BIOCONVERSION OF BIODEGRADABLE MUNICIPAL SOLID WASTE (BMSW) TO GLUCOSE FOR BIO-ETHANOL PRODUCTION

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Abstract

Municipal solid waste (MSW), as an emerging biomass source, presents a unique opportunity for large-scale second-generation bioethanol production. Feedstock supply is reliable and in sufficient quantity, making it a promising biomass source but the conversion yield is currently too low to make it financially attractive. This work presented in this thesis provides a better understanding of bioconversion systems, in particular of pre-treatment and hydrolysis processes which contribute to more than 60% of ethanol selling price. This thesis also presents a technique of bioconversion which allows conversion of MSW to bioethanol to be carried out more efficiently than with existing techniques.

This thesis starts with an assessment of the feasibility of using MSW to replace primary agricultural products as biomass sources. It presents an efficient MSW to ethanol bioconversion process which includes pre-treatment and enzymatic hydrolysis, and provides detailed quantitative information on the conditions that maximise the glucose yield to 80% after 24-h hydrolysis reaction. This thesis also presents the result of the characterisation of the complex substrate features of the selected MSW fractions which have lignin and cellulose crystallinity, and an evaluation of the effects of MSW-substrate features on the conversion process. Finally, it presents the first model of the effects of substrate features in cellulase-cellulose adsorption; cellulase-cellulose adsorption is recognised as a crucial step that controls the enzymatic hydrolysis rate.

This study shows that lignin, crystallinity, cellulose content and their interaction have an important influence on enzyme adsorption capacity. It is concluded that both lignin content and crystallinity play a greater role in cellulose-cellulase adsorption than cellulose content. Finally the presence of lignin has a greater effect than crystallinity on both the maximum enzyme adsorption capacity and steady-state enzyme adsorption, whereas crystallinity has a greater effect on the latter one.

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Abbreviations

AFEX: Ammonia fibre explosion

AIL: Acid insoluble lignin

ANOVA: Analysis of variance

ASL: Acid soluble lignin

CP: Carrot peelings

BMSW: Biodegradable municipal solid waste

CDP: Consolidated BioProcessing

CHB: Cellobiohydrolase

CrI: Crystallility Index

E^a: Enzyme adsorption

GOW: Garden organic waste

GR: Grass

HPLC: High performance liquid chromatography

KOW: Kitchen organic waste

MOW: Mixed organic waste

MSW: Municipal solid waste

MW: Microwave treatment

NP: Newspaper

PCW: Paper and card waste

PP: Potato peeling

SE: Steam treatment

SP: Scrap paper

SHF: Separate hydrolysis and fermentation

SSCF: Simultaneous saccharification and co-fermentation

SSF: Simultaneous saccharification and fermentation

T. virid: *Trichoderma virid*

T. reesei: *Trichoderma reesei*

Chapter 1

Introduction

1.1 Introduction

In a context where energy security, food security and waste management are becoming some of the greatest challenges for many nations, bioethanol production from biodegradable municipal solid waste (BMSW) offers tremendous perspectives. This technique has a clear potential for commercialisation but the cost of this emerging process is currently too high to allow its implementation on an industrial scale. Indeed, a number of studies have attempted to identify specific technical opportunities to lower bioethanol production costs (Lynd, 1996; Wyman, 1999; Hamelink et al., 2005). In its simplest form, bioethanol production relies on three consecutive stages: pre-treatment, hydrolysis and fermentation. When examining closely ethanol production processes, it appears that reducing ethanol selling price could be more effectively achieved by devoting appropriate research efforts to: (i) identifying new low-cost and abundant feedstock, (ii) developing efficient pre-treatment technologies, (iii) creating genetically-engineered organisms for hydrolysis and fermentation processes and (iv) implementing process integration (Wyman, 1999). In addition, the current use of products such as corns and crops as primary biomass, that is to produce ethanol, already results in an increased demand of agriculture products and subsequently in high food prices. It is feared that further exploitation of primary biomass would ultimately threaten the provision of basic food in many nations. To reduce ethanol price and ensure food supply security, it is necessary to investigate alternative biomass sources, sometimes termed second generation biomass. These include forestry waste, agricultural waste, or municipal solid waste (MSW).

MSW, as emerging biomass resource, presents a unique opportunity for large scale biofuel production. It is highly concentrated within urban environments and far less seasonal in nature than primary biomass, providing a more reliable supply. Moreover, infrastructure and networks are already in place to collect and process MSW; this presents major opportunities for integrating new waste management options and reducing the cost of bioethanol. Finally, life-cycle based assessments of the major environmental impacts (or sustainability indicators) of MSW have highlighted a range of environmental benefits to be gained from MSW energy recovery. These include:

- Reduced greenhouse gas emissions,
- Reduced acid gas emissions,
- Reduced depletion of natural resources (fossil fuels and materials),
- Reduced impact on water (leaching), and
- Reduced land contamination.

In a recent study by Mtui and Nakamura (2005), MSW from dumping sites in Tanzania was used for hydrolysis/fermentation process (SHF) to produce ethanol; they reported a 21% final ethanol yield. This figure is too low for a sustainable industrial production but laboratory research works offers promising perspectives. Glucose yield of 80% has been reported using mixed solids waste (35% construction lumber waste, 20% almond tree prunings, 20% wheat straw, 12.5% office paper and 12.5% newsprint) as initial substrates for the production of bioethanol after 7-day hydrolysis (Nguyen *et al.*, 1999). A higher lignocellulosic content in the agricultural and wood residue mixed waste (~63%) (Nguyen *et al.,* 1999) than in MSW (~38%) (Mtui and Nakamura, 2005) may explain the relatively lower product yields and long reaction time obtained using MSW. These findings suggest that MSW can be a promising biomass source, providing it is possible to have a better understanding of the physical and chemical factors that affects bioconversion process.

Although research into pre-treatment technology development and genetically engineered organism development has attracted lots of attention, pre-treatment process and hydrolysis process remain the critical steps of the larger ethanol conversion processes. Different pre-treatment methods have been developed but no existing one meets the requirements in terms of both performance and cost. Moreover,

most of the pre-treatment techniques and technologies developed are based on primary biomass sources with single substrate. As researchers (Hamelinck *et al.*, 2005) pointed out that the most appropriate pre-treatment method depends upon the type of biomass used, it is unclear whether current pre-treatment technologies can be used for MSW. For these reasons it is clear that further research is needed in these areas to develop an effective and low cost MSW-based bioconversion technique.

With more than 30% of the ethanol cost associated to the hydrolysis stage, it seems reasonable to think that the total cost can be reduced significantly by improving the product yield (i.e. glucose yield) of this process. Although optimisation has been carried out by some researchers, there is no definitive conclusion on what factors significantly affect the performance of the hydrolysis process. It is known though that hydrolysis is influenced by biomass properties (Mandels *et al.*, 1981; Nutor and Converse, 1991; Wang and Converse, 1992; Yang *et al.*, 2006). However, as most of the research in the field is concerned with single substrate process, it is also currently difficult to draw any conclusions from the existing body of literature about the effect of the biomass physical structures on the bioconversion process. Finally, scarce information is available on the chemical composition of MSW as a biomass source for ethanol production, making the calculation of the yield extremely difficult. With the advantages of low cost and large quantity, MSW feedstock is likely to become economically viable, if enhancement of bioethanol processes and technologies allows conversion of MSW to bioethanol to be carried out more efficiently.

1.2 Research aims and objectives

The research work described in this thesis is ambitious. It aims at enhancing the field of MSW-to-ethanol bioconversion mechanisms, and shall pave the way for the development of economically viable second-generation bioethanol production plants that could generate affordable energy and contribute to mitigate emerging threats to energy and food supply security. The research aims are threefold: (i) assessing the feasibility of using MSW as biomass sources, (ii) developing an efficient bioconversion process using available technology to convert MSW to ethanol and

determine the conditions required to maximise the glucose yield, and (iii) improving the existing level of understanding of the MSW feedstock-based hydrolysis mechanisms.

These research aims were achieved in consideration of the following five objectives:

1. Calculating an estimate of the quantity of ethanol that can be produced from MSW by considering both the quality and quantity of available MSW-feedstock in London, and show that MSW, as one of the promising biomass sources has the potential of replacing primary biomass sources.

2. Characterise the chemical composition and physical structure of selected BMSW.

3. Develop an efficient pre-treatment process for MSW-feedstock using available results obtained by analysing existing pre-treatment techniques for primary biomass feedstock.

4. Optimise the bioconversion process by identifying the main factors influencing the hydrolysis process performance (glucose yield) and determining the optimal enzymatic hydrolysis process conditions using fractional experimental design.

5. Improve our understanding and model the effects of substrate features on cellulase-cellulose adsorption mechanism during the enzymatic hydrolysis process.

1.3 Thesis outline

The following paragraphs are intended to give a quick overview of the content of each chapter of this thesis.

Chapter 2 presents an analysis of the potential biomass resources. It starts from the emerging UK biofuel industry, resulting in the large demand of biomass, followed with a review of current biomass situation. Starting by examining the chemical and physical properties of lignocellulosic materials, attention is then brought on understanding the broader technical and economic barriers of ethanol production. Finally, BMSW, as potential biomass, is analysed with an evaluation of its availability and potential use. The review and analysis of biomass resources in this chapter should provide an insight of using BMSW as biomass sources. It provides the reader with a contextual evidence of the importance and significance of replacing primary biomass sources with BMSW.

Chapter 3 presents a body of relevant research works on the broader biomass-to-ethanol conversion technology. A comprehensive review on the process involved including pre-treatment technologies, hydrolysis process, fermentation, waste disposal and process integration are included. A considerable portion is presented with the current pre-treatment methods and enzymatic hydrolysis process; these processes are the most critical steps of the whole bioethanol conversion process. Promising directions of research in these areas are presented at the end of this chapter; all supporting the research aims of this thesis.

Chapter 4 is devoted to a review of the existing studies on cellulase-cellulose adsorption during enzymatic hydrolysis. A considerable portion of this review is dedicated to the mechanisms of cellulose hydrolysis and their kinetics. Thereafter, the review focuses on cellulase-cellulose adsorption systems; an attention is given to features of the widely studied *Trichoderma* cellulase system and substrate's structure. The Section 'Quantitative Models' is interested in properties of various models reported in the literature. The next section outlines a number of challenges associated with understanding and modelling non-complex cellulase systems.

Finally, a model is selected to simulate the experimental results from this work with the theory, assumptions and equations are also given in that section. Finally, a regression model is introduced in order to model the effects of substrate features on cellulase-cellulose adsorption for MSW-biomass.

Chapter 5 is concerned with the experimental aspect of this work. It describes materials, apparatus and procedures that were used for this study. These include experiments aimed at determining the composition of biodegradable MSW and understanding how they will be used for the glucose production. Additionally this chapter describes the procedures of the glucose developed and used in glucose production. Pre-treatment processes are also described including chemical and physical pre-treatment, followed by the enzymatic hydrolysis process. The work was carried out by following the standard procedures as described in the National Renewable Energy Laboratory (NREL) manual. Other sources are also referenced as appropriate.

Chapter 6 explores the main areas of the knowledge on waste that is currently lacking in the literature. This together with biomass analysis should give the reader a clear indication of the feasibility of using MSW as a potential source of biomass for the production of bioethanol. In this work, biodegradable municipal solid wastes (BMSW) were classified into three groups: kitchen organic waste (KOW), green organic waste (GOW), and paper and card waste (PCW). For each model waste, moisture content and chemical composition including cellulose, hemicellulose, lignin, and ash content were examined to identify the potential glucose yield. The discussion also considers any other technical barriers that prevent further improvement of this process. Further analysis into cellulose crystallinity, bulk density, and particle size were carried out as they provide useful information on the structural properties of cellulose in the waste streams. Literature information on this aspect is scarce and there is a real shortage of useful conclusion. Detailed information on the characteristics of this part of MSW together with potential applications implies that the results described in this chapter should bring the scientific community a step closer into addressing this important issue.

Chapter 7 focuses on results of pre-hydrolysis experiments. The main purpose of

these methods is to increase the hydrolysis process efficiency by (i) removing lignin and hemicellulose that surround cellulose, and (ii) reducing the cellulose crystallites. In this work, various treatment techniques were carried out; they include steam treatment, microwave treatment, dilute acid treatment, two-step combination of acid treatment and steam treatment or microwave treatment, and acid-impregnated steam treatment or microwave treatment, on selected BMSW fractions. The most efficient method (acid-impregnated steam treatment) is chosen for further study. Therefore, in later parts of the chapter, detailed discussion focused on the selected pre-hydrolysis technique. The various factors that affect the process and their effects are analysed. It follows a composition analysis (for glucose yield) and crystallites determination. Finally, the selected treatment method was compared with other methods presented in the literature.

Chapter 8 investigate how the process can be optimised in order to improve the product yield and to reduce its cost. It starts from selecting cellulase for hydrolysis by comparing the two most commonly used enzymes (*Trichoderma virid* and *Trichoderma seerei*). The selected cellulase is used for all the enzymatic hydrolysis involved in this work. Furthermore, the different factors that were considered to be important from the literature review are selected for further study. The purpose is to identify the limiting factors in order to optimise the process and to set the value of non-significant factors in such a way that the cost associated with these factors (e.g. enzyme concentration) can be reduced. In order to study the interaction of factors, two-level fractional experimental designs with ANOVA analysis are introduced. After the effects of each factor are discussed, the optimal conditions for each type of selected waste fraction or their combination are given. Experimental results from these conditions were compared with the predicted values generated from the ANOVA model.

Chapter 9 provides fundamental understanding of the effects of substrate features on cellulase-cellulose adsorption. It starts from the hydrolysis kinetics and protein adsorption observed from the selected BMSW fractions. A model from the literature developed for pure cellulose is selected and fitted to the experimental data. This indicates whether the cellulose-cellulase adsorption for the BMSW-biomass follows the similar mechanisms as first generation biomass. It extends the understanding of

the theoretical model by developing new parameters for data fitting. The role of substrate features during cellulose-cellulase adsorption is also investigated. This provides understanding on how the substrate features affect and interplay during the adsorption process. Finally, a regression model is developed based on the experimental data obtained from this work in order to simulate the effects of substrate features on enzyme adsorption.

Chapter 10, after a brief reminder of the research aims, provides a comprehensive summary of the research work undertaken during this doctoral research training at UCL. It highlights the main empirical results obtained from the author, and the scientific conclusions that have been drawn by the author from this empirical work. Finally, it contains a few suggestions for future works in this promising area, with the aim of encouraging new exciting research work.

