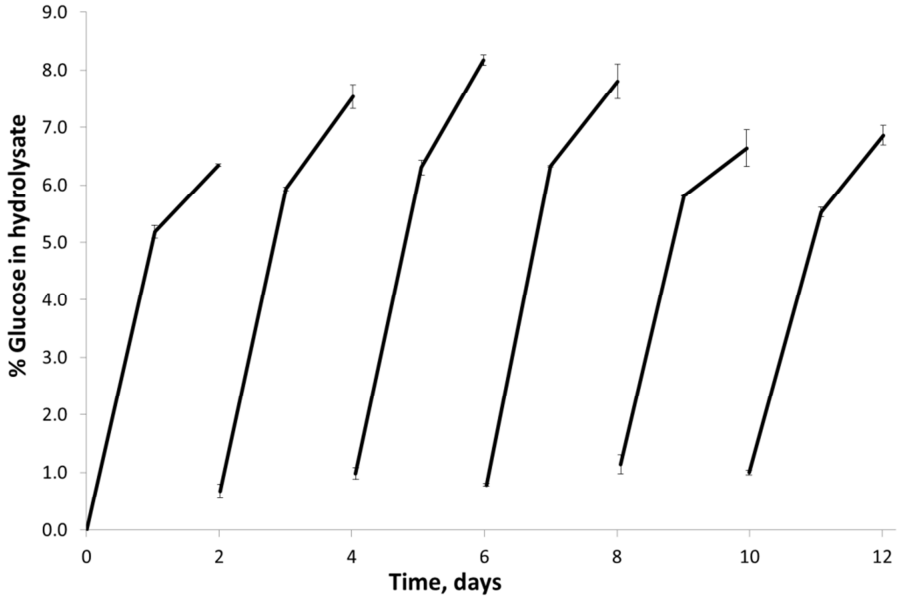


Figure 7.3-1. Glucose concentration in hydrolysate for 6 consecutive hydrolysis rounds with MSW pulp, using alkaline washwater recycle. Washwater at pH 9 from the previous round was readjusted to pH 5 and used as dilution water for the subsequent round of hydrolysis.

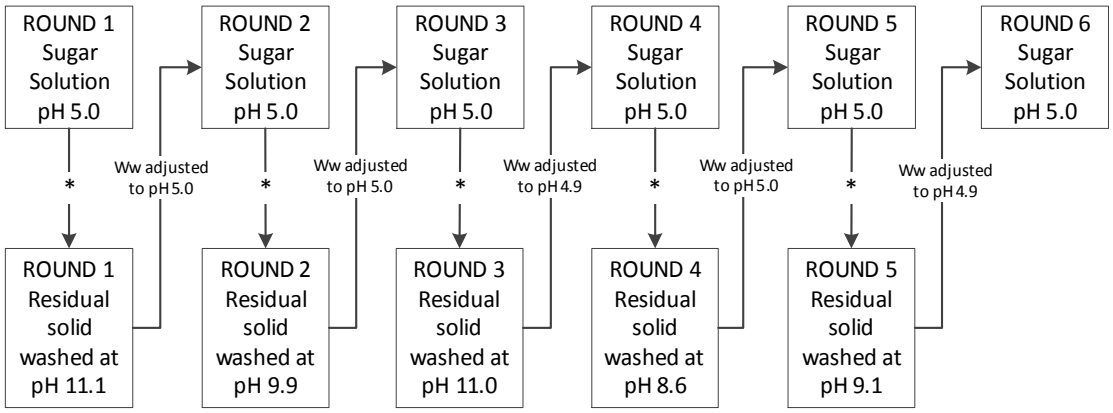


Figure 7.3-2. Schematic diagram of the alkaline washwater recycle scheme. Nomenclature: Ww – washwater

In round 1 of hydrolysis a glucose concentration of 6.3 wt% was achieved, equivalent to a glucan conversion of 45%, and the pH of the sugar solution was 5.0. This sugar solution was then removed by centrifugation and washwater was added. The pH of the residual solid plus washwater was then adjusted once with alkali and left to wash so that after an hour of washing the pH of the residual solid and washwater was 11.1. The washwater was then removed by centrifugation and adjusted to pH 5 before being used as dilution water in round 2 of hydrolysis (figure

7.3-2). In round 2 7.5 wt% glucose was obtained in the hydrolysate, equivalent to a glucan conversion of 49%. This high conversion was achieved despite the very high washwater pH in round 1. Although the washwater was then adjusted to pH 5 before being added into round 2, it was thought that the high pH might have denatured the enzymes from round 1 thus preventing any carryover into round 2. From the sugar data, however, it seems that some enzyme carryover did occur.

As noted above, the sugar data was not available during the process sequence, and it was thought the pH of the wash in round 1 was too high; thus the pH of the wash in round 2 was adjusted to a slightly lower value of 10. In round 3 a glucose concentration of 8.2 ± 0.1 wt% was achieved equating to a glucan conversion of 50%. This conversion seems low in view of the sugar concentration, but this was enhanced by the increased sugar augmentation in round 3. The same amount of alkali was added to the wash in round 3 as in round 2; however in round 3 it increased the wash pH to 11. Irrespective of this high pH, the glucan conversion achieved in round 4 was 50% with a glucose concentration of 7.8 ± 0.3 wt% in the hydrolysate. Based purely on the high pH of the wash and not on the sugar yield achieved, the alkali added to the residual solid and washwater in round 4 was again reduced to try and maintain a wash pH between 9 and 10. This reduction in added alkali in round 4 resulted in a final wash pH, after an hour of washing, of only 8.6. This was then adjusted to pH 5 before use in round 5. As can be seen in figure 7.3-1 this lower pH did not seem to be beneficial in carrying over enzymes: in round 5 a glucose concentration of only 6.6 ± 0.3 wt% was achieved, equating to 39% glucan conversion. Up until this point successive hydrolysis rounds appeared to show increased conversion, perhaps due to carryover of enzymes or other compounds in the washwater recycled as dilution water. In round 6 an average pH of 9.1 was used in the wash and this led to average glucose concentrations and glucan conversions of 7.0 ± 0.1 wt% and 43%, respectively.

The relatively low conversion in round 5 again supports the hypothesis that a washwater pH greater than 9 is beneficial for recycling enzymes. A pH of 9, however, is still perhaps a little low to ensure carryover of a large amount of enzyme. An overall 5% increase in conversion was seen between round 1 and 3 or 4

indicating carryover of either enzymes or beneficial compounds from the previous rounds' washwater. This is similar to the maximum increase in conversion found in the pH 5 wash recycle. In the current process sequence a glucose yield of more than 8 wt% was achieved in the third round which is a very promising result.

The soluble solids content of the washwater in round 4 was measured and found to be 2.7 wt%. This was higher than the soluble solids in the washwater of any of the other rounds, which averaged only 1.6% TS, most of which could be accounted for by the soluble sugars. By measuring the total solids and ash it was found that this extra 1% TS in the washwater used as dilution water in round 5 was organic material and not ash (table 7.3-2). From this result it seems that an alkali wash at below pH 9 resulted in a higher amount of soluble organic solid in the washwater than a wash at above pH 9. Further support for this is provided by the results for the pH 7 washwater process in section 7.2.1 (figure 7.2-4) where the average soluble solids content of the washwater was 1.9 ± 0.2 wt%, which is higher than in the alkali washwaters in rounds 1, 2, 3, and 5 of the current process strategy (see table 7.3-2). It is possible that an alkaline wash above pH 9 helps to precipitate out compounds that interfere with hydrolysis, as well as recovering enzyme activity which is then passed on to following rounds.

Table 7.3-2. Soluble solid in washwater from the pH 7 and alkali washwater recycle strategies.

	R 1	R 2	R 3	R 4	R 5
Alkaline washwater % total soluble solids	1.6	1.7	1.6	2.7	1.6
Alkaline washwater % ash of soluble solids	0.1	0.2	0.1	0.1	0.2
pH 7 total soluble solids	2.0	2.0	1.7	----	----

The pH 7 results are from Figure 7.2-4 figure, section 7.2.1. Nomenclature; R-Round, Av- Average

It is most likely that washing at high pH did assist in the transfer of enzymes, in agreement with the results of other studies (Rodrigues et al., 2012; Wang et al., 2012b). These previous studies were carried out with 1-5% solids, but current work further demonstrates that alkaline washing can also increase enzyme carryover in a high solids hydrolysis process.

It is interesting to note that the enzymes did not seem to denature at a pH higher than 10. This may be to do with the buffering capacity of the system. It was noted in preliminary experiments (not shown here) that even when the initial pH of a

washwater was 9, after half an hour this reduced to 8, after an hour 7.5, and pH continued to fall to about 7 as time proceeded. Thus even though an initial pH of 10 or above was observed in the first few rounds of the strategy used in figure 7.3-1, this may not have been the equilibrium pH of the system, and thus the enzymes were protected from denaturing.

The wash in this study was carried out at 25-30 ^{o}C for 1 hour. A very recent study by Shang et al., 2014 looking at desorption behaviour of enzymes on corn stover showed that for desorption at pH 10 the optimum time and temperature were the same as those selected in this work (Shang et al., 2014); this suggests that the conditions used here were adequate.

As sodium hydroxide was used for pH adjustment a significant amount of sodium accumulated in the system through the rounds; however this did not seem to have a detrimental effect on hydrolysis.

Although this strategy of an alkaline wash shows promise, sodium hydroxide is an expensive chemical to use at industrial scale e.g. US$ 300-500 per tonne in July 2014 (Alibaba, 2014). It is possible, however, that a similar result could be achieved using calcium hydroxide as a cheaper alternative, e.g. US$ 100-130 per tonne in July 2014 (Alibaba, 2014)

7.3.1 pH 9 wash on filter paper

Objective: To determine whether the increase in enzyme activity seen in the alkaline wash strategy was due to increased enzyme carryover or to the accumulation of beneficial compounds from the MSW pulp. A secondary objective was to determine whether a pH 9 wash removes crucial enzymes from the residual solid.