Chapter 2

Biomass resources analysis

2.1 Introduction

Biomass refers to biological material that can be used as fuel or for industrial production. Most commonly, biomass refers to plant matter grown for use as biofuel; it also includes biodegradable wastes that can be burnt as fuel. It excludes organic material which has been transformed by geological processes into substances such as coal or petroleum.

Using biomass as an energy source creates a 'closed carbon cycle'. This is because when biomass energy source grows, CO_2 is absorbed from the atmosphere, and when it is burnt the CO_2 stored by the biomass is released. Hence, the use of biomass as an energy source has huge potential to reduce the climate change problems caused by greenhouse gas emissions. Biomass can be used via different technologies for different products. With the interest of this work in bioethanol, the biomass analysed below refers to suitable sources for ethanol feedstock

The purpose of this chapter is to analyse the potential of using MSW as biomass source for ethanol production. For this purpose, the analysis starts from the emerging UK biofuel industry resulting in the large demand of biomass, followed by a review of current biomass situation and potentials. With the lignocellulosic materials' structures and properties, attentions are brought on understanding the technical and economic barriers of ethanol production as an alternative biofuel. Finally, biodegradable municipal solid waste, as potential biomass is analysed with an evaluation of the availability and quality of BMSW. The quantities of ethanol from BMSW are also estimated by considering London as a case study.

2.2 The emerging UK biofuel industry

According to European commission (EC, 2007) about 98% of the EU transport sector is dependent on oil. As supply increases to meet rising demand it is only a matter of time before global oil production reaches its geologically defined peak and goes into irreversible decline (Aleklett and Campbell, 2004). However, it is uncertain when the "Peak Oil" will occur, or the subsequent rate of decline in supply. To many, it is believed to be occurred in the next 10 to 15 years (POTF, 2007), and thus a 20 year "crash program" of mitigation measures is required to minimise economic costs (Hirsch *et al.*, 2005). A range of above ground factors compounds declining supply capacity. The International Energy Agency (IEA) anticipates increasing tight market beyond 2010 (MTOMR, 2007). The UK North Sea Oil peaked in 1999 and its high annual production decline rates caused the UK to become a net oil importer in 2006. As reliance on foreign oil increases the energy security of the UK is becoming a major political and economic priority.

Climate Change is widely accepted as the greatest environmental challenge facing the world today. The scientific consensus is that most of the observed increases in globally averaged temperatures since the mid-20th century are very likely due to the increase in anthropogenic (human) greenhouse gas (GHG) concentrations (IPCC, 2007). The UK's Climate Change Programme sets out a range of measures to ensure the UK delivers its legally binding target under the Kyoto Protocol to reduce greenhouse gases emissions to 12.5% below 1990 levels by 2008-2012, and to move the UK towards its domestic goal of a 20% reduction below 1990 levels by 2010 (HMG, 2006). The transport sector accounted for about 24% of UK greenhouse gas emissions in 2002, making it the second largest source of UK end-user emissions (UNFCCC, 2007). Road transport is by far the largest contributor to transport emissions (Baggott *et al.*, 2007). The UK Government Energy 2003 White Paper indicates a 10% reduction in the transport sector compared to 1990 levels for the year 2020 is to be met by improved vehicle efficiency and biofuels (DTI, 2007).

The drivers for the introduction of renewable transportation fuels (RTFs) into the

UK are to: 1_ reduce transport sector dependency on non-renewable fuels; 2_ reduce GHG emissions from transportation fuel chains; 3_ reduce the impact on air quality and health for transportation use; 4_ improve energy security in the transport sector; and 5_ contribution to rural development through domestic production of biomass-based fuels (DTI, 2007). Moreover, the EU Biofuel Directive (2003/30/EC) requires from all EU Member States a minimum proportion of transport biofuels or other renewable fuels to be sold on their markets, with a target of 5.75% by 2010 and 10% by 2020 (DEFRA, 2007a). In 2008 the UK will introduce the Renewable Transport Fuels Obligation (RTFO) that will require suppliers of road transport fuels to source increasing percentages of fuel from renewable sources. This starts at 2.5% in 2008 and will increase to 5% by volume in 2010 (DTI, 2006).

As stated above, the European Union Directive (2003/30/EC) has setted a target of 5.75% ethanol mixture with gasoline by 2010. This could result in an increase in the European ethanol demand. The great growth in ethanol demand is expected with an estimation of the ethanol market being worth up to 18.9 billion litres by 2012, approximately 150% greater than the market in 2004 (as represented on Figure 2.1).

Presently, fossil fuels namely petroleum and natural gas dominate the EU fuel market, however, both are limited in supply. In the UK, the price of petroleum and natural gas is largely dependent on the price of oil, which is rising at an alarming rate and has reached highs of up to £5 per gallon.

Ethanol therefore is a very attractive alternative for use in fuel applications resulting in a decrease in the dependence on oil producing countries and trade deficit. Moreover, it can provide a more stable market on which to base future revenue and profit margin predictions allowing less risk on investment decisions and so provide many benefits.

Study (Lynd, 1996) has shown that the lignocellulosic-ethanol fuel cycle has a high thermodynamic efficiency and could provide positive environmental impacts. There are currently no commercial scale lignocellulosic-ethanol plants operating in the world; however, a small number of different first-of-their-kind commercial and

demonstration scale facilities are due to become operational within the next few years.

Figure 2.1 EU Ethanol Demand (DTI, 2006)

2.3 Current biomass situation

Biomass is a biologically renewable resource, from which ethanol is directly or indirectly produced. It can be derived from products, residues and wastes from agriculture, forestry and related industries, as well as from the biodegradable fraction of industrial, commercial and municipal wastes. Meeting the increasing demand of ethanol in the bio-fuel industry will therefore require large biomass resources.

Conventional, bioethanol, also known as "first generation", is produced sugar, starch or oil biomass feedstock mainly cultivated as dedicated energy crops. About 3.4 billion gallons of ethanol are generated annually from cane sugar in Brazil (DEFRA, 2007b), but at currently controlled levels, prices are too high for sugar to be a viable feedstock. Even in Brazil, cyclical world sugar prices result in widely fluctuating ethanol production, disrupting supplies and prices in the fuel market. In 1998, more than 1.3 billion gallons of fuel ethanol made from starch crops, mostly corn, were consumed in the United States (GLA, 2007a, b). However, competing

demands for corn, its greater value for food and feed, and limitations in coproduct uses are projected to limit the market to 3–5 billion gallons (GLA, 2007c). In addition, federal and state incentives are required, even at current production levels, to support ethanol use, and controversy continues to surround these subsidies even though such practices were common in the emergence of the oil industry from one dedicated to making kerosene for lighting homes to the production of a full slate of fuels and petrochemicals (DEFRA, 2007c). It is important to realise that the widespread use of corn ethanol has fostered an acceptance and infrastructure that is poised for and vital to major expansion in ethanol use.

Second generation ethanol is made from plentiful lignocellulosic materials such as forestry and agricultural residues, significant portions of municipal solid waste (e.g. paper waste and yard waste), and woody and grassy crops grown to support fuel production. Unlike food crops, these sources of biomass have not competing uses as they are often dumped in landfill site. The lignocellulosic materials also have the advantages such as source available locally and in large quantity. Hence, the second-generation bioethanol is expected to make a major impact on transportation fuel markets.

Several types of promising biomass are suggested as energy conversion feedstock, including wood, agricultural and forest product residues, municipal solid waste and industrial waste. Except for the production of corn-derived ethanol as a transportation fuel, there are few large-scale efforts to grow crops intensively for conversion to energy carriers. It has been proposed that fast growing species such as switchgrass or sugar cane would be an economical option for energy-dedicated crops (McLaughlin *et al.*, 2002). High quality woody feedstock such as hybrid poplar and pine has also been considered (Tharakan *et al.*, 2003). This feedstock could be combusted, gasified, or biologically digested, depending on the composition of the fuel and the desired energy carrier product. Many plant species are potentially viable energy feedstock and can be selected based on cost, net greenhouse gas emissions, and appropriateness to the intended energy conversion process and growing environment.

However, as explained in the next section, lignocellulosic biomass is a more

complex material that requires more complex technology for conversion. Bio-ethanol produced from lignocellulosic biomass, known as "second generation" biofuels, is not yet commercially viable.

2.4 Lignocellulosic biomass properties

2.4.1 Lignocellulosic Biomass composition

In order to improve existing or develop new energy conversion processes for biomass, it is important to understand the composition. Lignocellulosic or woody biomass is composed of carbohydrate polymers (cellulose and hemicellulose), lignin and a remaining smaller part (extractives, acids, salts and minerals). The cellulose and hemicellulose, which typically comprise two thirds of the dry mass, are polysaccharides that can be hydrolysed to sugars and eventually be fermented to ethanol. The lignin cannot be used for ethanol production. Table 2.1 provides the compositions of representative types of biomass fuel and the carbon content and higher heating value (HHV) of the components.

Table 2.1 Biomass composition and chemical properties (MTOMR, 2007)

Cellulose (40–60% of the dry biomass) is a linear polymer of cellobiose (glucose–glucose dimer). The orientation of the linkages and additional hydrogen bonding make the polymer rigid and difficult to break (Hamelinck *et al.*, 2005). In hydrolysis the polysaccharide is broken down to free sugar molecules by the addition of water. This is also called saccharification. The product, glucose, is a six-carbon sugar or hexose.

Hemicellulose (20–40%) consists of short highly branched chains of various sugars: xylose, arabinose, galactose, and mannose. It also contains smaller amounts of non-sugars such as acetyl groups (Lightfoot and Green, 2002). Due to its banched, hemicellulose is relatively easy to hydrolyse (Hamelinck *et al.*, 2005).

Lignin (10–25%) exists in all lignocellulosic biomass. Therefore, ethanol production process will have lignin as a residue. It is a large complex polymer of phenylpropane and methoxy groups, but a non-carbohydrate polyphenolic substance that encrusts the cell walls and cements the cells together. It is degradable by only few organisms, into higher value products such as organic acids, phenols and vanillin (Hamelinck *et al.*, 2005).

Via chemical processes valuable fuel additives may be produced. Although these by-products can significantly enhance the competitiveness of ethanol technology (Watson *et al.*, 2000), the current available study deploys lignin only for power generation. The combination of hemicellulose and lignin provides a protective sheath around the cellulose, which must be modified or removed before efficient hydrolysis of cellulose can occur, and the crystalline structure of cellulose makes it highly insoluble and resistant to attack. Therefore, to economically hydrolyse (hemi) cellulose, more advanced pre-treatment technologies are required than in processing sugar or starch crops. After the cellulose and hemicellulose have been saccharified, the remainder of the ethanol production process is similar to grain-ethanol. However, different sugars require different enzymes for fermentation. The costs of ethanol production are highly sensitive to the delivered feedstock cost and the operating scale. But, unlike for biofuels from gasified biomass, the biochemical biomass composition plays a very important role in process performance, since the feedstock influences the ethanol yield via its (hemi) cellulose and sugar composition. Hence, this work will investigate the influence of biomass composition and structure in both pre-treatment process and hydrolysis step. This should provide some deep understanding from the view of biomass in waste-to-ethanol process.

Lignocellulosic perennial crops (e.g. short rotation coppices and grasses) are promising feedstock because of high yields, low costs, good suitability for

low-quality land (which is more easily available for energy crops), and low environmental impact (Hamelinck *et al.*, 2005). Most ethanol conversion systems encountered in the literature have been based on a single feedstock. Nevertheless considering the hydrolysis fermentation process, it is possible to use multiple feedstock types. Table 2.2 presents biochemical compositions for several suitable feedstocks. Pine has the highest combined sugar content, implying the highest potential ethanol production. The lignin content for most feedstock is about 27%, but grasses contain significantly less, and may thus coproduce less electricity.

Table 2.2 Biochemical compositions for several suitable feedstocks (% dry basis) (Hamelinck et al., 2005)

Note: [a] Low molecular weight organic materials (aromatics, terpenes, alcohols), some of which may be toxic to ethanol fermenting organisms and cause deposits in some pre-treatment.

2.4.2 Physiochemical properties of cellulose

Cellulose is a linear condensation polymer consisting of D-anhydroglucopyranose joined together by D-1, 4-glycosidic bonds. Anhydrocellobiose is the repeating unit of cellulose, since adjacent anhydroglucose molecules are rotated 180 °C with respect to their neighbours (Fig. 2.2a). This rotation causes cellulose to be highly symmetrical, because each side of the chain has an equal number of hydroxyl groups. Coupling of adjacent cellulose molecules by hydrogen bonds and van der Waal's forces result in a parallel alignment and a crystalline structure. Cellulose exists as sheets of glucopyranose rings lying in a plane with successive sheets stacked on top of each other to form a three-dimensional particle. Because of this

arrangement, the surface of a cellulose particle has distinct "faces" that interact with the aqueous environment and cellulase enzymes. The six carbons in the glucopyranose ring and internal β-glucosidic bonds lie in the ab plane or "110" face, whereas the ac plane or 110 face consists of the edges of rings (see Fig. 2.2b). Additional faces present reducing and nonreducing ends, respectively. The repeating unit of the 110 face is the cellobiose lattice, which measures 1.04 nm along the axis of the cellulose molecule and 0.54 nm in the perpendicular direction. About 100 cellulose glucans are aggregated into elementary fibrils with a crystalline width of 4 – 5 nm (O'Sullivan, 1997), and bunches of elementary fibrils are embedded in a matrix of hemicellulose with a thickness of 7 – 30 nm. The lignification process occurs late in the process of synthesising natural fibers, so lignin is located primarily on the exterior of microfibrils where it covalently bonds to hemicellulose (Fig. 2.2c; Klein and Snodgrass, 1993).

The complex structures have important influences on hydrolysis process. Studies (Cowling and Kirk, 1976; Fan *et al.*, 1980; Zhu, 2006; Tatsumi *et al.*, 2006) have shown that there is relationship between structural features of cellulose and rates of enzymatic hydrolysis. Structural features of cellulose are commonly considered including crystallinity index, presence of lignin, and accessible area.

Figure 2.2 Physiochemical properties of cellulose: a. Structure of cellulose featuring repeating β-1, 4-linked anhydrocellubiose units; b. cellulose crystal (Mosier et al., 1999); c. Organisation of lignocellulose organization into elementary fibrils and microfibrils *(Klein and Snodgrass, 1993)*

2.4.2.1 Crystallinity index (CrI)

Crystallinity has often been thought of as providing an indication of substrate reactivity, and is prominently featured in the model of Wood (1975). The crystallinity of dried cellulose samples can be quantitatively measured from the wide-range X-ray diffraction pattern (Krassig, 1993). Although studies have been carried out with consideration of crystallinity index as a rate-limiting factor for enzymatic hydrolysis, it is still difficult to conclude at this time whether CrI is a key

determinant factor of the enzymatic hydrolysis rate (Lynd *et al.*, 2002; Mansfield *et al.*, 1999). Some studies (Enz *et al.*, 1990; Ohmine *et al.*, 1983; Puls and Wood, 1991) have found that crystallinity does not increase during enzymatic hydrolysis, which is in contrast with the finding from Fan *et al.* (1980) and Lee and Fan (1982) that crystallinity increases over the course of cellulose hydrolysis as a preferential reaction of amorphous cellulose (Betrabet and Paralikar, 1977; Ooshima *et al.*, 1983). Future studies should aim at studying the role of CrI in impacting hydrolysis.

2.4.2.2 Characteristics of pre-treated lignocellulose

Natural cellulose molecules occur in elementary fibrils closely associated with hemicellulose and other structural polysaccharides as well as lignin. Such lignocellulose typically contains cellulose (35–50 %), hemicellulose (20–35 %), and lignin (5–30 %) in dry weight basis (Chang *et al.*, 1981; Klein and Snodgrass, 1993; Lynd *et al.*, 2002; Mansfield *et al.*, 1999). Since enzymatic hydrolysis of native lignocellulose usually results in 20% solubilisation of the originally present glucan, some form of pre-treatment to increase amenability to enzymatic hydrolysis is included in most process concepts for biological conversion of lignocellulose. Pre-treatment, under appropriate conditions, retains nearly all of the cellulose present in the original material and allows close to theoretical yields upon enzymatic hydrolysis.

2.4.2.3 Presence of lignin

Hydrolysis of lignocellulosic biomass is more complicated than that of pure cellulose due to the presence of nonglucan components such as lignin and hemicellulose. Lignin removal or redistribution is thought to have a significant effect on observed rates of enzymatic hydrolysis (Chernoglazov *et al.*, 1988; Converse and Opekar, 1993; Lynd *et al.*, 2002). It has been implicated as a competitive cellulase adsorbent that reduces the amount of cellulase available to catalyse cellulose hydrolysis (Bernardez *et al.*, 1993; Ooshima *et al.*, 1990; Sutcliffe and Saddler, 1986). In addition, it has been suggested that lignin blocks the progress of cellulase down the cellulose chain (Eriksson *et al.*, 2002; Mansfield *et al.*, 1999).

2.5 Availability of suitable biomass and a potential source for bio-ethanol

Conventional biofuel technologies from sugar, starch and oil based biomass are already commercially available and are being expanded within EU Member States to meet the short-term directive targets. A major expansion in conventional energy crops from the agricultural sector might put "additional pressure on farmland and biodiversity as well as on soil and water resources" (EEA, 2006). In the long-term lignocellulosic perennial crops (e.g. short rotation coppices and grasses), from both the agricultural and forestry sectors, are considered to be a promising feedstock due to their high yields, low costs, good suitability for low-quality land, low environmental impact (Hamelinck *et al.*, 2005), and avoidance of conflict with land use for food and feed production (NILE, 2005).

Furthermore, agricultural residues can also be considered as an attractive feedstock due to their wide availability and potentially low cost. Residues can however be difficult and expensive to collect and transport, as they are seasonal in nature that limits availability or requires storage, and supply reliability is climate dependent. Competition also exists with other by-product end-uses, such as livestock feed, which could result in increasing food prices and create a dangerous relationship to the increasing oil price.

The European Environment Agency (EEA) published an assessment in 2006 on how much biomass can technically be available for energy production without increasing pressure on the environment. Projections for 2010, 2020, and 2030 (as shown in Figure 2.3) show that in the long-term energy crops from agriculture provide the largest potential but that in the short-term, the largest potential comes from the waste sector.

Figure 2.3 Future environmentally compatible bioenergy potential (in PJ) in the UK (EEA, 2006)

Larger portions of the residues and waste streams generated by agriculture or forest products could be utilised. Table 2.3 summarised the studies on waste biomass available in the literature.

Around 430 million tonnes of waste materials are produced annually in the UK (DEFRA, 2007d). The sources of waste vary considerably, as can be seen from Figure 2.4. A significant proportion, 40%, of all UK waste arisings consists of biodegradable material. A high proportion of biodegradable waste is currently sent to landfill at an increasing financial cost for the responsible public or private organisation and environmental cost for society and nature. In 2005, the waste sector contributed a 3.4% to the national total greenhouse gas inventory (Baggott *et al.*, 2007). These emissions are derived through transport, processing, treatment and degrading wastes in landfill.

The biodegradable material is concentrated within the agricultural, industrial, commercial, and municipal waste sectors as shown on Figure 2.5. Each of these sources presents opportunities for utilising the biomass resources for bioethanol production. MSW presents a unique opportunity for large-scale biofuel production, as it is highly concentrated within urban environments and far less seasonal in nature, providing a more reliable supply. Moreover, infrastructures and networks are already in place to collect and process MSW and present opportunities for integrating with new waste management options.

Table 2.3 Previous studies of conversion waste biomass into ethanol production

Type of waste	Treatment method	Efficiency (yield)			Source
		Glucose[3]	Ethanol[4]	Bioconversion[5]	
Agricultural waste					
Rice straw	Steam explosion, enzymatic saccharification	83%	NR[6]	NR[6]	(Moniruzzaman, 1996)
Sugar cane bagasse	Liquid hot water, SSF[1]	NR[6]	NR[6]	81-90%	(Laser *et al.*, 2002)
Corn stover	Dilute sulphuric acid, SSF[1]	NR[6]		80-87%	(Nilausen *et al.*, 2004)
Forest waste					
Olive mill solid residue	Enzymatic hydrolysis in a continuous membrane reactor	45%	NR[6]	NR[6]	(Mameri *et al.*, 2000)
Hybrid poplar	Hydrothermal pre-treatment, SSF[1]	60%	NR[6]	60%	(Blankenhorn *et al.*, 1984)
Municipal waste					
Recycled paper	Enzymatic hydrolysis, SSF[1]	43.8-47.9%	NR[6]	74.2-84.3%	(Ballesteros et al., 2002)
Used newspaper	ammonia-hydrogen peroxide solution, enzymatic hydrolysis	90%	NR[6]	NR[6]	(Kim and Moon, 2003)
Recycled paper sludge	SSF[(1)]	87%	NR[6]	72%	(Montesinos et al., 1995)
Municipal waste (organic fraction)	Enzymatic hydrolysis	>40%	NR[6]	NR[6]	(Clanet and Durand, 1998)
Mixed waste[7]	Dilute sulphuric acid hydrolysis, enzymatic hydrolysis, yeast fermentation	80-90%	80%	NR[6]	(Nguyen *et al.*, 1999)
Sorted MSW[2)]	MixAlco process	NR[6]	NR[6]	98%	(Holtzapple *et al.*, 1999)
MSW[2] from dumping sites	Dilute sulphuric acid, enzymatic hydrolysis, fermentation	NR[6]	21%	NR[6]	(Mtui and Nakamura, 2005)

Note: [1] SSF-Simultaneous Saccharification and Fermentation; [2] MSW-Municipal Solid Waste; [3] Glucose yield: ratio of glucose produced from hydrolysis to theoretical potential glucose; [4] Ethanol yield: ratio of ethanol produced from fermentation/SSF to theoretical potential ethanol; [5] Conversion yield: ratio of reducing cellulose content during the reaction to initial cellulose content before reaction; [6] NP: not reported; [7] Mixed waste: combination of construction lumber waste, almond tree prunings, wheat straw, office waste paper and newsprint

Life-cycle-based assessments of the major environmental impacts (or sustainability indicators) of MSW have shown the positive benefits to be gained from MSW energy recovery. These gains are in the form of (IEA, 2007):

1. Reduced greenhouse gas emissions;
2. Reduced acid gas emissions;
3. Reduced depletion of natural resources (fossil fuels and materials);
4. Reduced impact on water (leaching); and
5. Reduced land contamination.

2.6 Opportunities of using BMSW as a biomass source for bio-ethanol

2.6.1 MSW availability

2.6.1.1 Municipal solid waste

The definition of Municipal solid waste (MSW) varies between countries. In the UK, MSW is defined by Defra as "all household waste, plus commercial waste and recycling that is managed by the council, parks and gardens waste, non-household clinical waste, cleared fly tips and a number of other minor categories of waste managed by the council" (DEFRA, 2007f). MSW is highly heterogeneous, containing a wide range of materials that can be grouped into three broad categories:

Dry recyclables including inert matter, typically glass and metals, and stable organic matter such as plastics and other synthetic materials;
Biodegradable waste including readily biodegradable organic matter such as kitchen waste, food residues, paper cardboard, grass cuttings, tree clippings and other garden wastes, and slowly biodegradable organic matter such as wood;
Residual waste including a mixture of materials that can not be reused, recycled or composted, and typically consists of stones, sand, composite and contaminated materials.

The proportions of these categories vary between countries, regions and households but typically the biodegradable fraction of MSW, Biodegradable Municipal Solid Waste (BMSW), constitutes 40 to 70% of the whole MSW stream. In London it is around 68% (GLA, 2003). Only the biodegradable fraction of MSW can be converted to bioethanol and therefore it will be the focus of this work into London's waste resource.

Around 430 million tonnes of waste materials are produced annually in the UK (DEFRA, 2007e). Among of all of waste generated, about 73% goes to landfill and 19% to incineration. Only few (8%) are recycled as shown in Table 2.4. Although with the disposal, both the volume and weight can be reduced a lot, they also bring many environmental problems, such as green house emissions and landfill leachate.

Table 2.4 Various disposal methods for waste

Type of waste	Tonnes (million)	Landfill (%)	Recycled (%)	Incineration (%)	Other (%)
Municipal solid waste	4.4	73	8	19	0
Commercial/industrial	6.4	50	33	2	15
Construction/demolition	6.1	2	81	0	17
Special waste	0.4	66	7	1	26

2.6.1.2 Biodegradable municipal solid waste (BMSW)

As mentioned in Chapter 1, BMSW is the main focus of this work because it contains cellulosic compositions that are the source for ethanol products. In this work, BMSW is categorised into three groups: kitchen organic waste (KOW), green organic waste (GOW) and paper and card waste (PCW).

- KOW is the entire mixed composition of kitchen organic waste including uncooked fruits and vegetables, cooked fruits and vegetables, breads, tea-bags, eggs, cheese, cooked meat, uncooked meat and paper, as shown in Table 2.5.
- GOW is the entire mixed composition of green organic waste including grasses, leaves, prunnings and trimmings, and branches and stumps, as shown in Table 2.6.
- PCW is the entire mixed composition of paper & card waste including

newspapers, magazines, office paper, corrugated cardboard, non-recyclable paper, card and paper packaging, card non packaging, and liquid cartons, as shown in Table 2.7.

- Mixed Organic waste (MOW) is the entire mixed composition of kitchen and green organic waste.
- Mixed BMSW is assumed to be the biodegradable mixed composition of KOW, GOW and PCW wastes.

The waste types mentioned above are considered to be heterogeneous, both in physical and biochemical composition. Few studies have looked in detail at the material or biochemical composition of MSW in the UK (Burnley, 2007). In order to have a good reflection on the availability of the waste, this work is based on the available information of the waste in the UK as a whole, and uses London as a case study.

Table 2.5 Material composition of kitchen organic wastes (% wet-weight composition of individual materials)

Kitchen waste fractions	The University of Southampton and Greenfinch Ltd (USGL, 2007)	Merseyside (MWDA, 2006)	Essex (ECCSSBC, 2004)
Uncooked fruit & vegetables	60	54.6	46.2
Cooked fruit & vegetables	7		
Bread	7		
Tea bags	10		
Eggs	1	45.4	53.8
Cheese	1		
Cooked meat	12		
Uncooked meat	1		
Paper	1		

Table 2.6 Materials composition of green organic wastes (mean percentage wet-weight)

Green Waste Feedstocks	California (CPA, 2007)	United States (EPA, 2007)
Leaves	79.1	50
Grass		25
Prunnings and trimmings	15.9	25
Branches and stumps	5.1	

Table 2.7 Material composition of paper and card wastes (mean composition percentage wet-weight)

Paper & Card material categories	Merseyside (MWDA, 2006)	Essex (ECCSSBC, 2004)	Wales (Burnley, 2007)	GLA Model[a]	Estimate
Newspapers & magazines	35.2	53.1	42.7	38.6	40
Office paper	7.6	11.8	9.9	9.9	10
Corrugated cardboard	9.6	7.7	24.5	26.0	25
Non-recyclable paper	27.9	13.9			
Card and paper packaging	18.0	11.5	23.0	25.5	25
Card non packaging	-	1.2			
Liquid cartons	1.7	0.9			

a. *Calculated using (GLA, 2007a), and applying data from (National Statistics, 2007a)*

2.6.1.3 London's Waste Management Strategy

EU Landfill Directive has set mandatory targets for the reduction of Biodegradable Municipal Solid Waste (BMW) sent to landfill; the UK national targets are (DEFRA, 2007d):

- by 2010 to reduce BMW landfilled to 75% of that produced in 1995;
- by 2013 to reduce BMW landfilled to 50% of that produced in 1995; and
- by 2020 to reduce BMW landfilled to 35% of that produced in 1995.

Therefore, it is necessary to investigate an alternative way to dispose the waste generated.

Landfill capacity is limited and there are legal requirements to divert biodegradable waste from landfill. National targets stipulate the required proportion of household waste that must be recycled or composted and the total proportion of municipal waste from which value must be recovered. The Mayor's Municipal Waste Management Strategy presents a 'preferred' waste management option (GLA, 2003, option 5), consisting of a combination of front end recycling linked to a systemic and integrated technology mix designed to provide both flexibility of approach and certainty of supply capacity.

The core of the strategy is to:

- Exceed national targets, London wide MSW recycling to reach 45% by 2015;
- Retain incineration capacity at current rates; and
- Manage residues and capacity shortfall through a combination of mechanical biological treatment (67%), gasification and pyrolysis (22%), anaerobic digestion (11%), or other new and emerging technologies.

This option was projected against a 'central' waste growth scenario, of 3.5% between 2001 and 2004 followed by a 2% growth after that, as shown in Figure 2.6. However, growth actually declined between 2001/02 and 2005/06 in line with a national decline. The reasons behind annual fluctuations in municipal waste arisings are complex and could reflect a range of seasonal factors (GLA, 2003). The DEFRA states a longer term national trend is for waste growth with total municipal waste

increasing by 0.5 % per annum on average (DEFRA, 2007b). These differences clearly demonstrate the need for further research and consensus to enable effective waste management development. This study will assume the Mayor's 'central' waste growth scenario as the best estimate for future availability. Under this scenario, new treatment processes are expected to be operational from 2010, see Figure 2.5. The Mayor's strategy sets out that where waste cannot be reused, recycled or composted, value should be recovered in the form of new and emerging advanced conversion technologies or treatment methods, which may include the production of biofuel to be used in London (GLA, 2003).