Method Summary:

(1) The process sequence described in section 7.3 above was repeated using Fisher brand filter paper in place of MSW pulp. The washwater recycle procedure was carried out for 3 rounds using 20% TS and 30 mg C-Tec3 g^{-1} pulp.

(2) The residual solid obtained after removal of the concentrated sugar solution and washing in round 2 was diluted to 13% TS with washwater from that round, and left to undergo a 3 day second-stage hydrolysis. The amount of washwater used in this second-stage hydrolysis was 50% of the total washwater from round 2.

Results: Glucose concentrations in the hydrolysate over time are shown in figure 7.3-3. Table 7.3-3 gives the final glucose concentrations in the hydrolysate, the percentage glucan conversions after 48 hours of hydrolysis, and the pH of the sugar solution, washwater and dilution water in each round.

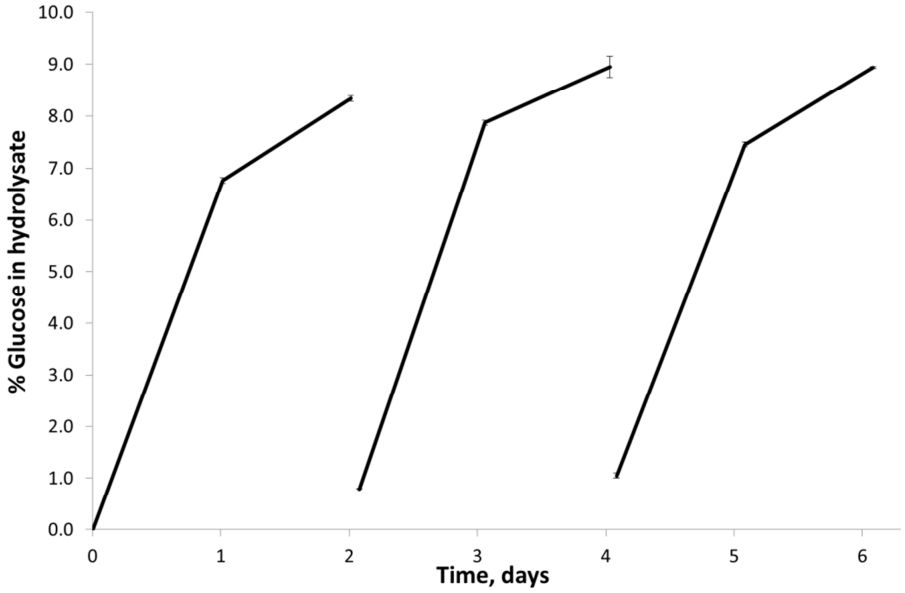


Figure 7.3-3. Glucose concentration in hydrolysate for 3 consecutive hydrolysis rounds with filter paper, using pH 9 washwater recycle . Washwater at pH 9 from the previous round was readjusted to pH 5 and used as dilution water for the subsequent round of hydrolysis.

Table 7.3-3. Glucan conversion, glucose yield and pH of the hydrolysate from pH 9 washwater recycle strategy on filter paper.

	Round 1	Round 2	Round 3
% Glucose in hydrolysate	8.3 ± 0.1	9.0 ± 0.2	8.9 ± 0.2
% Glucan conversion	40 ± 0	40 ± 2	38 ± 1
pH of hydrolysate after 48 hours	5.3	5.0	5.0
pH of washwater	9.6	9.2	-------
Adjusted pH of washwater (dilution water)	4.9	4.9	-------

The experiment in method (2) was undertaken to determine whether the pH 9 wash had removed crucial enzymes, thereby limiting the conversion achievable in a second-stage hydrolysis and confirming that these enzymes had transferred into the

washwater. In the second-stage hydrolysis it was found that an extra glucan conversion of 8.4% was achieved. This is much less than the 27.5% increase in conversion in the second stage which was achieved in section 7.2.2 when using MSW pulp. This result indicates that perhaps the pH 9 wash does remove crucial enzymes.

Unlike the alkaline washwater strategy using MSW pulp (section 7.3), no buffering activity or pH change was seen once the washwater was adjusted to pH 9 in the current experiment.

Another interesting observation from this experiment was the dramatic reduction in turbidity when the washwater pH was adjusted from 9 to 5 for use as dilution water (figure 7.3-4). This change in turbidity may be related to the re-folding of the enzymes in the washwater after the pH 9 wash. A similar observation was made by Shang et al., 2014 when they adjusted the pH of a wash medium.

Figure 7.3-4. Image of pH adjusted washwater. Washwater at pH 9 (right) and washwater adjusted to pH 5 (left).

Discussion: This washwater recycle process was carried out on filter paper with pH 9 washwater. Unlike the process strategy on MSW pulp (section 7.3) the results

showed no increase in conversion, and in fact there was a slight decrease in conversion in successive hydrolysis rounds. It is possible therefore that in the previous strategy the beneficial effect is due to compounds being transferred from the MSW pulp rather than enzyme activity carryover in the pH 9 washwater. This hypothesis is somewhat hampered by the fact that the soluble solids measured in the alkaline washwater from the MSW pulp strategy were almost all accounted for by the sugar present (table 7.3-2) It is also possible that, in the MSW pulp strategy, the enzymes carried over were those that had been unproductively bound to lignin. These enzymes would not have been used while unproductively bound, and hence when transferred to a subsequent hydrolysis may still have maintained a high level of activity.

The results presented here are contrary to those found by Rodrigues et al., 2012 & 2014, who used a model substrate and wheat straw to perform alkali enzyme desorption studies and enzyme recycle. In their experiments they desorbed enzymes from the residual solid and used the free enzyme activity in the sugar solution. Their study noted a high amount of enzyme carryover with an alkaline wash; however it was carried out at low solids content (5%) and this may have meant that the enzymes were better able to access the substrate and easier to remove during desorption. The lack of enzyme carryover seen in the current experiment may be due to the fact that when using filter paper there is no unproductive binding of enzymes. This means that more enzymes are free in solution during hydrolysis and are therefore probably removed along with the sugar solution, and thus not available for carryover in the washwater.

7.4 pH 5 & 7 wash water recycle with PEG 6000 over 3 rounds

Objective: To assess the performance of a steady state process configuration where pH 5 or 7 washwater containing PEG 6000 is used as dilution water for the subsequent round of hydrolysis.

Method summary: Hydrolysis was carried out at 20% TS with 30 mg C-Tec3 g^{-1} pulp for 48 hours, after which the concentrated sugar solution was removed by centrifugation from the hydrolysate and the residual pulp was washed with pH 5 or 7 water containing 1% PEG. After washing the washwater was removed by centrifugation from the residual solid and its pH was readjusted to 5. This water was then used as dilution water for the subsequent round of hydrolysis. See section 3.9.5 for a more detailed method.

The initial process was carried out in 3 rounds. In round 1 the dilution water containing 1% PEG was added half an hour after the enzyme. In the following two rounds the PEG solution was added 1.5 hours after the enzyme. In round 1 the wash was performed with a 1% PEG solution which was adjusted to pH 5; in round 2 the wash was performed with a 1% PEG solution at pH 7.

It should be noted that adding a washwater containing 1% PEG to a pulp at 40% TS gave a final PEG concentration in the solution of 0.6%.

Results: The results are shown in figure 7.4-1. Rounds 1, 2 and 3 achieved glucose concentrations of 8.3 ± 0.3, 7.7 ± 0.1, and 8.9 ± 0.3 wt% which equated to conversions of 59 ± 2%, 48%, and 56 ± 2%, respectively. It was thought that the lower conversion in round 2 might be due to detrimental effects caused by the pH 5 wash. It was noted, however, that this conversion in round 2 was still higher than conversion without any additive (see results from section 7.2 figure 7.2-1 and 7.2-4).

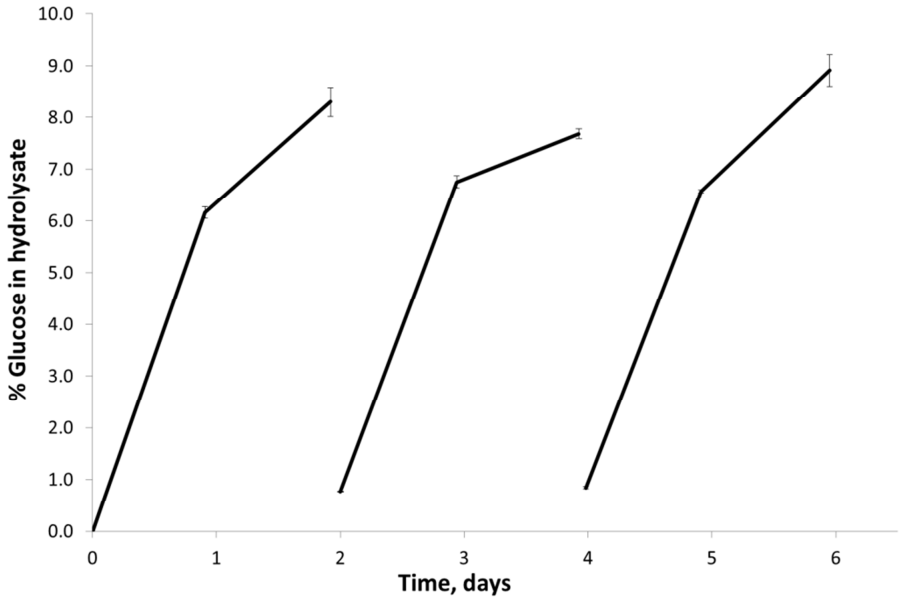


Figure 7.4-1. Glucose concentration in hydrolysate for 3 consecutive hydrolysis rounds with MSW pulp, using pH 5, 7 and PEG washwater recycle. Washwater with PEG 6000 at pH 5 from round 1 and 7 from round 2 was adjusted to pH 5 and used as dilution water for the subsequent round of hydrolysis.

Discussion: A high glucose concentration of 8.9 wt% was achieved in the third round of hydrolysis and the use of PEG in this recycle strategy seems to hold great promise.

As the conversion in round 1 was higher than that of round 2 and 3, it is thought that no enzyme carryover occurred in this scenario, and the increase hydrolysis yield was only to do with the positive action of PEG.