Figure 2.6 Estimated capacities of waste management facilities for Option Five, Strategy Report Recycling and Balanced Technology Mix at 'central growth rate', adapted from (GLA, 2003)

2.6.1.4 Estimating feedstock availability

An understanding of MSW at four different scales is required in order to understand its availability and quality as a bioethanol feedstock as explained in Figure 2.7.

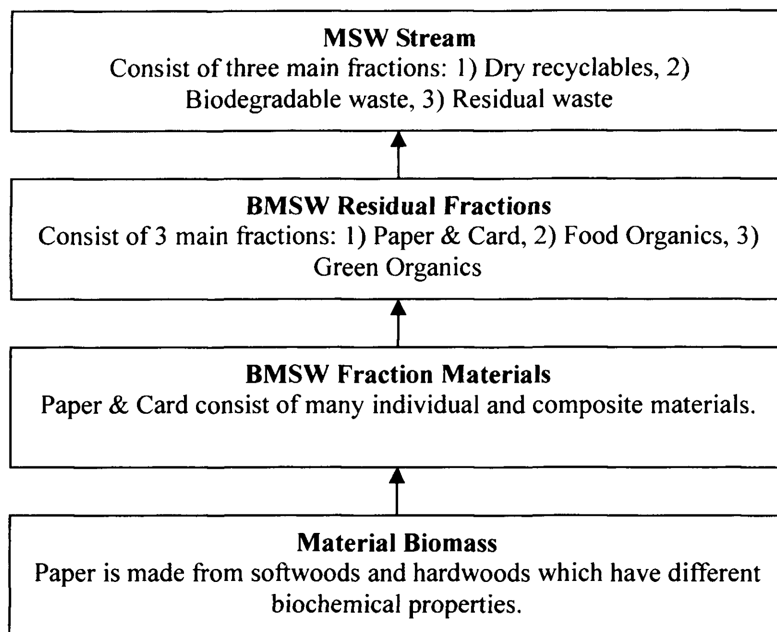


Figure 2.7 Hierarchy of MSW compositional data

The biodegradable fraction is itself a composition of many materials that can be classified into three main fractions: Paper and Card (PCW), Kitchen Organics (KOW), and Green Organics (GOW), which account of an estimated 54.5% in England and 57.4% in London of the total wet weight of all MSW arisings (Table 2.8). Green organics are estimated to be significantly lower in London compared to the rest of the UK and the Greater London Authority (GLA) Waste Scoping Study (GLA, 2007a) concluded that this is due to a smaller number of people per household and a higher proportion of properties with little or no garden. Other, smaller fractions that will not be evaluated in this study are wood, fines and textiles. Composition data for England is considered of limited and poor quality and a comprehensive government survey is expected to improve this situation in the near term (DEFRA, 2007e). Currently the best data are from DEFRA (DEFRA, 2007e), who has consolidated estimates from a number of studies (ranging from 2002 to 2005) and made a number of assumptions regarding less well surveyed sub-streams to form an overall estimate for the BMSW fractions. These are presented in Table 2.8. A project supported by the GLA investigated how factors affecting waste composition may influence the waste composition in London and developed a model that enables an approximation of household waste composition by ethnicity and type of property (GLA, 2007b). This model has been used to estimate the BMSW composition of London as shown in Table 2.8. Based on this estimated proportion of BMSW fractions within London,

Table 2.9 presents a projected estimate of the available arisings for recovery options after recycling and composting targets have been met.

A number of assumptions have been made to form this projection; two important ones are:

- The composition of MSW waste arisings remains constant; and
- The composition of materials sent to recycling or composting increases linearly. This is an area of risk as the recycling and composting of PCW is more mature than KOW and GOW. The latter is likely to increase at a greater rate through collection system and infrastructure improvements.

This assessment must be considered rough (+/-50%), however it provides some important insights into the quantity and type of waste resources that new and emerging technologies could utilise. From Figure 2.8 it can be seen that the availability of BMSW is likely to decrease until 2015 as the recycling and composting target increases from current levels of 10.3% to 35%. This decrease may affect PCW more than KOW and GOW unless the recovery rate for individual fractions changes. After 2015 all waste fractions increase in availability as the increased recycling and composting target of 45% is counteracted by expected increases in total MSW. KOW is projected to be the main feedstock resource by 2020, accounting for 37% of waste available for new recovery technologies.

Table 2.8 Estimated proportions of MSW fractional arisings (% non-dry weight)

Waste Fraction	England Arisings Proportion of MSW steam[a]	London 2003/04 MSW arisings[b]	London Waste Streams			
			Household waste[c]	All Recycling[d]	Civic amenity sites[e]	Other waste streams[f]
PCW	18.0	23.6	27.8	36.0	2.0	24-38
KOW	17.2	26.1	26.5[g]	12.4[g]	0.0	16-22
GOW	19.2	7.7	7.8[g]	4.2[g]	8.0	2-16
BMSW	54.5	57.4	62.1	52.0	10.0	-

a. Based on (DEFRA, 2007e, Table A1.24]
b. Calculated using (GLA, 2007c), and applying data from (National Statistics, 2007a) Table 1.9 excluding 'mixed' the ethnicity equates to 12.8% Asian and 10.9% Black. Applying data from (National Statistics, 2007b) counting all London entries for 'Flat; maisonette or apartment' equates to 48.8%.
c. Based on (GLA, 2007a) Table 4 Average Wt%.
d. Based on (DEFRA, 2004) Table 4.
e. Based on (GLA, 2007a) Table 8
f. Based on (GLA, 2007a) guesstimate from section 2.3 'other waste streams' survey results.
g. Adapted to separate KOW and GOW values based on the estimated proportion for total arisings. From 'London 2003/04 MSW arisings' KOW+GOW = 33.8%, of which KOW = (100/33.8)*26.1=77.2%, and GOW = (100/33.8)*7.7=22.8%. Therefore the adjusted values for 'Household waste' are: KOW = (34.3/100)*77.2= 26.5% and GOW = (34.3/100)*22.8=7.8%. The adjusted values for 'All recycling' are: KOW = (16/100)*77.2=12.4% and GOW = (16/100)*26.5=4.2%.

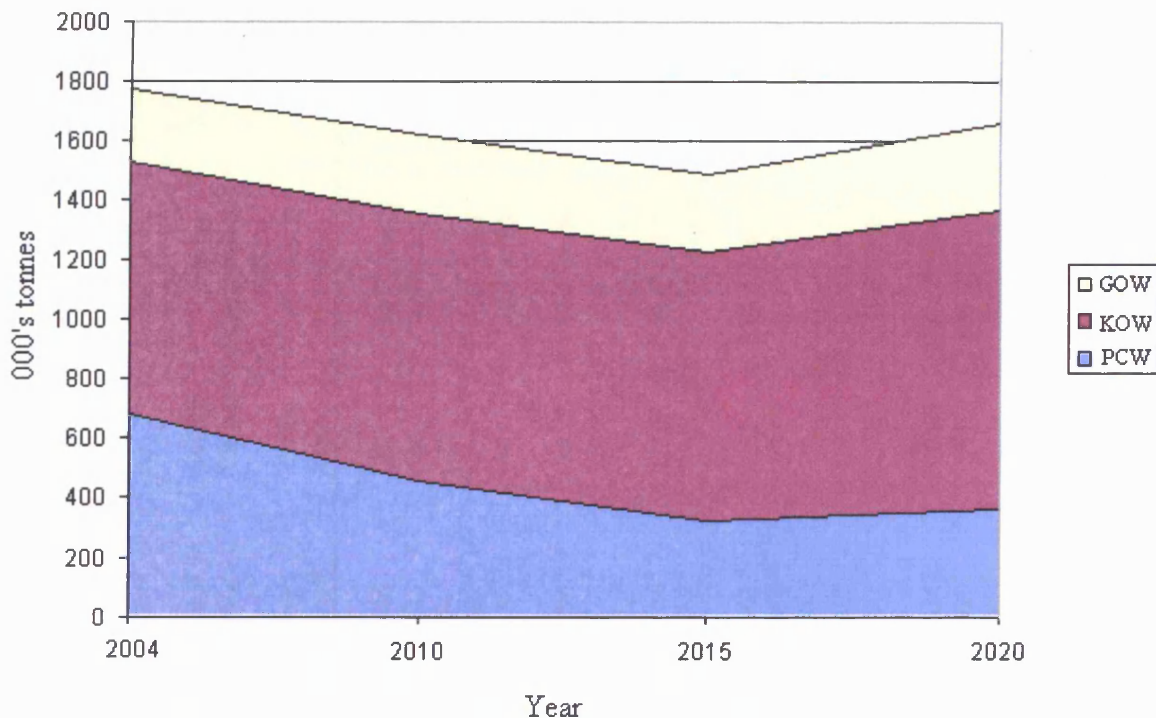


Figure 2.8 Available waste fractions, trends for new recovery technologies

Table 2.9 Projected estimates for BMSW arisings and management routes

Waste Fraction	Total MSW arisings (000's tonnes)	Recycling / Composting: Available Arisings ('000s tonnes)	Recycling / Composting: Proportion of arisings (proportion of stream) (% non-dry weight)	Recovery or Disposal: Available Arisings[f] ('000s tonnes)	Recovery or Disposal: Proportion of arisings (proportion of stream)[g] (% non-dry weight)	Incineration: Available Arisings ('000s tonnes)	Incineration: Proportion of arisings (proportion of stream) (% non-dry weight)	New Recovery Options or Disposal: Available Arisings (000s tonnes)	New Recovery Options or Disposal: Proportion of arisings (proportion of stream) (% non-dry weight)
2003/04									
PCW	1,025 [c]	161 [b]	15.7[b] (36.2)[k]	864	84.3 (22.2)	184 [h]	18.0[h] (22.2)[k]	682 [h]	66.5[h] (22.2)[k]
KOW	1,133 [c]	56 [b]	4.9[b] (12.6)[k]	1,077	95.1 (27.6)	228 [h]	20.1[h] (27.6)[k]	847 [h]	74.8[h] (27.6)[k]
GOW	334 [c]	17 [b]	5.1[b] (3.8)[k]	317	94.9 (8.1)	67 [h]	20.1[h] (8.1)[k]	249 [h]	74.6[h] (8.1)[k]
BMSW	2,492 [c]	234 [b]	9.4[b] (52.6)[k]	2,258	90.6 (57.9)	479 [h]	19.2[h] (57.9)[k]	1,778 [h]	71.3[h] (57.9)[k]
Total MSW	4,342 [d]	445 [e]	10.3[e] (100)[k]	3,897	89.7 (100)	827 [e]	19.1[e] (100)	3,070	70.6 (100)
2010									
PCW	1,294 [c]	691 [j]	53.4[i] (36.0)[k]	603	46.6 (16.9)	147 [h]	11.4[h] (16.9)[k]	456 [h]	35.2[h] (16.9)[k]
KOW	1,435 [c]	244 [j]	17.0[i] (12.7)[k]	1,191	83.0 (33.4)	291 [h]	20.3[h] (33.4)[k]	901 [h]	62.8[h] (33.4)[k]
GOW	422 [c]	73 [j]	17.3[i] (3.8)[k]	349	82.7 (9.8)	85 [h]	20.1[h] (9.8)[k]	264 [h]	62.6[h] (9.8)[k]
BMSW	3,151 [c]	1,005 [j]	31.9[i] (52.3)[k]	2,146	68.1 (60.1)	523 [h]	16.6[h] (60.1)[k]	1,621 [h]	51.4[h] (60.1)[k]
Total MSW	5,488 [a]	1,921 [a]	35.0[a] (100)	3,567	65.0 (100)	870 [a]	15.9[a] (100)	2,697	49.1 (100)
2015									
PCW	1,403 [c]	963 [j]	68.6[i] (36.0)[k]	440	31.4 (13.4)	116 [h]	8.3[h] (13.4)[k]	323 [h]	23.0[h] (13.4)[k]
KOW	1,556 [c]	333 [j]	21.4[i] (12.4)[k]	1,223	78.6 (37.4)	322 [h]	20.7[h] (37.4)[k]	902 [h]	58.0[h] (37.4)[k]
GOW	458 [c]	102 [j]	22.3[i] (3.8)[k]	356	77.7 (10.9)	94 [h]	20.5[h] (10.9)[k]	263 [h]	57.4[h] (10.9)[k]
BMSW	3,417 [c]	1,404 [j]	41.1[i] (52.4)[k]	2,013	58.9 (61.5)	530 [h]	15.5[h] (61.5)[k]	1,483 [h]	43.4[h] (61.5)[k]
Total MSW	5,952 [a]	2,678 [a]	45.0[a] (100)[k]	3,274	55.0 (100)	862 [a]	14.5[a] (100)	2,412	40.5 (100)
2020									
PCW	1,524 [c]	1,047 [j]	68.7[i] (36.0)[k]	477	31.3 (13.4)	116 [h]	7.6[h] (13.4)[k]	361 [h]	23.7[h] (13.4)[k]
KOW	1,690 [c]	361 [j]	21.4[i] (12.4)[k]	1,329	78.6 (37.4)	322 [h]	19.1[h] (37.4)[k]	1,008 [h]	59.6[h] (37.4)[k]
GOW	497 [c]	111 [j]	22.3[i] (3.8)[k]	386	77.7 (10.9)	94 [h]	18.9[h] (10.9)[k]	294 [h]	59.2[h] (10.9)[k]
BMSW	3,711 [c]	1,524 [j]	41.1[i] (52.4)[k]	2,187	58.9 (61.5)	530 [h]	14.3[h] (61.5)[k]	1,657 [h]	44.7[h] (61.5)[k]
Total MSW	6,465 [a]	2,909 [a]	45.0[a] (100)	3,556	55.0 (100)	862 [a]	13.3[a] (100)	2,694	41.7 (100)

Notes for Table 2.9

a. Based on estimates from (GLA, 2003) Figure 22.
b. Based on (DEFRA, 2004) Table 4. Organics that account for 73 tonnes consist of KOW+GOW; have been adjusted to separate KOW and GOW into two values. Based on the estimated proportional calculation from Table 2 note 'g': KOW = 77.2% and GOW = 22.8% of the organic fraction. Therefore KOW = 73*0.772 = 56.4, and GOW = 73*0.228 = 16.6.
c. Based on Table 2 'London 2003/04 MSW arisings' (% non-dry weight).
Assuming upper recycling/composting limits: PCW = 85%, KOW = 75%, GOW = 90% (28) Table B1.2
d. Based on (DEFRA, 2005) Table 1, London 2003/04, Total municipal waste.
e. Based on (DEFRA, 2005) Table 2, London 2003/04, Incineration with EfW + Incineration without EfW.
f. Waste not recycled or composted is available for current recovery options (incineration), new recovery options, or landfill.
g. The proportion of waste available for recovery options (incineration), new recovery options, or landfill. In parenthesis is the proportional composition of this available waste. This shows how the composition of the MSW stream is modified by the recycling and recovery levels for different waste fractions.
h. The breakdown of arisings sent to 'incineration' or 'new recovery options' is calculated first by applying the proportion of the individual waste fraction to the estimated total arisings for the particular management route. I.e. for PCW available for incineration 2003/04, total arisings are 827 thousand tonnes, proportion of PCW within the 'recovery or disposal' waste stream is 22.2% (see note g), therefore the arisings of PCW available for incineration = 827*0.222 = 184 thousand tonnes. The proportion of PCW arisings managed by incineration can then be calculated based on the total MSW stream arisings of PCW: (100/1025)*184 = 17.95%. The same methodology is applied to the arisings and proportional breakdown for all other fractions, and for 'new recovery options'.
i. Proportion of waste sent to recycling is assumed to increase linearly from 2003/04 across all fractions. I.e. for 2010 KOW = (35/10.3)*5=17%.
j. The quantity of arisings is calculated based on the proportion from note 'i' and the projected fractional composition of MSW (based on note 'c'). E.g. for KOW: proportion of recycling and composting for KOW fraction equals 17.0% and the projected arisings of KOW waste equals 1,435 thousand tonnes, therefore the projected arisings sent to recycling and composting = 1,435*0.17=243.95 thousand tonnes.
k. The proportion of waste fractions available within a particular waste management route is calculated by taking the proportion of the arising of a particular waste fraction (e.g. PCW) from the total MSW arisings for the particular waste management route.

2.6.2 BMSW biomass quality

2.6.2.1 Introduction

From Section 2.6.1.4, it is obvious that 57.4 % of MSW in London are biodegradable. Among these, 23.6% are from PCW, 26.1% are from KOW and about 7.7 % from GOW. With this large quantity of BMSW waste (1.8 millions tonnes) available, this section investigates the potential quality of BMSW as biomass sources by looking at three categories that have been defined in Section 2.6.1.2.

Kitchen Organics waster (KOW)

Table 2.5 shows that more than 60% of KOW are uncooked fruits and vegetables (USGL, 2007); many of them are potato and carrot peelings.

Green Organics waste (GOW)

About 79% of green waste is estimated to be grass as shown in Table 2.6. Therefore, as a major portion of GOW, grass is chosen for experimental study in this work.

Paper & Card waste (PCW)

Detailed composition of PCW has been shown in Table 2.7. Based on the current available information, more than 50% of PCW are newspaper and office scrap paper. Hence, these two types of paper are selected as representatives of PCW.

Mixed BMSW: is assumed to be a mixed composition of PCW, KOW, and GOW, that is, 20% of each type pf selected waste (newspaper, scrap paper, carrot peelings, potato peelings, and grass) in this work.

These are heterogeneous feedstocks, both in physical and biochemical composition. Few studies have looked in detail at the material or biochemical composition of MSW in the UK (Burnley, 2007). The following brings together this limited research to provide the best available information on the potential for bioethanol production and considerations for conversion systems.

2.6.2.2 Paper & card waste (PCW)

From Table 2.8, PCW accounts for approximately 23.6% of all MSW arisings in London. Paper included in a mixed domestic collection system is unacceptable for papermaking due to the high risk of contamination even when the paper is bagged separately, but collected with the general waste (Woodland Trust, 2000). Before going to paper mills, paper must be segregated into various grades by a Materials Recovery Facility (MRF) that also removes contaminants such as glass, staples, paperclips, adhesives, etc (Woodland Trust, 2000). However, even after this cleaning process, there can remain both physical and chemical contamination. Contaminants of similar density to cellulose fibers may not be identified and extracted, including: shards of glass, metals, plastics, adhesives, grease (CPA, 2007).

The current collection system presents barriers to higher PCW recycling rates and possibly high rates of reject that can be used for other recovery options. BS EN 643 is the UK version of the European Standard EN 643, which defines grades and combinations of acceptable types of recovered paper (CPA, 2007). As paper recovery from MSW increases the UK paper industry are likely to specify this standard in new contracts and will be increasingly unwilling to accept recovered paper from co-mingled sources (CPA, 2007). Therefore the collection and source segregation systems are likely to become more advanced over time with aspirations for achieving the estimated upper recycling limit of 85% (DEFRA, 2007e).

Table 2.10 presents the results of research findings into the biochemical composition of different PCW. Most PCW are chemically processed to remove lignin from mixtures of hardwoods and softwoods. There are exceptions to this, most notably newsprint that is primarily mechanically processed spruce and pine, and therefore has the same composition on a dry-weight basis as native wood (Wyman, 1996). The pulping process alters the biomass structure of paper and card and a number of sources indicate that this could improve the conversion process and reduce the need for pre-treatment (Rivers and Emert, 1988; Dale, 2004; Clanet *et al.*, 1998). Enzymatic hydrolysis of separated paper has been shown to convert quickly and nearly completely to bioethanol and the lower levels of five carbon sugars reduce the dependence on higher hemicellulose hydrolysis efficiency (Dale and

Musgrove, 2004). Different pre-treatment methods have been shown to improve the conversion process for different PC materials and an optimal process would have to be developed based on site-specific samples (Rivers and Emert, 1988).

Taking into consideration the quality of paper for recycling, three PC waste feedstocks can be broadly identified and their biochemical composition and theoretical ethanol yield estimated from Table 2.11. Moisture content of urban segregated waste paper has been estimated to be 5% (CIWMB, 2004). Optimum PC is assumed to be segregated high quality paper such as white office paper that has a higher proportion of polysaccharides and low proportion of lignin. Low demand PC is assumed to be segregated corrugated cardboard which is towards the bottom of the hierarchy of recovered paper (Paperchain, 2007). The targeting of low-grade waste paper for ethanol production is a strategy currently being pursued by Universal Entech and Bio-Process Innovation (Dale and Musgrove, 2004). Mixed PC is assumed to be at the bottom of the hierarchy of recovered paper (Paperchain, 2007). The prospects for recycling mixed paper are more limited, making it potentially available for other recovery options; however consideration of contamination issues would be required.

Table 2.10 Biochemical composition of paper & card waste materials (percentage mean dry weight)

No.	Material Fraction	Cellulose	Hemicellulose				Lignin	Ash
		Glucan	Galactan	Mannan	Xylan	Arabinan		
1	Newsprint[3]	54.7		30.1			14.2	1.0
2	Newsprint[4]	48.5		9.0			23.9	-
3	Newsprint[7]	64.4	0.0	16.6	4.6	0.5	21.0	0.4
4	Newspaper[5]	44.3	0.6	4.9	5.2	0.6	29.3	3.5
5	Newspaper[8]	35.1	2.3	10.7	5.0	3.9	39.1	1.0
6	Newsprint[2]	63.8	0.6	5.0	5.3	0.6	21.3	3.5
7	Coated Paper[4]	42.3		9.4			15.0	-
8	Coated Paper[5]	46.8	0	2.3	7.0	0.7	19	24.1
9	Paper[6]	33.8		12.9			12.2	12.5
10	Office Paper[4]	87.4		8.4			2.3	-
11	White Office Paper[8]	65.4	0.0	0.0	14.4	0.0	9.5	14.0
12	White Office Paper[1]	71.7		16.4			-	-
13	Un-coated Free Sheet[5]	74.9	0.3	2.7	8.9	0	5.3	7.7
14	Packaging Paper[5]	66.2	0.6	3.2	6.6	0.6	15.6	0.7
15	Corrugated Cardboard[9]			75			-	-
16	Corrugated Cardboard[3]	73.5		13.7			11.8	1.0
17	Corrugated Cardboard[4]	57.3		9.9			20.8	-

Note: 1. Capek-Menard *et al.*, 1992; 2. Kemppainen and Shonnardm, 2005; 3. Rivers and Emert, 1988; 4. EPA, 2005; 5. CEC, 1999; 6. Eklind and Kirchmann, 2000; 7. Lee , 1997; 8. Foyle *et al.*, 2007; 9. Kadar *et al.*, 2004.

Table 2.11 Biochemical composition of potential paper & card waste feedstocks

PCW feedstocks	% wet weight				% moisture content	PCW (% wet weight)	Theoretical Ethanol yield (L/dry tonne)*
	Cellulose	Hemicellulose	Lignin	Ash			
Optimum [a]	72.3	12.7	7.8	12.8	5	10	584
Low Demand[b]	68.8	12.4	17.2	1.1	5	25	559
Mixed [c]	59.1	14.3	18.6	5.8	5	100	505

*calculated using (DOE, 2007)

a. Calculated by averaging values from case study numbers 10-15 from Table 8.
b. Calculated by averaging values from case study numbers 18-19 from Table 8.
c. Calculated by applying the estimated material composition from Table 6 to the following averaged chemical compositions of Table 8. Newspaper & magazine = numbers 1-9; Office paper = numbers 10-15; corrugated cardboard = numbers 18-19; others = numbers 10 and 16.

2.6.2.3 Kitchen Organic Waste (KOW)

KOW waste accounts for approximately 26% of all MSW arising in London as presented in Table 2.8. Fruit and vegetables (cooked and uncooked) constitute the majority of KOW. Table 2.12 presents the range of KOW materials and their chemical composition and combines the weighting system to arrive at a final mixed KOW composition. The theoretical yield from this composition is 363 L/dry tonnes (DOE, 2007).

Fruits and vegetables contain lower levels of cellulose and lignin than wood; however there is a significant difference in composition between the pulp and the peelings of fruit and vegetables. The peelings contain higher proportions of lignin and lower proportions of carbohydrates. New research by Waste and Resources Action Programme (WRAP) has revealed that about half of the food thrown away by households in the UK is edible food, with the rest comprising of peelings, meat bones etc (WRAP, 2007). Therefore since KOW may consist of either the peelings or whole fruit or vegetable and this has been considered in Table 2.12.

A small proportion, approximately 2 %, of KOW will be of non-putrescible material, usually consisting of the plastics bags used to contain the waste (USGL, 2007). In general, meat contains no carbohydrates unless other carbohydrate-containing ingredients are added. As meat, cooked and uncooked, could constitute a significant

proportion 13% of KOW (USGL, 2007)). This should be taken into account along with contamination from non-putrescible materials. Therefore an estimated 14% of KOW by wet weight will be assumed to be non-biodegradable.

Most fresh vegetables and fruit are high in water content, generally greater than 70%, and frequently greater than 85% (Dimambro *et al.*, 2006). The moisture content has been estimated at about 70% for mixed KOW.

Table 2.12 Biochemical composition of kitchen organic waste materials (% wet weight)

Kitchen Organic waste materials	Mono-saccharides (%)	Di-saccharides (%)	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Ash (%)	Moisture Content (%)	KOW waste proportion (%)
Carrot, raw [a]	40.8	12.2	3.9	1.3	23.9	8.3	88.3	0.3
Nuts, mixed nuts, oil roasted, with peanuts, without salt added [a]	0	0	8.8	2.9	10.1	3.5	2	1.8
Orange peel, raw [a]	0	0	39.3	13.1	38.6	2.9	72.5	1.8
Beans, snap, green, raw [a]	14.4	0	18	6	34.9	6.8	90.3	2.4
Peas, green, raw [a]	26.9	0	33.3	11.1	24.2	4.1	78.9	2.4
Tomatoes, red, ripe, raw, year round average [a]	47.8	0	1.2	0.4	21.8	9.1	94.5	4.2
Potatoes, white, flesh and skin, raw [a]	6.2	73.3	0	0	13	5.1	81.6	4.5
Orange, raw, with peel [a]	20.2	20.2	15.4	5.1	25.4	3.4	82.3	5.4
Lettuce, green leaf, raw [a]	15.8	0	10.8	3.6	26.4	12.6	95.1	8.4
Banana Whole [a]	48.9	21.52	7.9	2.6	10.4	3.3	74.9	12.3
Apple with skin [a]	72	0.4	5	1.7	16.6	1.3	85.6	13.5
Potatoes, white, flesh and skin, baked [a]	5.7	73.2	0	0	8.5	5.1	75.4	0.7
Tomatoes, red, ripe, cooked [a]	44	0	10.9	3.6	12.4	10.6	94.34	0.2
Biscuits, plain or buttermilk, prepared from recipe [a]	3.1	0	43.3	14.4	2.1	4.5	28.9	0.2
Crackers, standard snack-type, regular [a]	1.8	0	44.5	14.8	1.6	2.9	3.5	0.2
Cake, fruitcake, commercially prepared [a]	40	0	28.2	9.4	5	1.3	25.3	0.2
Cake, sponge, commercially prepared [a]	52.1	0	25.5	8.5	0.7	1.7	29.7	0.3
Potatoes, french fried, all types, salt not added in processing, frozen, oven-heated [a]	0.7	52.3	11.1	3.7	6.8	4.9	63.1	0.3
Potato chips, plain, salted [a]	0.4	22.95	17.2	5.7	4.5	4	2.3	0.4
Carrots, cooked, boiled, drained, with salt [a]	35.1	1.7	12.2	4.1	30.5	6.8	90.2	0.9
Beans, baked, canned, plain or vegetarian [a]	28.4	26.3	4.6	1.5	14.6	6.3	72	0.4
Cereals ready-to-eat, KASHI 7 Whole Grain Flakes by KELLOGG [a]	8.9	32.2	24.2	8.1	11.8	2	3.4	1.6
Rice, white, long-grain, regular, cooked, enriched, with salt [a]	0.2	43.95	33	11	1.3	1.3	68.4	0.6
Pasta, fresh-refrigerated, plain, cooked [a]	0	39.7	29.7	9.9	0	1	68.6	0.5
Pizza, cheese topping, regular crust, frozen, cooked [a]	6.6	34.9	6.3	2.1	4.1	3.8	46.3	0.5
Bread, white, commercially prepared (includes soft bread crumbs) [a]	6.7	63.9	5.2	3.8	3.2		36.4	7.0
Tea leaves [b]	17.6	6	2.7	11.6	5.1	1.3	69.7	10.0

Table 2.13 (continued) Biochemical composition of kitchen organic waste materials (% wet weight)

Kitchen Organic waste materials	Mono-saccharides (%)	Di-saccharides (%)	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Ash (%)	Moisture Content (%)	KOW waste proportion (%)
Egg, whole, raw, fresh [a]	3.2	0	0	0	0	3.6	75.8	1.0
Cheese, cheddar [a]	0.8	0	0.9	0.3	0	6.2	36.8	1.0
Meat, bones and other non-biodegradable materials	-	-	-	-	-	-	65.0	14.0
Consolidated Kitchen Organic Waste (%)*	15.8	18.4	10.7	5.2	9.3	2.6	-	-

a. *Figures obtained from (USDA, 2007). Where the breakdown of values between mon- di- and poly- saccharide components is not know it is assumed to be in the ratio of 33/33/33, Where the breakdown of values between disaccharide and polysaccharide components is not know it is assumed to be in the ratio of 50/50, Where breakdown of values between polysaccharide components is not known, they have been assumed to be in the ratio of 75% cellulose and 25% hemicellulose.*

b. *C Figures obtained from (Ohkouchi and Inoue, 2007).*

**Calculated through multiplying each component value by the weighting score and taking the sum total for each component.*

***Calculated by taking the wet weight and converting it to dry weight by using the consolidated wet weight of 69.8%.*

2.6.2.4 Green Organic Waste (GOW)

GO waste accounts for approximately 8% of all MSW arisings in London as shown in Table 2.8. Table 2.13 presents a rough consolidated estimate for GOW based on the biochemical composition and proportion of its major fractions based on U.S. Figures (EPA, 1999). The higher lignin content of GOW implies the need for greater pre-treatment to separate the cellulose and hemicellulose components (Lissens *et al.*, 2004). However the lignin in grass is not as restrictive to microorganisms as the lignin in other components such as branches (EPA, 2005). Moisture content is estimated to be 17.7%; however this must be treated with caution as other studies have estimated 30% (CEC, 1999). The theoretical yield of about 420 L/dry tonne, derived using (DOE, 2007), is also much higher than that estimated by NREL of 192 ltr/dry tonne (OOE, 2000).