In round 1 the PEG was added half an hour after enzyme addition. When the result for this strategy is compared with that in section 5.3.3, where PEG was added at the same time as the enzyme, it can be seen that the conversion with simultaneous addition was 4% higher. This may indicate that simultaneous addition is more effective, perhaps because it prevents the adsorption of too many cellobiohydrolases onto a cellulase fibril which can cause enzyme jamming and limit hydrolysis.

As the results for the pH 7 wash seemed promising it was decided to repeat the trial in a subsequent process sequence with 5 rounds of hydrolysis to see if a steady state conversion could be achieved.

Unlike nitrogen derivatives, there are differing views as to the mechanism underlying the effect of PEG. It has been suggested that PEG enhances conversion on lignocellulosic substrates but not on avicel (pure cellulose). This would indicate that it has a similar mode of action to proteins, interacting hydrophobically with lignin to reduce unproductive binding of cellulases to the lignin (Borjesson et al., 2007). Other studies, however, have shown that the addition of PEG increases the hydrolysis of avicel (Kumar & Wyman, 2009a; Li et al., 2012a), suggesting that a different mode of action is also present. In a study by (Li et al., 2012a) on corn stover it was shown that not only did PEG decrease the unproductive binding of cellulases to lignin, but also that after 24 hours stability of the enzyme was 10% higher in the presence of PEG than without the additive. Addition of PEG either simultaneously or 1 hour after the cellulase addition gave an increase in enzyme activity compared to no additive; however a more pronounced increase in conversion was seen when the additive and enzyme were added simultaneously. The results also showed that increase in cellulose conversion from the addition of 1% PEG was accompanied by a decrease in enzyme adsorbed onto the cellulose, indicating that PEG prevents unproductive binding of cellulase on cellulose, which can cause enzyme jamming or overcrowding along a cellulose fibril (Igarashi et al., 2011; Xu & Ding, 2007) and in turn results in reduced hydrolysis yield (Li et al., 2012a; Yu et al., 2013).

7.5 pH 7 washwater recycle with PEG 6000 over 5 rounds

Objective: To assess whether enhanced conversion could be achieved by performing rounds of hydrolysis with a washwater recycle process where the washwater is at pH 7 and contains PEG 6000.

Method summary: Hydrolysis was carried out at 20% TS with 30 mg C-Tec3 g^{-1} pulp for 48 hours, after which the concentrated sugar solution was removed from the hydrolysate by centrifugation and the residual pulp was washed with pH 7 water containing 1% PEG. After washing the washwater was removed from the primary hydrolysis by centrifugation and its pH was readjusted to 5. This water was then used as dilution water for the subsequent round of hydrolysis. The dilution water in

this process strategy was added 1.5 hours after the addition of enzyme. This strategy was carried out for 5 rounds of hydrolysis. See section 3.9.5 for a more detailed method.

It should be noted that adding a washwater containing 1% PEG to a pulp at 40% TS gave a final PEG concentration in the solution of 0.6%.

Results & Discussion: The glucose concentrations for each round of hydrolysis were 6.5 ± 0.2 wt%, 7.6 ± 0.5 wt%, 7.1 ± 0.3 wt%, 7.9 ± 0.3 wt%, and 6.7 ± 0.4 wt% respectively. This corresponded to conversions of 47 ± 2 %, 49 ± 4 %, 45 ± 4 %, 47 ± 4 %, and 41 ± 2 % respectively.

As can be seen in figure 7.5-1 the conversion achieved in this initial round was much lower than that in round 1 in section 7.4 above. This may be due to the fact that PEG was added too late to have a positive effect.

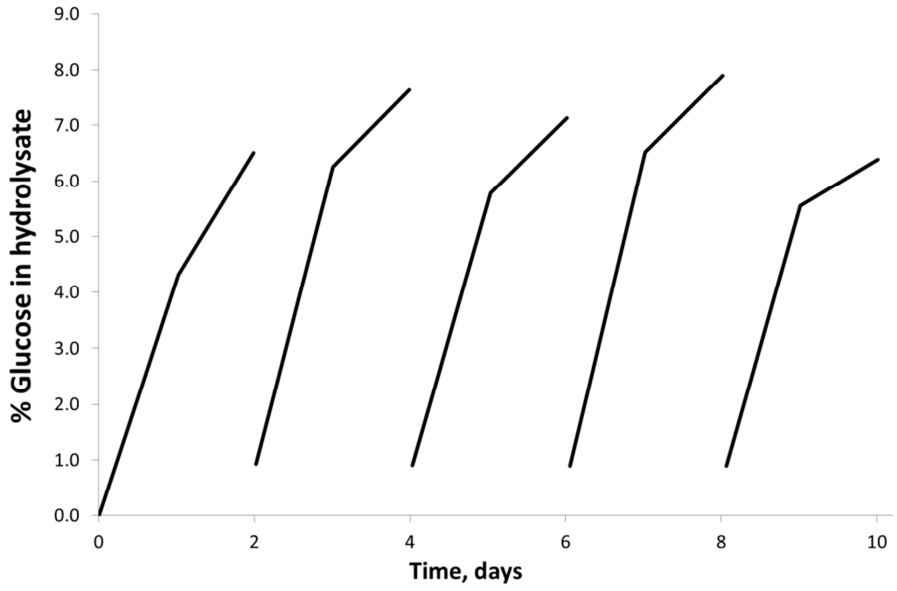


Figure 7.5-1. Glucose concentration in hydrolysate for 5 consecutive hydrolysis rounds with MSW pulp, using pH 7 and PEG washwater recycle. Washwater with PEG 6000 at pH 7 was adjusted to pH 5 and used as dilution water for the subsequent round of hydrolysis.

The pH results for the strategy are given in table 7.5-1 below. In all of the previous washwater recycle strategies the pH of the sugar solution after 48 hours of hydrolysis remained at pH 5, whereas in this strategy this was not the case. The pH of the sugar solution decreased in successive rounds of hydrolysis, falling to below

5, even though the washwater was adjusted to pH 5 with phosphoric acid before use as dilution water.

Table 7.5-1. pH of the hydrolysate sugar solution and washwater through the rounds of hydrolysis in the pH7 & PEG washwater recycle strategy.

	R 1	R 2	R 3	R 4
pH of sugar solution	5.0	4.9	4.6	4.8
pH of washwater after wash	5.8	5.6	5.2	5.4
Adjusted pH of washwater (dilution water)	5.0	5.0	5.2	5.4

Nomenclature: R- Round

Rounds 2 and 4 achieved better conversion yields than in the other rounds, and this may have been due to the slightly higher pH of the hydrolysate as compared to the other rounds. Due to the falling sugar solution pH in the first three rounds of hydrolysis, it was decided not to adjust the pH of the washwaters used as dilution water in round 3 and 4. The pH of the final sugar solution (round 5) was not measured, but as the pH of the washwater going into round 5 was quite high it may have settled at above the optimal range, possibly accounting for the low glucose concentration in this round. Figure 7.5-2 shows the effect of pH on product performance for C-Tec3 (Novozymes, 2012), and it can be seen that if the pH in round 5 buffered at above 5.4 this could have a large effect on hydrolysis.

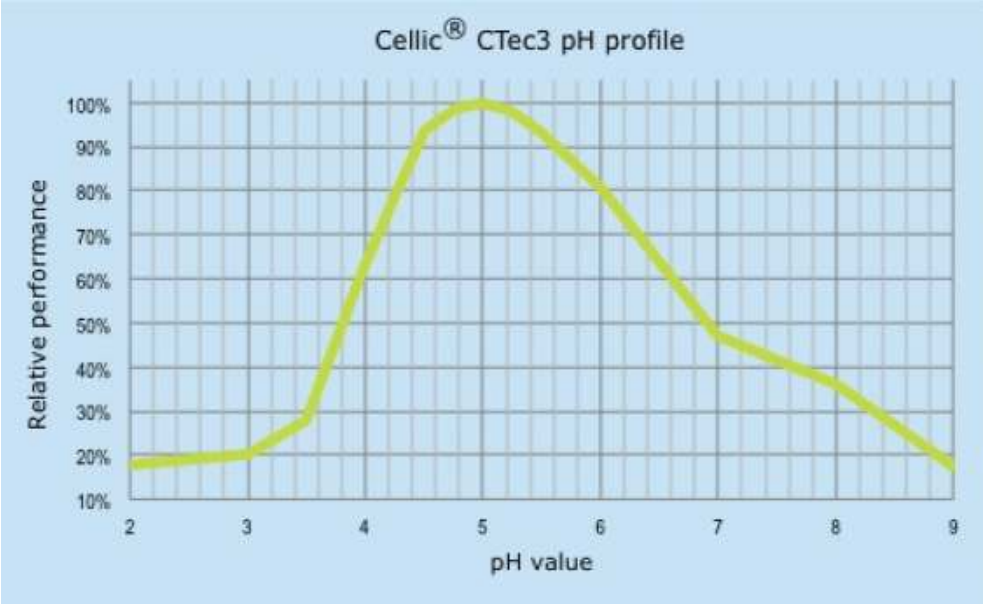


Figure 7.5-2. Relative product performance of C-Tec3 with respect to pH of reaction for unwashed acid pretreated corn stover (PCS) at 18%TS for 3-5 days; temperature 52.5 °C for all data points (Novozymes, 2012)

A further point of interest in the results was the calcium content in the sugar solution and washwater, which was found to increase significantly in each round of hydrolysis (see figure 7.5-3 and 7.5-4). This was not the case in previous experiments without PEG, in which the calcium content of the hydrolysates and washwaters remained fairly constant (see section 7.2.2 & 7.3).