Table 2.13 Biochemical compositions of green organics waste materials (% wet weight)

GOW	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Ash (%)	Moisture Content (%)
Leaf [a]	16.7	11.5	47.7	unknown	8.3
Leaf [b]	17.8	23.1	26.1	33.6	8.3
Grass [a]	34.9	13.3	37.5	unknown	24
Branch [a]	39.3	20.4	36.2	unknown	10
Woody Yard Waste [c]	35.4	19.6	31.5	0.1	30
Hardwood [b]	28.4	17.9	14.5	0.4	5.4
Softwood [b]	35.1	17.1	18.6	0.8	6.5

a. *Figures from (EPA, 2007b) Table 1, assumed to be stated in dry weight basis. Figures for Leaf, Grass and Branch adjusted for wet weight basis using assumed moisture content of 8.3%, 24%, and 10% respectively.*

b. *Figures from (Eklind and Kirchmann, 2000) Table 4, adjusted for ash content then for moisture stated in Table 1.*

c. *Figures from (Lissens et al., 2004) adjusted for an assumed moisture content of 30%.*

2.7 Conclusions

The emerging biofuel industry demands large amount of biomass feedstock. Although commercial ethanol has been produced from primary biomass sources

such as corns, the global food crisis caused by the use of primary biomass has been increased. With The increase of bioethanol demand and the pressure of food supply security caused by first generation ethanol, the use of lignocellulosic materials, known as second-generation biomass, has been encouraged. However, its more complex structure requires further advance technology in order to be fully utilised. Hence, in order to make lignocellulosic ethanol competitive with the current fossil fuels, it is the time to investigate the conversion process of lignocellulosic feedstock.

MSW, as one of the promising biomass sources has the potential benefits of replacing primary biomass sources. By analysing the available national MSW data, about 60% of MSW generated is biodegradable and readily available in London. Moreover, the requirement of EU landfill directives and current national waste management strategy requires the alternative solution of BMSW disposal. Hence, using BMSW as biomass source for ethanol production, it will not only bring the economic benefits of fuel production but also prevent the pollution from waste to the environment.

This work is an attempt to classify the BMSW into three types: paper and card waste (PCW), kitchen organics waste (KOW), and green organics waste (GOW). Newspaper, scrap paper, potato peelings, carrot peelings, and grass as the representatives of different types of waste streams are selected for further experimental study. Based on the case study in London, the analysis of BMSW biochemical quality shows that KOW (accounting for 26% of total MSW) has a theoretical ethanol yield of 363 L/dry tonne; GOW (accounting for 8% of all MSW theoretically) can yield 420 L/dry tonne; and PWC (accounting for 23.6% of MSW generated) has a theoretical yield of 505 L/dry tonne.

With this analysis, it is obvious that the large amount of BMSW has the potential of becoming bioethanol sources. With the both potential economic viables and environmental benefits, further investigation is necessary.

Chapter 3

Ethanol conversion processes

3.1 Introduction

Typical ethanol process starts from pre-hydrolysis treatment process which is also called pre-treatment. Two alternative routes are being developed for producing bio-ethanol from lignocellulosic biomass: biochemical processes and thermochemical processes. Biochemical process consists of hydrolysis of the polysaccharides to their monomeric constituents (or their derivatives) and subsequent fermentation to bio-ethanol. Thermochemical process is via liquefaction (either direct or indirect) of biopolymers to basic elements that are then recombined into Synthetic Gas (SNG). SNG can subsequently be fermented to produce bio-ethanol. Thermochemical processes have advantages such as shorter resident time, generally a few days less than biochemical process depending on hydrolysis process; however, biochemical processes are estimated to be more cost-effective in the long-term, with the development of enzyme production (representing about 30% of the ethanol selling price) and future improvement of the process (accounting about 50% of the ethanol selling price), as reported by Hamelinck *et al.* (2005) for process scales within the 400 - 2000 MW. According to the techno-economic performance study by Hamelinck *et al.* (2005), the total capital investment per KW_{HHV} installed ethanol production may decrease from 2.1 k€ in short term up to 0.9 k€ in the long term; in this study, the short term system consists of the currently commercially available configurations with 400 MW_{HHV} biomass input by using dilute acid treatment followed by the Simultaneous Saccharification and Fermentation (SSF) process, while the long term system inclines toward the theoretical possibilities with 2000 MW_{HHV} biomass input by using liquid hot water

treatment followed by Consolidated BioProcessing (CBP). Further research is needed to understand the potential of biochemical processes; this will be the focus of this chapter.

A typical biochemical ethanol conversion process is shown in Figure 3.1. It includes pre-treatment, hydrolysis, fermentation, residual disposal and ethanol recovery. The following sections will review the current studies in the literature related to this process. More attention will be placed on the biomass ethanol technology that is based on enzymatic hydrolysis of cellulose, since the application of modern biotechnology offers the greatest potential for cost reductions that could make ethanol ultimately competitive with conventional fuels on a large scale without subsidies. The emphasis is on technologies, process steps, and configurations used in similar studies (DOE, 1993; Hinman *et al.*, 1992); although those selected are believed to be frontrunners, a variety of other options could prove equally or more cost effective with further development.

Studies on conversion of lignocellulosic materials to ethanol in the last twenty years are extensive (Dale *et al.*, 1982; Wright, 1998; Bjerre *et al.*, 1996; Duff and Murray, 1996). The conversion includes two processes: (i) hydrolysis of cellulose in the lignocellulosic materials to fermentable reducing sugars, and (ii) fermentation of the sugars to ethanol. The hydrolysis is usually catalysed with cellulase enzymes, and the fermentation is carried out with yeasts or bacteria. This chapter reviews the past and ongoing studies on bio-ethanol process, including pre-treatment technology, hydrolysis, fermentation, and process integration. With such a comprehensive review, the promising study areas in this field will be identified and hence the research questions will be identified and listed.

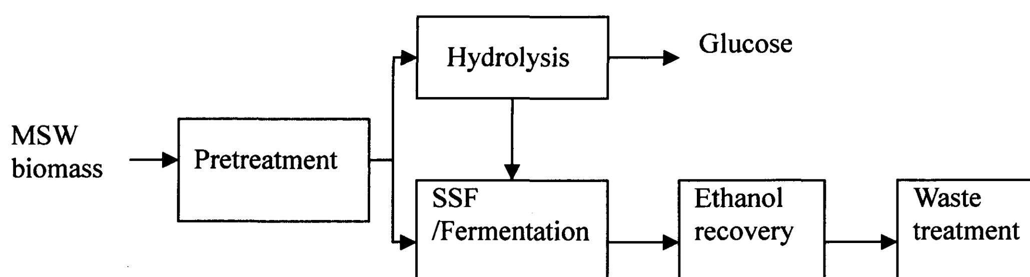


Figure 3.1 Bio-ethanol conversion processes

3.2 Pre-treatment

3.2.1 Introduction

Pre-treatment is used to alter the structure of cellulosic biomass to make it more accessible to the enzymatic conversion (Mosier et al., 2005). According to the evaluation study by Lynd (1996), pre-treatment is one of the most costly steps in cellulosic ethanol production, accounting for 33% of total processing costs (exclusive of feedstock) based on the National Renewable Energy Laboratory (NREL) design base-case. This base-case system uses a poplar energy crop as feedstock, assumed to cost $42 per delivered dry tonne, and an SSF-based process via yeast and cellulase production by *T. reesei*; it produces 60.1 million gallons ethanol from 658,000 dry tonnes feedstock per year with $150.3 million installed capital cost. There are several categories of pre-treatment methods: mechanical, physical, chemical, and biological. Physical pre-treatments, typically demand large amounts of energy and are expensive, employ purely mechanical means to reduce feedstock particle size, thus increasing surface area. Chemical pre-treatments use a variety of chemicals-acids, alkalis, organic solvents, oxidizing agents, supercritical fluids, and ligninase enzymes. Among these, dilute acid pre-treatment, ammonia fibre explosion (AFEX), and lime pre-treatment have emerged as particularly effective chemical methods. Hydrothermal pre-treatment refers to the use of water-as liquid or vapour or both-and heat to pre-treat biomass. The potential advantages of hydrothermal treatment include no requirement for purchased acid, for special non-corrosive reactor materials or for preliminary feedstock size reduction, and it produces much lower quantities of hydrolysate neutralization residues (Laser et al., 2002).

The purpose of the pre-treatment is to remove lignin and hemicellulose (with the common target of 80%), reduce cellulose crystallinity, and increase the porosity of the materials. In order to achieve certain pre-treatment efficiency of lignocellulosic materials (McMillan, 1994), pre-treatment must meet the following requirements: (1) improve the formation of sugars or the ability to subsequently form sugars by enzymatic hydrolysis; (2) avoid the degradation or loss of carbohydrate; (3) avoid

the formation of by-products inhibitory to the subsequent hydrolysis and fermentation processes; and (4) be cost-effective.

Pre-treatment is vital for the efficiency of all enzymatic configurations and typically, hydrolysis yields in the absence of pre-treatment are less than 20% of theoretical yields, whereas yields after pre-treatment often exceed 90% of theoretical. Chemical, physical, biological or combinations of pre-treatment are being developed; each has its advantages and disadvantages, as shown in Table 3.1.

Table 3.1 Comparison of pre-treatment processes

Feature	Dilute acid	Steam explosion	Ammonia Fibre Explosion (AFEX)	Liquid hot water
Reactive fibre	Yes	Yes	Yes	Yes
Particle size reduction required	Yes	No	No	No
Hydrolyzate inhibitory	Yes	Yes	No	Slightly
Pentose recovery	Moderate	Low	High	High
Low cost materials of construction	No	Yes	Yes	Yes
Production of process residues	Yes	No	No	No
Potential for process simplicity	Moderate	High	Moderate	High
Effectiveness at low moisture contents	Moderate	High	Very high	Not known

3.2.2 Mechanical Comminution

Mechanical comminution is a series of processes involving chipping, grinding, milling, and/or a combination of them employed to reduce the particle size of biomass. The particle size and biomass type have strongly influence on power requirements for comminution. With the decreasing particle size required, more power is needed. Also, the energy that is required for milling coarse biomass chips into fine particles strongly depends on the substrates. It has been shown that more than 25% of total energy requirement for substrate is needed spent on milling the material to particle size below 150 μm (Ballesteros *et al.*, 2000).

It has been observed that the reactivity of cellulose and external surface area can be

increased by milling processes, especially vibratory ball milling. Millett *et al.* (1976) also observed that complete digestion of milled biomass is possible after vibratory ball milling breaks down the crystallinity of cellulose to an extent. Fan *et al.* (1980) found that although the efficacy of ball milling is strongly dependent upon the material type being milled, a reduction in cellulose crystallinity is caused. It is now also known that the density of ball-milled material is higher than that of native material that results in a higher solids loading for ball-milled material.

Most of studies suggested that, in order to achieve efficient product yield, it is necessary to reduce the particle size less than 12mm (Negro *et al.*, 2003; Finkelstein and Davison, 1999; Ballesteros *et al.*, 2000). Hence, mechanical comminution can be considered as an essential step of the ethanol conversion process.

3.2.3 Physical treatment

3.2.3.1 Steam explosion

In the last decade, steam-explosion has come to be considered one of the most effective pre-treatment, characterized by low use of chemicals and low energy consumption (Excoffier *et al.*, 1991; Heitz *et al.*, 1991; De Bari *et al.*, 2002). It involves the treatment of material with saturated steam for a specified time and pressure, followed by rapid decompression. The biomass is heated at elevated temperature using high pressure steam for a few minutes, and then subjected to explosive decompression in order to physically and chemically modify the biomass: hemicellulose is hydrolysed, lignin is solubilised, and the accessibility of the cellulose to cellulase enzymes is improved (Vlasenko *et al.*, 1997; Fan *et al.*, 1982). However, the hydrolysate contains, in addition to fermentable sugars, a broad range of compounds, such as weak acids, furan and phenolic derivatives, some of which present inhibitory effects on the cellulase components and/or are toxic to the yeast used in the fermentation step. The composition of these inhibitors depends on the lignocellulosic properties and the pre-treatment conditions (Ando *et al.*, 1986; Delgenes *et al.*, 1996; Palmqvist *et al.*, 1996; Clark *et al.*, 1984).