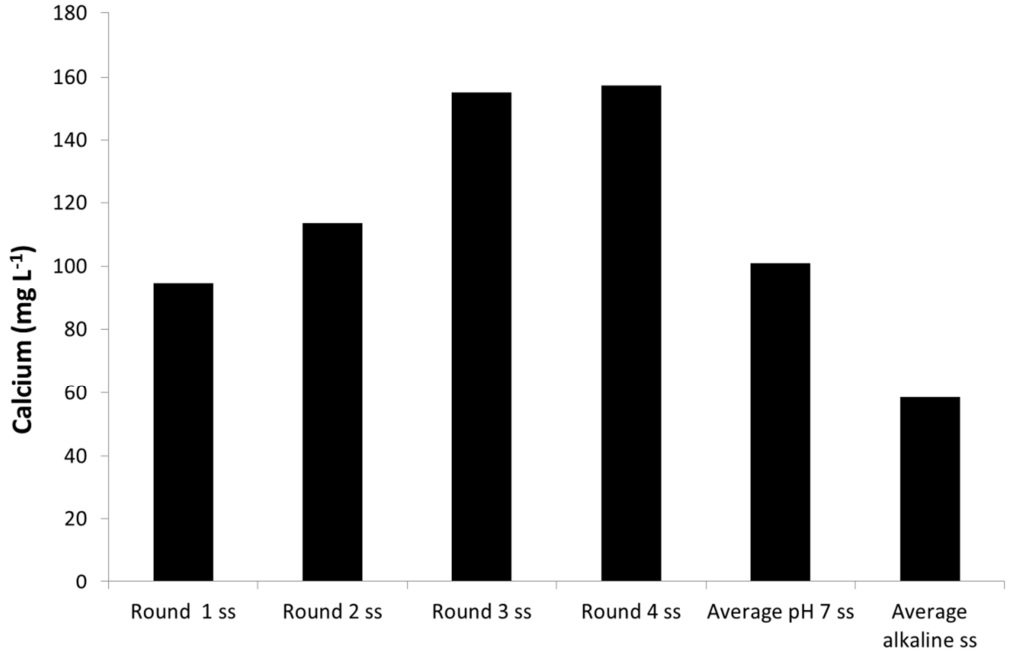


Figure 7.5-3. Calcium content in the sugar solution of the 48 hours hydrolysis with PEG as compared to the average calcium content of the pH 7 (section 7.2.1) and alkaline (section 7.3) washwater recycle strategies.

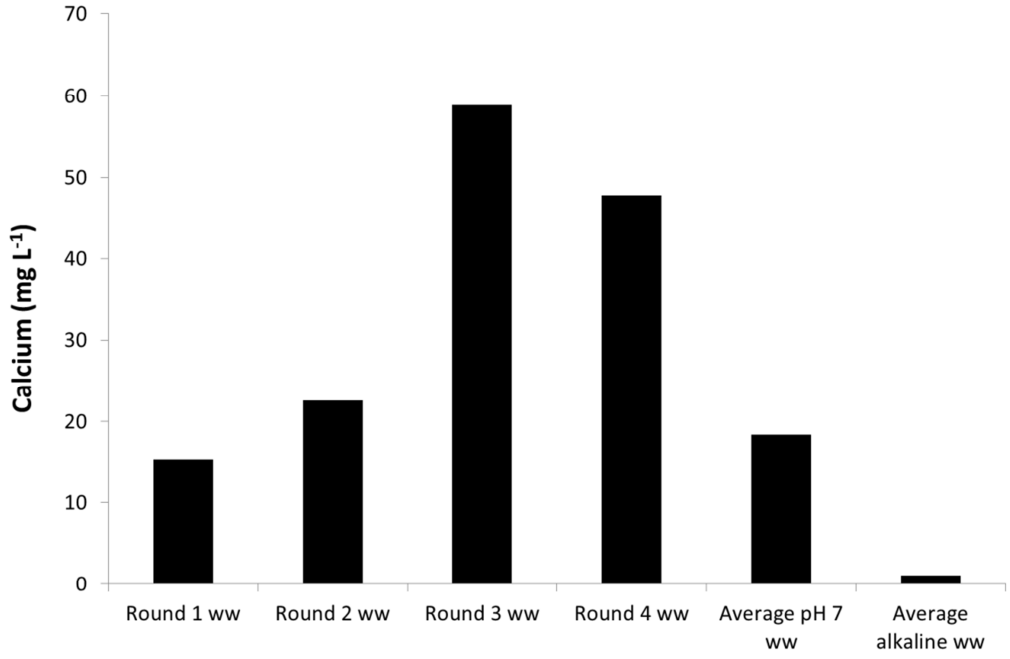


Figure 7.5-4. Calcium content in the washwater of the 48 hours hydrolysis with PEG as compared to the average calcium content of the pH 7 (section 7.2.1) and alkaline (section 7.3) washwater recycle strategies.

It has been found that the presence of PEG can help stabilise calcium in solution and prevent it from precipitating out (Cölfen & Qi, 2001; Polowczyk et al., 2013), and this may be a reason for the calcium build-up seen in this strategy. It is surprising that the pH buffered at < 5, as the increasing calcium content should have led to a rise in pH. It was noted, however, that in the first two rounds of hydrolysis the calcium concentration was similar to that without PEG and so it is possible that some other factor was responsible for the low pH.

Addition of PEG 6000 was not effective at increasing conversion in the hydrolysis process with intermediate wash step (section 6.7.1.2), perhaps due to a high enzyme dosing and increased hydrophobic interference. A similar process may have operated here, where a large number of hydrophobic compounds could have been carried over through the rounds in a manner detrimental to hydrolysis. Thus in the next process strategy the amount of PEG was reduced to see if this led to any improvement. This inability of PEG to enhance the hydrolysis of a MSW-derived substrate has also been observed by other researchers (Jensen et al., 2011; Kemppainen et al., 2014). In the study by Kemppainen et al. (2014) it was speculated that the reason for this lack of enhancement was due to the absence of an alkyl chain on the PEG molecule: their findings showed that surfactants Softanol and Lutensol, which do contain this chain, did enhance hydrolysis. It may be, however, that the conditions used in those previous experiments were not optimum for enzyme enhancement with PEG. As evidenced in the current research, various factors seem to influence the efficacy of PEG such as: the concentration used, its addition in relation to the time enzyme is added and the amount of enzyme added.

Using this sequence of unit processes it was found that throughout all the rounds of hydrolysis it was possible to remove 52.5% of the liquid by centrifugation from the hydrolysate, a similar result to that in section 6.7.1.2. This is up to 9% more liquid removal than when using 50 mg C-Tec3 g^{-1} pulp and no PEG (section 6.7). The result

again supports the view that this compound is either increasing the endoglucanase activity or acting as a polymer to increase water removal (section 6.7.1.2).

7.5.1 Repeat of PEG 6000 washwater recycle with 0.25-0.4 wt% PEG 600

Objective: To determine whether the glucan conversion achieved by addition of washwater containing PEG 1.5 hours after enzyme addition in section 7.5 above could be improved upon by adding PEG before enzyme addition and at a lower PEG concentration.

Method Summary:

(1) The experimental conditions used were the same as in section 7.5 above, except that the amount of PEG was reduced. In Round 1 and 2 PEG was added to give a concentration of 0.41% in the liquid portion of the hydrolysate (i.e. the 80% of the hydrolysate that was liquid contained 0.41% PEG). In rounds 3, 4 and 5 PEG was added so as to give a concentration 0.25% PEG in the liquid portion of the hydrolysate. Half of the PEG used was added to the 40% solid before enzyme addition, and half with the washwater 1.5 hours after enzyme addition. For a more detailed method see section 3.9.5.

(2) A separate hydrolysis process was run at 20% TS with 30 mg C-Tec3 g^{-1} pulp and 0.25% PEG in the liquid portion of the first-stage hydrolysate. Hydrolysis was carried out for 48 hours after which the concentrated sugar solution was removed by centrifugation.

Results and Discussion: The glucose concentration in the hydrolysate for each 48-hour hydrolysis (rounds 1-5) is shown in figure 7.5-5. The glucan conversion and glucose in hydrolysate for rounds 1-5 and for the separate hydrolysis are given in table 7.5-2.

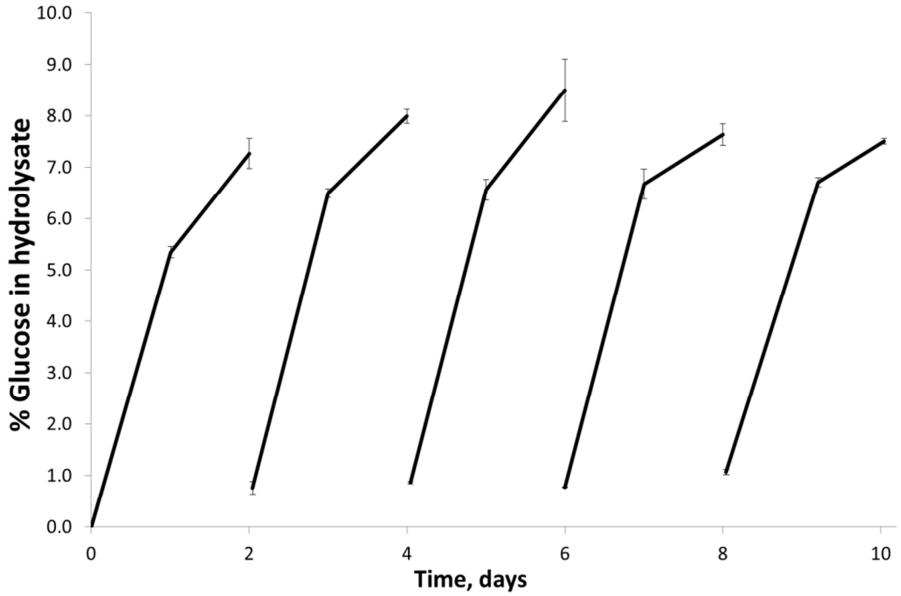


Figure 7.5-5. Glucose concentration in hydrolysate for 5 consecutive hydrolysis rounds with MSW pulp, using pH 7 and PEG washwater recycle. Washwater with PEG 6000 at pH 7 was adjusted to pH 5 and used as dilution water for the subsequent round of hydrolysis.

Table 7.5-2. Glucose concentration in the hydrolysate and glucan conversion after 48 hours in the repeat of pH 7 & PEG washwater recycle strategy.