As a promising pre-treatment converting low cost biomass into value chemical end-products such as fuel, chemical products (Duff and Murray, 1996; Vlasenko *et al.*, 1997), steam explosion has several types such as, with high pressure, dilute acid, and so on. Pre-treatment with steam explosion at atmospheric pressure produced auto-hydrolysis of the hemicellulose and a good substrate for enzymatic hydrolysis (Nidetzky and Steiner, 2001). It partially hydrolysed the hemicellulose to water-soluble oligomers or to individual sugars, which is responsible for the large increase in accessibility of the cellulose to enzymatic hydrolysis. Fernandez *et al.* (1998) determined that the water-soluble phenolic fraction generated during steam explosion is a function of the severity of the steam treatment.

The materials pre-treated with steam explosion are slightly acidic (pH 3.5 - 4.0) due to water soluble lignin and acetic acid (McKenzie, 1991). It has several attractive potential features, such as lower environmental impact, lower capital investment, energy efficiency, less hazardous process chemicals, and more complete recovery of all wood biopolymers (Avellar and Glasser, 1998).

Many studies on biomass material treated by steam explosion have been carried out, such as Lignocellulosic material with steam pre-treatment for enhance enzymatic hydrolysis (Brownell *et al.*, 1985; Gregg and Saddler, 1996), wood pre-treated by steam explosion (Nunes and Pourquie, 1996), and plant biomass (Sawada *et al.*, 1995). The work reported that steam explosion is an effective pre-treatment for biomass materials. However, the high temperature involved in the process has cause the increase of crystallinity for cellulose (Amash and Zugenmaier, 2000). This has been seen as the biggest disadvantage of steam explosion since hydrolysis of cellulose is inhibited by high crystallinity (Ladisch, 1989)

3.2.3.3 Liquid hot water

Liquid hot water (LHW) pre-treatment, where biomass is exposed to pressurized hot water (solids concentration < 20%), appears to have the potential: (i) to generate reactive fibre (> 90% conversion) (van Walsum *et al.*, 1996); (ii) to recover most of the pentosans (> 90%) (Mok and Antal, 1992), and (iii) to produce hydrolysate that

results in little or no inhibition of glucose fermentation (van Walsum *et al.*, 1996; and Allen *et al.*, 1996). Van Walsum *et al.* (1996) attempted to examine all three-performance metrics but did not present results for pentosan recovery due to difficulties in obtaining reproducible data at 10% solids. Poor reproducibility resulted from high transient temperatures (up to 260 °C) that were necessary to achieve 220 °C at these solids concentrations (the upper design limit for the reactor). Allen *et al.* (2001) have examined the three metrics for LHW and steam pre-treatment of corn fibre. At a solids concentration of 5%, they obtained 86% SSF conversion, 82% xylan recovery, and no hydrolysate inhibition of fermentation yield.

As stated by Allen *et al.* (1996), hot liquid water treatment requires biomass to be separated into its components for the complete conversion to the highest value products. Regardless of whether a steam or liquid water process is used, the unique properties of hot, compressed, liquid water need to be exploited to fractionate biomass. This breaking of chemical bonds may be enhanced by the increased disproportionation of water at elevated temperatures. Although hemicellulose is partially deacetylated as well as depolymerized under these conditions (Bouchard *et al.*, 1991), some evidence suggests that the cleavage of glycosidic bonds may not depend on the presence of hemicellulose-derived organic acids. A mechanism other than acid hydrolysis is necessary to be followed (Bobleter *et al.*, 1986)

The major advantages of hot-water pre-treatment compared to dilute-acid are avoiding the use of mineral acid (with its myriad of disadvantages) and reducing sugar degradation products. However, corn fibre treated with hot water requires further processing than that treated with dilute-acid because the former is not severe enough to saccharify xylan sugars, as needed for their fermentation. About 80% enzymatic digestibilities have been reported by using this method (Allen *et al.*, 1996). However, current commercial xylanases is ineffective for saccharifying corn fibre. Studies on new enzyme are necessary if continue using this pre-treatment method. Like steam explosion, high temperature also results in high crystallinity for the cellulose.

3.2.4 Chemical pre-treatment

3.2.4.1 Dilute-acid hydrolysis

Dilute acid hydrolysis was originally used to directly saccharify lignocellulosic materials, however, direct saccharification suffered from low yields because of sugar decomposition. It has been used as pre-treatment techniques that include dilute sulphuric acid, dilute nitric acid, dilute hydrochloric acid, dilute phosphoric acid, and peracetic acid. Of all acid-based pre-treatment methods, sulphuric acid has been most extensively studied, because it is inexpensive and effective. Lots of researches with feedstock materials such as hardwood and softwood, herbaceous crops, agricultural residues and wastepaper pre-treated by dilute acid have been carried out.

Dilute acid pre-treatment has several advantages; of which the major is that significantly higher xylose yields can be obtained. Grohmann *et al.* (1985) reports xylose yields of 80% of theoretical with using a batch dilute-acid process. Torget *et al.* (1994) conducted a two-temperature dilute-acid pre-treatment with a percolation process and obtained a xylose yield of 90%. Esteghlalian *et al.* (1997) stated that the dilute sulphuric acid pre-treatment could achieve high reaction rates and significantly improve cellulose hydrolysis. At moderate temperature, direct saccharification suffered from low yields because of sugar decomposition. Despite of the advantages, dilute acid pre-treatment usually has higher cost than some physico-chemical pre-treatment processes (such as steam explosion) because of the need of acid recovery. A neutralisation of pH is necessary for the downstream enzymatic hydrolysis or fermentation processes. Hence there is a need to investigate more effective methods by reducing the use of acid.

3.2.4.2 Alkaline pre-treatment

Alkaline pre-treatment techniques are basically delignification processes, together with a significant amount of hemicellulose solubilised. A number of alkaline pre-treatments have been reported (McMillan, 1994; Bjerre et al., 1996; Chosdu et al., 1993); most of them involve the use of sodium hydroxide, or sodium hydroxide

in combination with other chemicals such as peroxide, or others.

The efficiency of alkaline pre-treatment varies with the factors such as substrate and treatment conditions. Generally, it is more effective using alkaline pre-treatment on agricultural residues and herbaceous crops than on wood materials (Sun and Cheng, 2002). Fan *et al.* (1987) and McMillan (1994) used alkaline hydrolysis to pre-treat lignocellulosic materials and reported that the effect of alkaline pre-treatment depends on the lignin content of the materials. The mechanism of alkaline hydrolysis is believed to be saponification of intermolecular ester bonds crosslinking xylan hemicelluloses and other components, for example, lignin and other hemicellulose (McMillan, 1994). The porosity of lignocellulosic materials increases with the removal of the crosslinks (Tarkow and Feist, 1969).

Dilute NaOH treatment is also reported in order to reduce the amount of alkaline used. Fan et al. (1987) reported that the structure changes, including an increase in internal surface area, a decrease in the degree of polymerization, a decrease in crystallinity, separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure, caused by dilute NaOH treatment. The digestibility of NaOH-treated hardwood increased from 14% to 55% with the decrease of lignin content from 24–55% to 20%. However, no effect of dilute NaOH pre-treatment was observed for softwoods with lignin content greater than 26% (Millet *et al.*, 1976). Dilute NaOH pre-treatment was also effective for the hydrolysis of straws with relatively low lignin content of 10–18% (Bjerre *et al.*, 1996). Chosdu *et al.* (1993) used the combination of irradiation and 2% NaOH for pre-treatment of corn stalk, cassava bark and peanut husk with a glucose yield of 43% for corn stalk but 3.5% for cassava bark and 2.5% for peanut husk.

3.2.4.3 Lime treatment

There are several alkaline pre-treatment techniques with different reactants such as sodium, potassium, calcium, ammonium hydroxide, and lime. Of these techniques, lime has been most extensively studied because of its benefits with low reagent cost and safety. Playne (1984) reported that the enzyme digestibility of sugarcane

bagasse was improved from 20% before pre-treatment to 72% after pre-treatment with lime at ambient conditions for up to 192 h. It was also shown with higher temperatures and shorter reactions times to effectively pretreat lignocellulose with lime. Chang *et al.* (1998) obtained the digestibility result for bagasse treated with lime at 120 °C for 1 h.

Kim and Hotlzapple (2005) used corn stover as feedstock biomass to study the lime pre-treatment. During the treatment, an excess of calcium hydroxide (0.5 g/g raw biomass) were used. After 4 weeks at 55°C with aeration, some cellulose and hemicellulose were solubilised as monomers and oligomers in the pre-treatment liquor. When considering the dissolved fragments of glucan and xylan in the pre-treatment liquor, the overall yields of glucose and xylose were 93.2% and 79.5%. It has been pointed out that the pre-treatment liquor has no inhibitory effect on ethanol fermentation. However, the solid weight loss is significant due to additional delignification. Although the treatment process has a high recovery of glucan and xylan, more than 50% of the lignin still remains in the pre-treated corn stover after the non-oxidative treatment, which negatively affects the enzymatic hydrolysis.

3.2.5 Biological pre-treatment

In biological pre-treatment processes, microorganisms such as brown-, white- and soft-rot fungi are used to degrade lignin and hemicellulose in waste materials (Schurz, 1978). Brown rots mainly attack cellulose, while white and soft rots attack both cellulose and lignin. White-rot fungi are the most effective basidiomycetes for biological pre-treatment of lignocellulosic materials (Fan *et al.*, 1987). Hatakka (1983) studied the pre-treatment of wheat straw by 19 white-rot fungi and found that 35% of the straw was converted to reducing sugars by Pleurotus ostreatus in five weeks. Similar conversion was obtained in the pre-treatment by Phanerochaete sordida 37 and Pycnoporus cinnabarinus 115 in four weeks. In order to prevent the loss of cellulose, a cellulase-less mutant of Sporotrichum pulverulentum was developed for the degradation of lignin in wood chips (Ander and Eriksson, 1977).

Akin *et al.* (1995) also reported the delignification of Bermuda grass by white-rot fungi. The biodegradation of Bermuda grass stems was improved by 29 - 32% using Ceriporiopsis subvermispora and 63% - 77% using Cyathus stercoreus after 6 weeks. The white-rot fungus P. chrysosporium produces lignin-degrading enzymes, lignin peroxidases and manganese- dependent peroxidases, during secondary metabolism in response to carbon or nitrogen limitation (Boominathan and Reddy, 1992). Both enzymes have been found in the extracellular filtrates of many white-rot fungi for the degradation of wood cell walls (Kirk and Farrell, 1987). Other enzymes including polyphenol oxidases, laccases, H_2O_2 producing enzymes and quinone-reducing enzymes can also degrade lignin (Blanchette, 1991).

Biological pre-treatment has the advantages of requiring no chemicals and low energy supply. However, biological processes are slow, and most lignin-solubilising microorganisms also solubilise or consume hemicelluloe and cellulose. This technique is facing the challenge to be more time effective.

3.2.6 Combination of two or more pre-treatment processes

3.2.6.1 Advantage of combined pre-treatment process

Combination of several pre-treatment processes can take advantages from different types of process such as chemical and physical process, which can lead to higher lignin and hemicellulose removal. However, combination of two or more processes often requires longer residential time and it is likely that some part of cellulose decreases during the long process. Three typical combined pre-treatment processes are shown as follow:

3.2.6.2 Steam explosion with carbon dioxide

As mentioned in Section 3.2.31, steam explosionhas been investigated extensively (Bender, 1979; Shimizu *et al.*, 1994). It involves exposure of the lignocellulosic feed to a high-pressure steam to let water molecules penetrate the substrate structure. The pressure is then suddenly released to let the water molecules escape in an explosive fashion. In the process, the lignocellulosic structures are disrupted to

increase the accessibility of cellulose to the enzymes. This explosion pre-treatment enhances cellulose hydrolysis rate and increased glucose yield from about 40% to 80% (Gregg and Saddler, 1996; Tsao, 1987). However, high temperature associated with the high-pressure cause extensive damage to xylose (Tsao, 1987).

Steam explosion but using carbon dioxide (also called supercritical carbon dioxide explosion), as an alternative method, relies on a lower temperature compared with steam explosion and can reduce expense compared with ammonia explosion. In this process, carbon dioxide is also called supercritical fluid. The term, "supercritical fluid" refers to a fluid that is in a gaseous form but compressed to a liquidlike density at temperatures above its critical point. The fluid possesses the characteristics of mass transfer of a "gaslike" with the solvating power of a "liquidlike" (Larson and King, 1986). In this case, carbon dioxide (critical temperature, 31.1 °C; critical pressure, 73 atm) is used. It has the advantage of free supply from fermentation processes where carbon dioxide is released. Once dissolved in water, carbon dioxide will form carbonic acid. Even though it is a weak acid, it should be helpful in hydrolysing hemicellulose as well as cellulose. Moreover, the low temperature will prevent any appreciable decomposition of monosaccharides by the acid. Upon an explosive release of the carbon dioxide pressure, the disruption of the cellulosic structure should increase the accessible surface area of the substrate to enzymatic hydrolysis.

Zheng (1998) reported that the higher carbon dioxide pressure the more effective the explosion pre-treatment. Other gases including nitrogen and helium had been tried in the explosion test, but carbon dioxide appeared to be the most effective. It is also suggested that there is some mechanism beyond just the simple destruction of the anatomical structures of the cellulosic materials. It should be noted that the moisture (water) content in the cellulosic materials would have an important effect on the carbon dioxide explosion pre-treatment. There are two explanations for this Zheng (1998): (i) the carbon acid, which is formed when carbon dioxide is dissolved in the water, might have helped the hydrolysis;(ii) the carbon dioxide molecules were able to get into some small cavities in the crystalline structures and, thus, upon explosion, more cellulose became accessible.

3.2.6.3 Ammonia explosion

Ammonia explosion is a process involving putting ammonia solution (5%-15%) through a column reactor packed with biomass at elevated temperatures (160 °C-180 °C) and a fluid velocity of 1 ml*$cm^{-2}min^{-1}$ with residence times of 14 min. It is also known as ammonia recycled percolation (ARP) since ammonia is separated and recycled.

Ammonia fibre explosion (*AFEX*), as one of the techniques, has been reported to yield optimal hydrolysis rates from pre-treated lignocelluloses at low enzyme loadings (< 20 FPU/g cellulose) (Dale and Moreira, 1982; Holtzapple *et al.*, 1991; Dale *et al.*, 1996; Moniruzzaman *et al.*, 1997; Foster *et al.*, 2001). A recent study showed that the enzymatic digestibility if the ammonia recycled percolation treated corn stover to be 90% with an enzyme loading of 10 FPU/g-glucan which is higher than that required for a cellulose (Kim *et al.*, 2002).

The major advantage of ammonia pre-treatment is the low cost of ammonia. Moreover, the ammonia can be recovered to further drive the cost of this pre-treatment (Holtzapple *et al.*, 1992). However, studies have shown that AFEX is more effective on Herbaceous and agricultural residues but only moderately well on hardwoods, and is not attractive for softwoods (McMillan, 1994).