	ROUND					
	1	**2**	**3**	**4**	**5**	**Separate hydrolysis**
Glucose concentration (wt%) in hydrolysate after 48 hours	7.3 ± 0.3	8.0 ± 0.1	8.5 ± 0.6	7.6 ± 0.2	7.6 ± 0.1	8.0 ± 0.1
% Glucan conversion after 48 hours	51 ± 3	51 ± 2	54 ± 6	48 ± 2	46 ± 1	54 ± 1

The results show that the glucan conversion in the first two rounds of hydrolysis was the same, at an average of 51%; however the sugar content in round 2 was higher at 8 wt% due to sugar augmentation from the previous round. The PEG content in these rounds was 0.41%. PEG addition was reduced in the third and subsequent rounds to 0.25%, and this seemed to have a positive effect in the third round where the conversion increased to 54 ± 6% with a glucose concentration of 8.5 wt%. This result lends support to the suggestion in section 6.7.1.2 that too many hydrophobic interactions may be a hindrance to the hydrolysis system. Here it was seen that when the PEG content was reduced the conversion increased. The presence of PEG in the system prior to the addition of enzyme may also have contributed to this positive effect.

Changes in the pH of the hydrolysate during this washwater recycle strategy are shown in table 7.5-3. In the first round of hydrolysis the pH increased from the starting pH of 5 to 5.6 after 48 hours of hydrolysis. At the end of the first-stage hydrolysis in rounds 2 and 3, however, the hydrolysate buffered at a pH of 4.5. In order to counteract this buffering, instead of adjusting the washwater produced from rounds 4 and 5 to pH 5, the washwater from round 4 (dilution water for round 5) was adjusted to pH 6 and the washwater from round 5 (dilution water for round 6) to pH 7. Unexpectedly, the system then buffered at even lower pH values of 4.3 and 4.2 by the end of the first-stage hydrolysis in rounds 4 and 5.

Table 7.5-3. pH changes in the repeat of pH 7 & PEG washwater recycle strategy

	R 1	R 2	R 3	R 4	R 5
pH of hydrolysate at 48 hrs of hydrolysis	5.6	4.5	4.5	4.3	4.2
pH of washwater after wash	6.3	5.1	5	4.6	------
Adjusted pH of washwater (dilution water)	4.9	5.1	6	7	------

Nomenclature; R - Round

It is unclear why this buffering effect occurred, and to provide further insight into the system the anion and cation contents in the hydrolysate were measured. Figures 7.5-6 and 7.5-7 show the most prominent ions found. In addition to this it was found that the hydrolysate and washwater (section 3.6) contained a significant amount of nitrate and in the hydrolysate this increased from 4 to 5.3 g L^{-1}. This high level of nitrate was present due to the process steps employed to produce the pulp at Fiberight.

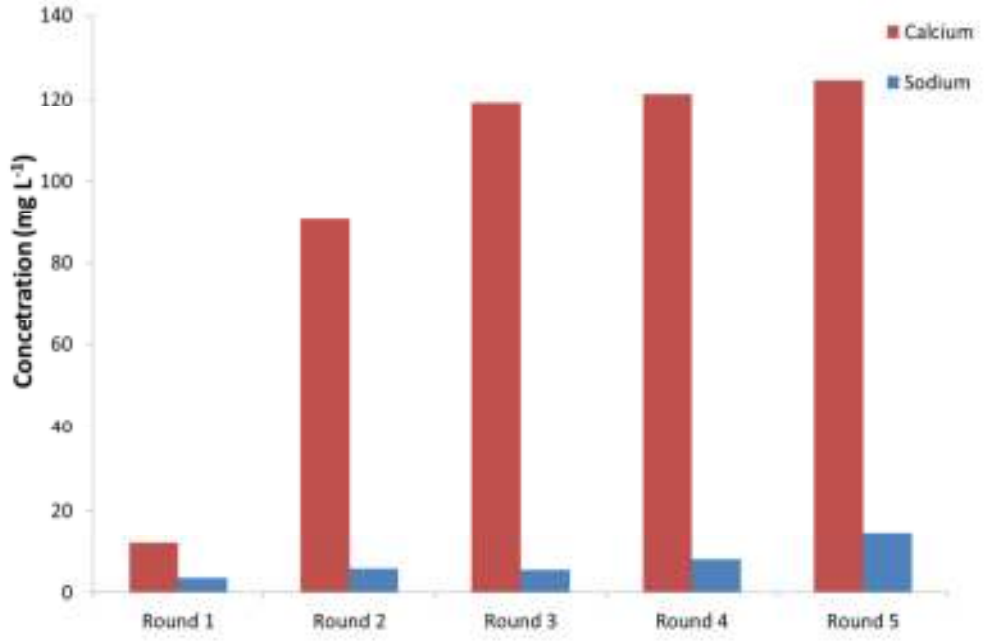


Figure 7.5-6. Calcium and sodium in washwater of washwater recycle strategy with pH 7 & PEG

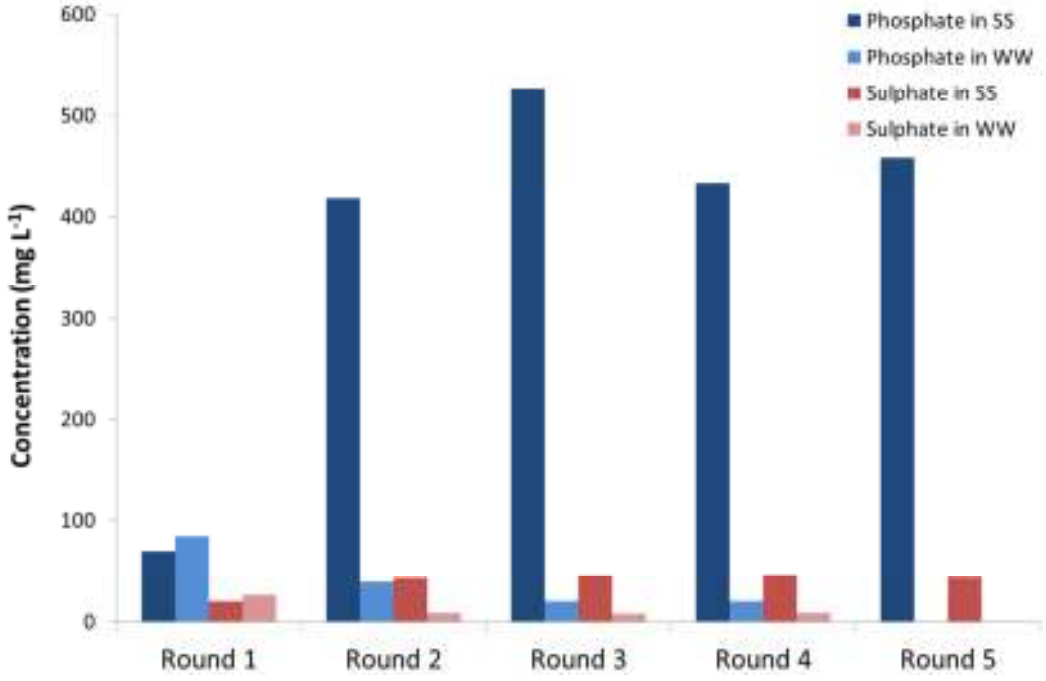


Figure 7.5-7. Phosphate and sulphate content in the hydrolysate and washwater of washwater recycle strategy with pH 7 & PEG

From these results it appeared that there may have been some interaction between the calcium, phosphate, sulphate, nitrate and PEG in the system which caused this buffering effect. Although there was a slight build-up of sodium through the rounds, this is not thought to have had any major effect on the hydrolysis. In the alkaline washwater recycle strategy (section 7.3) the sodium concentration was much higher

and yet did not seem to affect hydrolysis or lead to a low buffering point in the hydrolysate. It was previously shown (section 7.2.1) that calcium acts to increase the pH of the system; however this does not seem to be the case here. It is also of interest to note from the cation analysis that phosphate and sulphate concentrations in the washwater decreased through successive rounds. This result was unexpected, especially for sulphate, as this seemed to increase in the hydrolysate from round 1-5: meaning that less sulphate was added in the washwater in each round but more was produced in the hydrolysate than in the previous round.

The addition of the pH 6 and 7 washwaters to the MSW pulp in rounds 4 and 5 seemed to have a negative effect on conversion and pH. It is possible that the higher pH of these washwaters meant that there was decreased enzyme activity at the beginning of hydrolysis, leading to slow release of the compounds which caused the buffering effect and in turn a slow return to a lower and more suitable pH for hydrolysis. This would also explain why the pH 7 washwater was more detrimental than the pH 6 washwater. In addition, it was found that the higher pH washwaters were associated with an increase in suspended solids as can be seen in figure 7.5-8. This was not the case for the pH 5 washwater. These extra solids entering rounds 4 and 5 of hydrolysis may have had an inhibitory effect on conversion.

Another interesting point noted in the separate hydrolysis was that 56% of the available liquid was removed with centrifugation after the first stage. This is 13% more than without PEG and 4% more than when using 0.60% PEG (table 7.5-4). Therefore it is thought that 0.25 wt% is a more optimal PEG loading than 0.41 or 0.60 wt%, not only to increase glucan conversion but also to improve the dewaterability of the pulp. Given this improved dewaterability it would be of interest to investigate what total solids content could be achieved if filter pressing was used rather than centrifugation after hydrolysis.

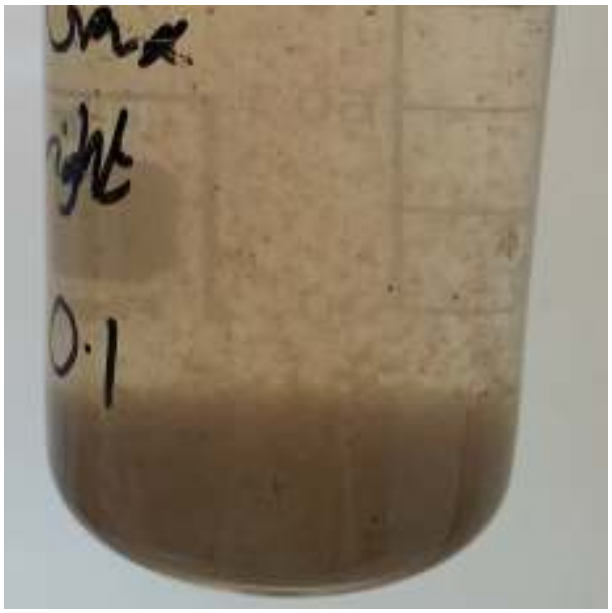


Figure 7.5-8. pH 7 washwater used for dilution of pulp in Round 5 of washwater recycle strategy.

Table 7.5-4. Percentage of extractable liquid/dewaterability of MSW pulp across various experiments in the current research

(Section) Experiment	Enzyme dose mg C-Tec3 g^{-1} pulp	Glucan conversion after 48 hours of hydrolysis (%)	Average liquid removed from hydrolysate (%)
(6.7.1)	50	67	45
(07.2) Round 1	30	45	43-45
(7.5) Round 1	30	47	53
(7.5.1) Separate hydrolysis	30	54	56

Liquid extracted by centrifugation

It is unclear whether enzyme carryover occurs when using the additive PEG: there was a slight increase in conversion (~3%) between round 1 and 3, but this may been due to the decrease in PEG concentration from 0.41% to 0.25%.

7.5.1.1 Second-stage hydrolysis of residual solids from section 7.5.1

Objective: To quantify the effect on glucose concentration and overall glucan conversion of increasing the residual liquid in a second-stage of hydrolysis.

Method Summary:

(1) 3-day second stage hydrolysis was carried out with (i) the residual pulp from the first stage hydrolysis in rounds 1, 2 and 3 of the washwater recycle strategy with 0.25-0.41 wt% PEG (section 7.5.1); and (ii) the separate hydrolysis experiment in section 7.5.1. After the concentrated sugar solution was removed by centrifugation from the hydrolysate of the separate hydrolysis process, the residual pulp was washed with pH 7 water containing 0.25% PEG. After washing the washwater was partially removed to leave a residual solids content of 14.5% TS. This residual solid and washwater was adjusted to pH 5 with phosphoric acid and left to undergo a 3-day second stage hydrolysis without the addition of extra enzyme.

Details of the standard method for second stage hydrolysis can be found in section 3.9.4.

Results and Discussion: Each of these process-sequences began with different amounts of total solids at the beginning of the second stage hydrolysis (table 7.5-5). Glucose concentrations in the hydrolysate from these processes are given in figures 7.5-9 to -12 and table 7.5-5 and total glucan conversion for the overall first and second stage hydrolyses are shown in table 7.5-5.

Table 7.5-5. Second-stage TS concentration, glucose concentration in the hydrolysate and total glucan conversion at end of second stage hydrolysis (after 5 days) in pH7 & PEG washwater recycle strategy

	ROUND			Separate
	1	**2**	**3**	**hydrolysis**
% TS for second-stage hydrolysis	23.2	21.4	17.4	14.5
Glucose concentration (wt%) at start of second-stage hydrolysis	1.2 ± 0.2	1.4 ± 0	1.2 ± 0	1.7 ± 0
Glucose concentration (wt%) at end of second-stage hydrolysis	8.3 ± 0.6	7.5 ± 0.1	7.4 ± 0.2	4.9 ± 0.2
% Glucan conversion after 2 stages of hydrolysis (5 days hydrolysis)	74 ± 2	72 ± 2	81 ± 5	71 ± 2

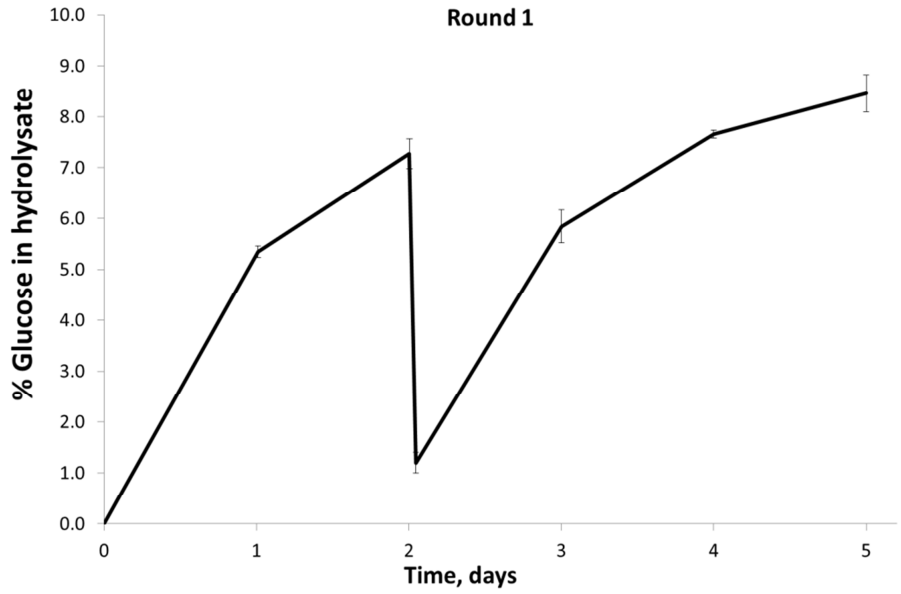


Figure 7.5-9. Glucose concentration in hydrolysate from first and second stage hydrolysis, round 1. The second stage TS was 23.2%. Error bars represent range.

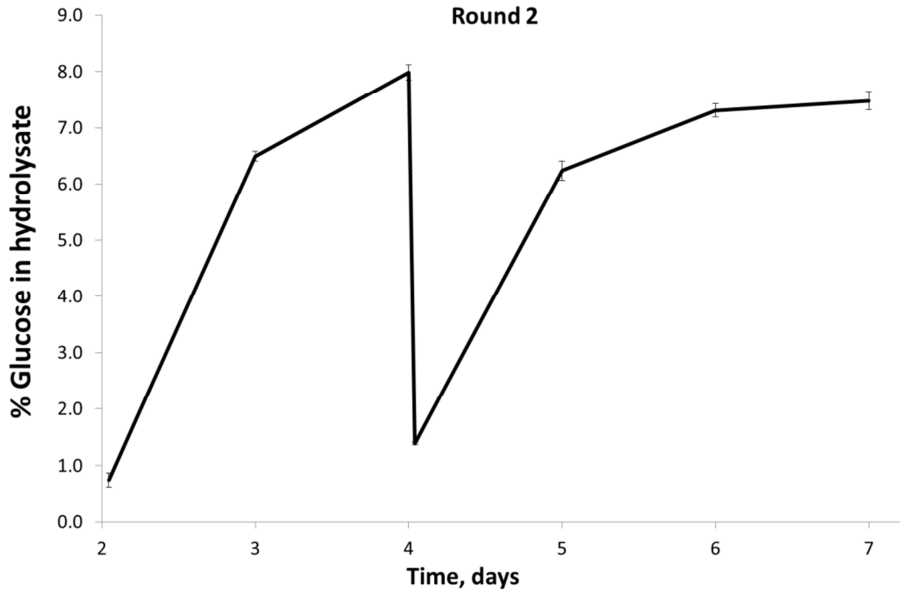


Figure 7.5-10. Glucose concentration in hydrolysate from first and second stage hydrolysis, round 2. The second stage TS was 21.4%. Error bars represent range.

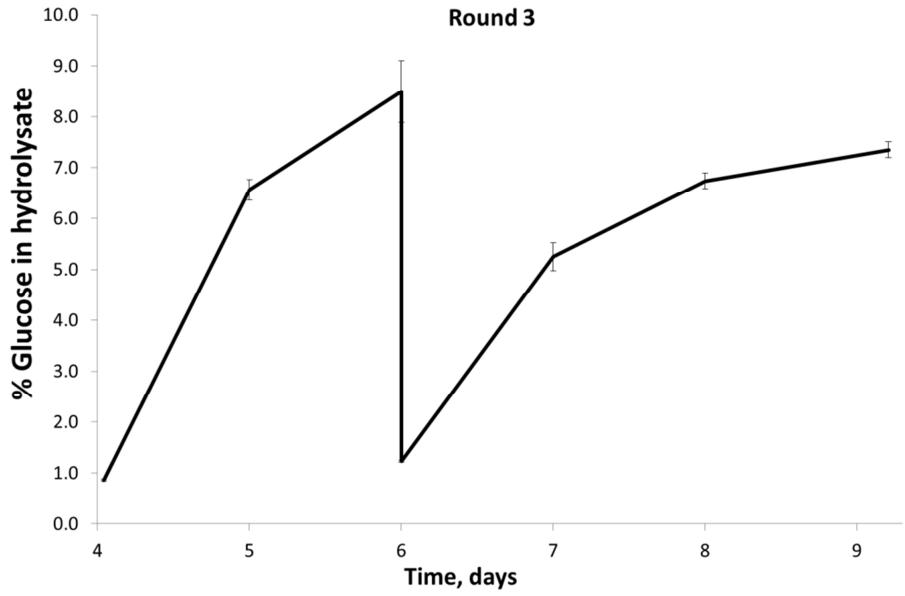


Figure 7.5-11. Glucose concentration in hydrolysate from first and second stage hydrolysis, round 3. The second stage TS was 14.4%. Error bars represent range.

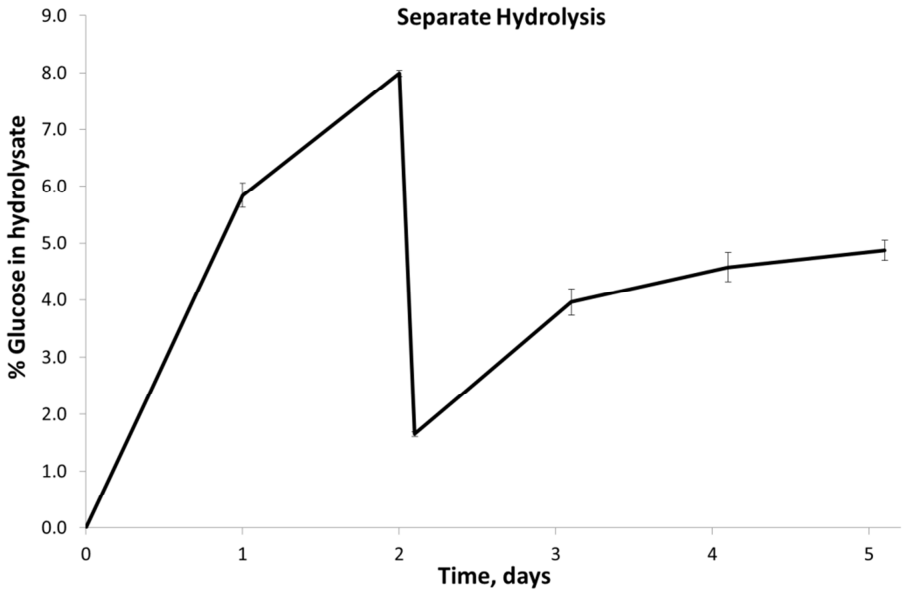


Figure 7.5-12. Glucose concentration in hydrolysate from first and second stage hydrolysis, in the separate hydrolysis. The second stage TS was 14.4%. Error bars represent range.

Discussion: As stated the objective of the second stage hydrolysis processes was to determine the optimum residual solids content for second-stage conversion. The aim was to achieve a high percentage of glucose in the hydrolysate of the second stage by adding just enough water to facilitate mixing. It was important to try and reduce the viscosity in the second stage, as the results of the original process in section 7.2.2 figure 7.2-5 showed that, although a high glucose concentration was

achieved in the second stage, the mixture did not adequately liquefy until after 24 hours.

The second stage hydrolysis was performed using the residual solids from rounds 1, 2 and 3 in section 7.5-1 as well as from the separate hydrolysis. The total solids content had been reduced through these rounds by the addition of increasing amounts of washwater as can be seen in table 7.5-5. The reason for not using the residual solid from rounds 4 and 5 for the second stage hydrolysis was that in those rounds the pH of the dilution water was adjusted to above pH 5 (table 7.5-3) at the beginning of the first-stage hydrolysis, and the amount of suspended solids going into those rounds was higher than in the first 3 rounds. Thus the conditions in rounds 4 and 5 were too different to those from rounds 1-3, and it was decided that rounds 4 and 5 should not be used in the second stage hydrolysis study.

From the results in table 7.5-5 it can be seen that the overall glucan conversions across rounds 1, 2, 3 and the separate hydrolysis were on average 74, 72, 81 and 71 % respectively. Thus the result from round 3 had the best overall conversion. The residual solids from round 3 were diluted to a content of 17% TS; due to the shorter fibre length (as a result of the first-stage hydrolysis) the pulp in this residual material was a readily mixable suspension. The total average glucan conversion of 81% for the first and second stage hydrolyses is equivalent to the conversion in section 6.7.1; however the strategy used here achieves this with a 40% decrease in enzyme loading. Furthermore, one of the process replicates in round 3 reached an overall glucan conversion of 86% which is even higher than that achieved in section 6.7.1. The average first and second stage glucose concentrations achieved in round 3 were 8.5 and 7.4 wt% respectively. If the hydrolysates from the first and second stages were mixed, this would result in an overall average glucose concentration of slightly over 8 wt%. Additionally in this strategy the initial solids content was 20% as opposed to the 18.5% used in section 6.7.1. This means that the actual amount of glucose produced here was higher than that in the previous process strategies. In future work it would be of interest to see what would occur if β-glucosidase was added in this second stage.

Conclusion: The two-stage hydrolysis strategy used here made it possible to obtain an 81% glucan conversion using 40% less enzyme than in section 6.7.1. Mixing of the liquid portions of the hydrolysate stage 1 and 2 would give a sugar solution containing > 8 wt% glucose. These results are highly significant in that they represent a practical means of achieving the desired combination of high conversion at high product concentration with low enzyme use. It is also likely that there is scope for further optimisation of the approach.

7.6 Conclusions from chapter 7

In this chapter a series of strategies were designed and tested, in which a first-stage hydrolysis was performed for 48-hours. A concentrated sugar solution was removed from this by centrifugation and the residual pulp was then washed. The washwater was removed from the residual solid and used as dilution water for a subsequent round of hydrolysis. The washwater recycle process was carried out for 3-6 rounds. In some of the strategies the residual solid which remained after washing was used to perform a second-stage hydrolysis with part of the residual washwater, but without additional enzyme. This washwater recycle strategy was carried out with wash conditions at pH 3-5, 7, 9+ and pH 5 and 7 with the addition of PEG 6000.

In the first strategy, using a wash between pH 3-5, it was found that continuous enzyme hydrolysis could be successfully performed over 6 rounds using the washwater from the previous hydrolysis round without any contamination occurring through the rounds. It was found that performing the wash at pH 5 or lower led to an increase in the amount of calcium that leached out from the MSW pulp and into the washwater. This calcium then drove up the washwater pH. Although the washwater was adjusted to pH 5 before use as dilution water for the subsequent round of hydrolysis, this acidic wash meant there was an increasing quantity of calcium in the dilution water through the rounds. This increase in calcium seemed to be beneficial for hydrolysis. In this first strategy using 30 mg CTec3 g^{-1} pulp it was possible to obtain a sugar solution with 7.7% glucose in the first-stage hydrolysis, and a 4% increase in glucan conversion was seen from the 1^{st} to the 5^{th} round, perhaps indicating enzyme carryover.

A second stage hydrolysis was performed from the residual solids in round 1 and 4 and total glucan conversions of up to 74% was achieved through the first and second stage of hydrolysis. If the hydrolysates from the first and second stages were combined then an overall glucose solution containing 7.2 wt% would be obtained.

When using an alkaline wash an increase in glucan conversion of 5% was seen between round 1 and round 3 or 4. In this strategy a glucose concentration of up to 8.2% was achieved in the first stage of hydrolysis. When using MSW pulp it was found that it was beneficial to perform the wash at pH 10 or higher, as this led to the greatest improvement in glucan conversion in a subsequent round. When this strategy was repeated on Fisher brand filter paper with a wash at pH 9 there was no evidence of enzyme carryover through the hydrolysis rounds. This possibly indicates that the beneficial action of the alkaline wash on the MSW pulp was afforded by the carryover of compounds that enhanced hydrolysis, or that a higher pH wash was needed to carryover the enzymes. As this control experiment was done on filter paper, however, it is also possible that there was no unproductive binding of enzymes, meaning that a larger number of enzymes were free in solution and were lost with the removal of the concentrated sugar solution.

Finally the strategy was repeated using PEG 6000 in the hydrolysis and washwater medium. Some strange results were seen when this compound was used which included high amounts of calcium suspended in solution and the pH of the system buffering at a pH of < 5, even though the system pH was adjusted to 5 or above at the beginning of first-stage hydrolysis.

Different concentrations of PEG were used in the various experiments testing this strategy and it was found that a concentration of 0.25 wt% was more effective in enhancing conversion than concentrations of 0.41 or 0.60 wt%. This lower concentration also led to an increase in the dewaterability of the hydrolysate, making it was possible to recover up to 56% of the liquid in the hydrolysate by centrifugation after the first-stage of hydrolysis, as compared to only 43% when the additive was not used. It was not clear whether the additive led to enzyme carryover.

Lastly 4 rounds of hydrolysis were performed using the PEG washwater recycle strategy, in which the total solids in the second stage hydrolysis were varied to reduce the viscosity. With 0.25 wt% PEG and solids contents of 20% TS in the first stage and 17% TS in the second stage it was possible to achieve a total glucan conversion of 81% at a 40% lower enzyme dosing than that used in section 6.7.1, where the same conversion was achieved. Furthermore mixing of the hydrolysates from the first and second stages of hydrolysis would give a final glucose solution containing over 8 wt% glucose, which is the desired concentration for fermentation and distillation if the product is used for ethanol production.

Chapter 8: Conclusions & Recommendations

8.1 Conclusions

The focus of this research was to find ways of improving enzyme usage and sugar yield in the hydrolysis of a lignocellulosic feedstock, with particular attention to methods that are potentially suitable for future large-scale application in an industrial bio-refinery. The lignocellulosic feed used in this research was paper pulp derived from municipal solid waste (MSW which had been processed to remove non-reactive products such as plastics, glass and metal contaminants). In the current research this pulp was hydrolysed by the enzymatic route to produce monomeric sugars which could then be used in a sugar platform bio-refinery.

In order to develop a strategy suitable for industrial application it was important to first understand the substrate characteristics and how these affected the enzymatic hydrolysis of this material. It was also crucial to determine whether the enzyme activity could be enhanced when using this material as well as the potential limitations of the chosen enzyme system which was the Cellic C-Tec family of enzymes from Novozymes.

The substrate provided by Fiberight contained batch to batch variations depending in part on process modifications which occurred in the plant through the duration of this work, and in part perhaps on variability in the input material. On average it was found that the substrate contained 56: 12: 27: 5 of Glucose: Hemicellulosic sugars apart from glucose: Lignin & pseudo lignin: Ash on total solids. Most of this pseudo lignin component could be removed through an acetone extraction or when performing a Fibrecap compositional analysis, and the true lignin content of the fibre was between 9-14% of total solids.

The hydrolysis behaviour of the MSW pulp was found to be similar to that of other lignocellulosic substrates, in that the hydrolysis rate was initially rapid but then decreased with time. This substrate was subject to the solids effect where increased solids content led to an overall decrease in percentage sugar conversion. Also, an

increase in enzyme dosing did not lead to a proportional increase in substrate conversion. Agitation is important in achieving higher hydrolysis yields and by investigating various mixing strategies it was found that tumbling or gravity mixing was superior to orbital shaking and rotational mixing. Using this mixing approach it was possibly to liquefy MSW pulp at high solids (>20%) in 24 hours and at enzyme doses of less than 50 mg g^{-1} pulp, whereas this was not possible with the other mixing strategies (orbital and rotational mixing).

Various substrate properties and their effects on hydrolysis were investigated. It was found that reducing the particle size by milling to ≤0.5 mm did not enhance conversion, and in fact hindered the process possibly due to the release of inhibitory compounds into the reaction matrix. Solvent extraction to remove extractives or pseudo lignin with acetone seemed to increase the substrate accessibility and led to an increase of up to 6% in conversion compared to un-extracted pulp. This increase in conversion, however, would most probably not offset the cost of employing a solvent extraction step in a full-scale process unless the extraction procedure was also able to recover value-added compounds or building blocks. Mild alkali treatment led to a significant decrease in conversion, possibly due to deposition of compounds on the surface of the cellulose making it inaccessible. Lignin extraction using sodium chlorite and acetic acid only showed limited success and SEM imaging of the material indicated that it led to surface deposits or structural changes which reduced cellulose accessibility. The complex nature of the substrate and the many processing steps it has been through prior to hydrolysis may mean the lignin is not susceptible to removal. From these various extraction treatments it was concluded that any extractives or lignin present in the substrate do not significantly hinder substrate conversion.

Steps were also taken to try and enhance enzymatic activity and it was found that using a nitrogen additive such as the amino acid glycine did increase substrate conversion up to 5%. This was not as significant as the polymer additive PEG 6000 which increased conversion at a high solids concentration (20% TS) by 15% over 48 hours, while allowing a 40% decrease in the enzyme requirement. Addition of 0.25 wt% PEG 6000 in the reaction medium also improved substrate dewaterability, with

an increase of up to 13% in the amount of liquid that could be removed by centrifugation after a 48-hour hydrolysis.

When comparing enzyme activity in different reaction media it was found that 5% higher conversion was achieved in water adjusted to pH 5 with phosphoric acid compared to a pH 5 citric acid buffer.

Substrate feeding strategies were also investigated and it was found that at high solids concentrations batch hydrolysis was superior to fed batch or split batch substrate and enzyme addition.

The current research focused on high solids processes due to their ability to produce high concentration sugar solutions and reduce the general overall energy demand. Thus a number of process sequences were set up at high solids contents to try and meet these criteria.

The main difficulty arising from the operation of a high solids hydrolysis process is the prevalence of the solids effect which may be caused by: insufficient mixing; product inhibition as a result of increasing sugar concentration; decreased water availability; irreversible binding of adsorbed enzyme to the substrate, including non-productive binding to lignin; inhibition of enzyme adsorption; and enzyme denaturation.

To overcome some of these limitations a number of process strategies were devised to alleviate product inhibition and increase water availability in the hydrolysis system. These parameters were manipulated so that it was possible to obtain higher overall sugar yields, substrate conversions and ethanol titres compared to those in a standard batch hydrolysis where the enzyme activity diminishes significantly after 48 hours. The results from various process configurations are given in table 8.1-1.

The first set of processes aimed to alleviate product inhibition by using an intermediate fermentation step at 30 ^{o}C between two hydrolysis stages at 50 ^{o}C. Using this method it was possible to reactivate the enzyme system. This strategy was however limited due to the unfermented soluble sugars released during

hydrolysis and the decreased water availability caused by their presence in the system. Further to this the ethanol present after fermentation was shown to decrease enzyme activity.

The second set of processes tested a strategy to relieve product inhibition and increase water availability by removing part of the concentrated sugar solution after a first hydrolysis stage of 48-hours. The residual solids were then washed and the washwater removed and replaced with an equal volume of pH 5 water. This procedure also reactivated the enzyme system and led to increased conversion compared to controls without a wash step. Hydrolysis was enhanced by the addition of β-glucosidase in the second stage, indicating that this enzyme may have been lost or denatured during the initial steps. The addition of β-glucosidase led to the simultaneous release of glucose and xylose: a possible explanation is that freeing the cellobiose makes the associated xylan available for conversion by hemicellulases in the system. The reactivation of the hydrolysis system achieved by β-glucosidase addition was greater than from adding C-Tec3 into the second-stage hydrolysis.

Table 8.1-1 compares the 4-day single stage results for glucan conversion with those obtained from both the intermediate fermentation and intermediate wash strategies. The use of an intermediate wash step that removed residual sugars from the substrate produced an even higher glucan conversion, confirming that water availability may have been a key factor in reducing the activity of enzymes retained on the solid fraction.

The most significant improvement was obtained when β-glucosidase was replenished after the first stage hydrolysis, as this apparently helped to reactivate cellulases that were bound to the substrate. Using this strategy gave a higher glucan conversion at a lower enzyme dose than in many other high solids hydrolysis studies (Xue et al., 2012b; Zhang et al., 2009; Zhang et al., 2012b). The value of obtaining a concentrated sugar solution from this high conversion yield should also be noted.

Table 8.1-1. Comparison of all two stage hydrolysis systems after 96 hours of hydrolysis.

Experimental condition	% Conversion in primary hydrolysis	% Total conversion after second-stage hydrolysis	% Conversion improvement compared to control (no fermentation or wash step)
A. Single stage hydrolysis (96 hours)	68.0 ± 5	-	-
B. Two-stage hydrolysis with intermediate fermentation (24-hour initial hydrolysis)	46.5 ± 0.36	76.5 ± 0.5	8.5 ± 0.5
C. Two stage hydrolysis with intermediate wash step	67. 2 ± 1.3	81.2 ± 0.7	13.2 ± 0.7
D. Two stage hydrolysis with intermediate wash step and β-glucosidase addition	67.2 ± 0.8	87.6 ± 0.4	19.6 ± 0.4

All strategies has an initial enzyme loading of 50 mg C-Tec3 g^{-1} pulp. Strategy E had a β-glucosidase loading of 25 mg enzyme g^{-1} pulp original in the second stage of hydrolysis.

A further strategy was trialled in which the washwater from the intermediate wash step was used as the dilution water in a subsequent batch hydrolysis. This showed no detrimental effects on glucan conversion, while the glucose concentration in the hydrolysate was increased due to augmentation by the 1 wt% glucose in the washwater. In addition, it was found that enzyme recovery in the washwater was increased if the residual solid was washed at pH 9 rather than 5.

From these results it was clear that a split hydrolysis (i.e. one with a first and second stage and an intermediate wash step) was promising in achieving high glucose yields and obtaining a high overall glucan conversion. A continuous hydrolysis process was therefore tested where washwater from one hydrolysis was used as the dilution water in a subsequent hydrolysis over 3-6 rounds. This was undertaken in order to maximise the yield of sugars and to determine whether any carryover of enzyme activity could be seen. This washwater recycle strategy was carried out with wash conditions at pH 3-5, 7, 9+ and pH 5 and 7 with the addition of PEG 6000.

Of these the two most promising strategies were those using an alkaline wash and that using a PEG 6000 wash at a concentration of 0.25 w% in the reaction medium. In addition to the continuous first stage hydrolysis process a second-stage hydrolysis was performed within some of the strategies without the addition of extra water at pH 5 and this gave an increase in glucan conversion of up to 28%.

An alkaline wash gave an increase in glucan conversion of 5 % between round 1 and round 3 or 4, possibly indicating enzyme carryover. In this strategy a glucose concentration of up to 8.2% was achieved in the first stage of hydrolysis.

When using PEG it was unclear whether enzyme carryover occurred between the rounds, and further investigation is needed to clarify this. Furthermore this additive caused a pH buffering effect which resulted in the pH of hydrolysate settling below the optimal level. Despite this, it was possible to obtain consistent glucose concentrations above 7.3 wt% in the hydrolysates of the first-stage and this could probably be improved upon with further process optimisation.

The most important result came from the third round of this strategy when using a pH 7 wash and 0.25 wt% PEG, with solids contents of 20% TS and 17% TS (after 48 hours of hydrolysis) in the first and second stages of hydrolysis. This gave a total glucan conversion of 81% with a 40% reduction in the required enzyme dosing. Mixing of the hydrolysates from the first and second stages would have given a final concentration of 8+ wt% glucose, which was above the target value in this research.

The research therefore achieved the main aims outlined in Chapter 1 by developing a process for effective hydrolysis of MSW pulp at a relatively low enzyme loading (30 mg C-Tec3 g^{-1} pulp) under high solids conditions (20% TS). A high glucan conversion (80+ %) was achieved with a high sugar content (8+ wt%) reducing the need for further concentration of the hydrolysate. Obtaining a high glucan conversion minimises the amount of unconverted cellulose in the substrate. This residual solid and associated sugar could be as used for biogas production, alternatively gasification or pyrolysis could be employed to create various value-added products. By integrating the production of sugar and chemicals this substrate can be used successfully as a feedstock for a sugar-lignin platform bio-refinery.

8.2 Recommendations

Although the outlined aims of this research were achieved a number of follow up studies could be performed to clarify some of the results obtained. This includes

further investigation into certain reaction mechanisms and verification of some outlying results.

As acetone extraction was shown to increase the glucan conversion at low solids it would be of interest to see whether this same effect is observed at high solids and what improvements in glucose yield, if any, can be achieved.

More conclusive evidence is required on the effects of disc milling to determine whether this is one of the major causes in the batch to batch differences in glucan conversion.

Although the alkali washwater recycle strategy worked well on MSW pulp this was not the case on filter paper with a pH 9 washwater recycle strategy. Thus further experiments could be carried out on filter paper with a pH 10 wash to see if this improves the results. Also it may be of interest to see the effects of adding PEG to the alkaline washwater recycle strategy on MSW pulp.

The first and second stage hydrolysis process strategy in the final chapter showed very good results and it would be of interest to see whether addition of a β-glucoisdase preparation in the second stage would either further enhance conversion in the second stage or increase the rate of hydrolysis in the second stage.

Finally the effect of PEG and the mechanism by which it acts merits further work. Firstly the mechanism of increased dewaterability when using PEG in the reaction medium needs to be investigated. This could be achieved by taking viscosity measurements and analysing the fibre length as hydrolysis progresses both, with and without the additive. If viscosity decreases more rapidly with PEG this indicates that most probably PEG is increasing the endoglucanase activity which could be further verified by the fibre length analysis.

Secondly, it needs to be ascertained if PEG addition leads to the suspension of ions in the hydrolysate and washwater and whether this causes a pH buffering effect. PEG could be added to a washwater recycle strategy on filter paper with the

addition of various ions either separately or together to determine if a certain combination causes the buffering effect.

Lastly the PEG washwater recycle should be repeated for 5 rounds on MSW pulp with 0.25% PEG to establish whether enzyme carryover occurs in this strategy.

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