3.2.6.4 Dilute acid treatment with steam explosion

As dilute acid pre-treatment and steam explosion are the two most popular methods, attention has been attracted on the combination of these two different processes (chemical and physical). Commonly, there are two types of combination: (i) acid treatment followed by steam explosion (called two-stage dilute acid treatment); (ii) adding acid into the reactor during steam process (also called acid catalysed or acid-impregnation).

In two-stage dilute acid hydrolysis process, biomass is firstly treated with dilute acid at relatively mild conditions resulting in hemicelluose fraction being hydrolysed, and then at higher temperature for depolymerisation of cellulose into

glucose. The liquid phase, containing the monomeric sugars is removed between the treatments, thereby avoiding degradation of monosaccharides formed. It is important to avoid monosaccharide degradation products for improving the ethanol yield. Sanchez *et al.* (2004) carried out the two-stage dilute acid hydrolysis using Bolivian straw material, Paja brava. In first stage, Paja brava material was pre-treated with steam followed by dilute sulphuric acid (0.5% or 1.0% by wt) hydrolysis at temperatures between 170 °C and 230 °C for a residence time between 3 and 10 min. The highest yield of hemicellulose derived sugars were found at a temperature of 190 °C, and a reaction time of 5 – 10 min, whereas in second stage hydrolysis considerably higher temperature (230 °C) was found for hydrolysis of remaining fraction of cellulose.

The benefits of acid catalysis during pre-treatment of lignocellulosic materials have been well documented in the literature. Thus, the use of acid in steam explosion has been reported to enhance the overall recovery yield of pre-treated fractions (including hemicellulose derived sugars), as well as the susceptibility of the insoluble residue to enzymatic hydrolysis which includes HNO_3 (Saddler *et al.*, 1982), SO_2 (Ramos *et al.*, 1992; Eklund *et al.*, 1995), formic acid (Sudo *et al.*, 1986), dilute H_2SO_4 (Torget *et al.*, 1991; Eklund *et al.*, 1995; Ramos *et al.*, 2000) and dilute H_3PO_4 (Deschamps *et al.*, 1996). Also, the acid impregnation decreases the steam requirements for best pre-treatment of wood chips, thus enhancing the economics of the process (Nguyen and Saddler, 1991).

Acid catalysed steam explosion is one of the most cost-effective processes for hardwood and agricultural residues, but it is less effective for softwoods (Hamelinck *et al.*, 2005). Limitations include destruction of a portion of the xylan fraction, incomplete disruption of the biomass structure, and generation of compounds that may inhibit microorganism's uses in downstream processes. Moreover, the addition of acid requires neutralisation that is water washing, which decreases the overall sugar yields

Sun and Cheng (2005) reported that dilute sulphuric acid pre-treatment at temperature of 121 °C was effective in solubilising hemicellulose in the biomass. The relatively lower temperature results in less increase of cellulose crystallinity

that affects the downstream process (hydrolysis). About 50 – 66% of xylan in the biomass was hydrolysed into monomeric xylose for sulphuric acid concentration higher than 1.2% and pre-treatment time longer than 60 min. The xylose yields in the filtrates of two agricultural residues were similar under the same pre-treatment conditions. Pre-treatment time of 30 min did not provide a good solubilisation of hemicellulose. The monomeric glucose yield in the prehydrolysate of bermudagrass increased with the increase of acid concentration and pre-treatment time. Approximately 27 – 33% of glucan from bermudagrass was converted into glucose when acid concentration and pre-treatment time were 1.2% (reported values are within the range of 1% - 4%) and 60 min respectively.

Mosier *et al.* (2005) examined the pre-treatment methods with temperatures of 140°C -180 °C were for the first stage while the second stage was run at 170 °C, 180 °C, 190 °C, 200 °C, and 204 °C, Times of 10, 15, and 20 min were used in each with sulphuric acid levels of 0.0735%, 0.4015%, and 0.735% by weight. About 83.0 of the hemicellulose and 26.3 – 52.5% of the lignin was solubilised. The pre-treated cellulose was highly digestible upon subsequent enzyme hydrolysis with up to 90% being attained (Torget *et al.*, 1990, 1991, 1994).

Despite achieving excellent hemicellulose sugar yields and highly digestible cellulose with low acid loadings, equipment configurations and the high ratio of water to solids employed in flow-through systems require significant energy for pre-treatment and product recovery. Practical systems that lend themselves to commercial applications have not been demonstrated. As other high temperature pre-treatment, dilute acid treatment with steam explosion has the same problem of crystallinity increase caused by high temperature.

3.2.7 Justification of research and gaps of knowledge

Numerous pre-treatment methods or combinations of pre-treatment methods are available, all having their specific advantages and disadvantages. The choice for a pre-treatment technology heavily influences cost and performance in subsequent

hydrolysis and fermentation. The ideal pre-treatment process would produce reactive fibre; require little or no feedstock size reduction; entail reactors of reasonable size (high solids loading), built of materials with a moderate cost; not produce solid residues; have a high degree of simplicity.

From a mechanistic standpoint, there are several similarities among these pre-treatment methods. Except for mechanical comminution, pre-treatment techniques always employ catalysts (acid, ammonia, alkali) that enable lower temperature and pressure operation. Another commonality is that many pre-treatment are performed at sufficiently high temperature to hydrolyse hemicellulose. Since the overall carbohydrate yield is the most important factor in commercial-scale biomass conversion processes, research to improve pre-treatment techniques focus on minimising, or preferably eliminating, degradation of the carbohydrate fraction of lignocellulosic biomass. However, the development of more effective and efficient pre-treatment methods is hindered by incomplete knowledge about lignocellulosic structure, the nature interactions between lignicellulose components and pre-treatment chemicals, and factors controlling enzymatic hydrolysis.

3.4 Hydrolysis

3.4.1 Introduction

Where lignin removal and hemicellulose hydrolysis are classed as pre-treatment, cellulose hydrolysis is abbreviated to hydrolysis: it is considered the major hydrolysis step. In hydrolysis, the cellulose is converted into glucose sugars:

$$(C_6H_{10}O_5)_n + nH_2O \rightarrow nC_6H_{12}O_6 \quad (3.1)$$

The main challenge of producing ethanol from renewable lignocellulosic biomass has been found to be the hydrolysis stage of the process. The reaction is catalysed by dilute acid, concentrated acid, or enzymes (cellulase). Without any pre-treatment,

the typical hydrolysis yield is less than 20%, whereas yields exceed 80% after pre-treatment.

3.4.2 Acid Hydrolysis

Acid hydrolysis of cellulose is a complex heterogeneous reaction between cellulose (solid) and water (liquid). A basic study of cellulose hydrolysis was concluded by Saeman (1945) that high temperature dilute acid hydrolysis of cellulose could be described as a pseudo-homogeneous consecutive first order reaction. The hydrolysis depends not only on the temperature and concentration of acid but also on the physical factors in the reaction, for example the physical state of the cellulose.

The dilute acid is the oldest available technology for converting cellulose biomass to glucose (with first commercial plant built in Germany in 1898). The major advantage of dilute acid hydrolysis is that it is ideally suited to continuous processing because it is very flexible and it can deal with many sources of lignocellulosic material with a short reaction time. Previous studies (DOE, 2003; Arkenol, 2003) showed that pure cellulose produced over 50% of hydrolysis yield with 1% sulphuric acid in a continuous reactor.

Acid hydrolysis is only applied in the so-called two-stage acid processes, following acid pre-treatment. Both dilute and concentrated versions occur. The first stage is essentially the hemicellulose hydrolysis as discussed above. If the reaction would continue, the sugars produced would convert into other chemicals - typically furfural. The sugar degradation not only reduces the sugar yield, but the furfural and other by-products can also inhibit the fermentation process. Therefore, the first stage is conducted under mild process conditions (e.g. 0.7% sulphuric acid, 190 °C) to recover the 5- carbon sugars, while in the second stage only the remaining solids with the more resistant cellulose undergo harsher conditions (215 °C, but a milder 0.4% acid) to recover the 6-carbon sugars. Both stages have a 3-min residence time. Yields are 89% for mannose, 82% for galactose, but only 50% for glucose. The hydrolysed solutions are recovered from both stages and fermented to alcohol (DOE,

2003; Graf and Koehler, 2000).

The concentrated acid process has a very high sugar yield (90%-quantitative), can handle diverse feedstock, is relatively rapid (10-12 h in total), and gives little degradation. Concentrated acids such as H_2SO_4 and HCl have been used to treat lignocellulosic materials. However, it is critical to the economical viability of this process to minimise the amount of acid (reported values are within the range of 40 - 77 % v/v (Wright, 1988; Sivers and Zacchi, 1995; Camacho *et al.*, 1996; Arkenol, 2003)). Furthermore the equipment required to perform (in order to prevent corrosion) is more expensive than for dilute acid (DOE, 2003; Graf and Koehler, 2000).

3.4.3 Enzymatic hydrolysis

3.4.3.1 Introduction

Enzymatic hydrolysis is a process by which enzymes (biological catalysts) are used to break down cellulose into sugar (i.e. glucose). The most common enzyme used is Trichoderma reesei; it in fact produced cellulase enzymes, which hydrolyses cellulose (Persson *et al.*, 1991). Enzymatic hydrolysis has the advantages of being energy sparing and avoids the use of toxic substances or corrosive acids because of the relatively mild reaction conditions. Therefore, cellulose hydrolysis catalysed by cellulase has been widely investigated. However, high costs of pre-treatment and cellulase production, and significant enzyme deactivation occur during the hydrolysis; these economic problems have hampered enzymatic hydrolysis of cellulose to glucose.

Enzymatic hydrolysis of cellulose is carried out with cellulase enzymes that are highly specific (Beguin and Aubert, 1994). There are three steps for enzymatic hydrolysis of cellulose: adsorption of cellulose enzymes onto the surface of the cellulose, the biodegradation of cellulose to fermentable sugars, and desorption of cellulose. In order to provide maximum contact for cellulose with enzyme, pre-treatment is required to open up the structure and to provide access for the

enzyme to active sites.

Compared with acid hydrolysis, enzymatic hydrolysis has several advantages: (i) the very mild process conditions give potentially high yields, and (ii) the maintenance costs are low (no corrosion problem). Moreover, the process is compatible with many pre-treatment options, although purely physical methods are typically not adequate (Graf and Koehler, 2000; Sun and Cheng, 2002). Many experts see enzymatic hydrolysis as key to cost-effective ethanol production in the long run (Wymand, 1999; Lynd, 2002; DOE, 2003). However, hydrolysis is negatively influenced by structural features such as crystallinity, degree of cellulose polymerisation, and lignin content, and positively by surface area (Sun and Cheng, 2002). A low substrate concentration gives low yield and rate, and a high cellulase dosage may increase the costs disproportional. It has been suggested that hydrolysis can be enhanced with adding certain surfactants (to facilitate desorption of cellulase after reaction), by using mixes of cellulase from different organisms, and by adding other enzymes (e.g. pectinase) (Sun and Cheng, 2002).

In order to improve the yield and rate of the enzymatic hydrolysis, current research mainly focuses on: (i) enhancing enzyme activity in distinctive hydrolysis and fermentation process steps (Sun and Cheng, 2002), and (ii) combining the different steps in less reactors to avoid intermediate and end products of the hydrolysis, cellobiose and glucose, inhibit the cellulase activity. Enzymes can be recovered and recycled, so that the enzyme concentration can be higher against lower enzyme cost, although the enzyme quality decreases gradually (Sun and Cheng, 2002).

There are many factors that have been identified to affect the hydrolysis rate of cellulose, which are: the porosity (accessible surface area) of the waste materials, cellulose fibre crystallinity, and lignin and hemicellulose content (McMillan, 1994). Other disadvantages are long reaction times (at least 24 h), large reactors (due to low concentration of substrate) and high cost of enzymes (about 30% of total ethanol price). Therefore, enzymatic hydrolysis requires more research before it becomes economically feasible process. The following are the factors affecting hydrolysis yield from previous study.

3.4.3.2 Cellulase

Cellulase enzymes are produced from organisms that live on cellulosic material; they may be produced in a separate reactor, or bought from industrial suppliers. Both bacteria and fungi can produce cellulase enzymes, but fungi get the most research attention because of their aerobic growth conditions and fair production rate (Sun and Cheng, 2002). The cost of enzymes has been considered as one of the challenges for enzymatic hydrolysis. Presently, they contribute significantly to the cost of ethanol, over 20% without recycling being taken into account (Wyman, 1999). Thus, the cost would need to be reduced considerably to make the process economically viable.

Cellulases are usually a kind of compound of several enzymes that work together synergistically to attack typical parts of the cellulose fibre (DOE, 2003; Sun and Cheng, 2002). There are at least three major groups of cellulases involved in the hydrolysis process: a. endoglucanase (EG, endo-1,4-D-glucanohydrolase, or EC 3.2.1.4) which attacks regions of low crystallinity in the cellulose fibre, creating free chain-ends; b. exoglucanase or cellobiohydrolase (CBH, 1,4-β-D-glucan cellobiohydrolase, or EC 3.2.1.91.) which degrades the molecule further by removing cellobiose units from the free chain-ends; c. β-glucosidase (EC 3.2.1.21) which hydrolyses cellobiose to produce glucose (Coughlan and Ljumgdahl, 1988). In addition, there are also some ancillary enzymes that attack hemicellulose, which are: glucuronidase, acetylesterase, xylanase, *β*-xylosidase, galactomannanase and glucomannanase (Duff and Murray, 1996).

Extensive studies (Goyal *et al.*, 1991; Gritzali and Brown, 1978; Knowles *et al.*, 1987; Kyriacou *et al.*, 1987; Nidetzky *et al.*, 1994) have been carried out on the effect of cellulase on hydrolysis yield. Sattler (1989) reported that all enyme-substrate ratios an initial fast reaction lasting for 10 hours is followed by a slow reaction. More review on the effects of cellulase on hydrolysis process is presented in Chapter 4.

3.4.3.3 Other factors

Substrate concentration

One of the main factors affecting hydrolysis efficiency is substrate concentration. An increase of substrate concentration normally will result in an increase of the product yield; however, high substrate concentration will cause the substrate inhibition which depends on the ratio of total substrate to total enzyme (Huang and Penner, 1991; Penner and Liaw, 1994). Cheung *et al.* (1997) reported that low substrate levels result in an increase of the yield and reaction rate of the hydrolysis. Huang and Penner (1991) found that the substrate inhibition occurred when the ratio of the microcrystalline substrate Avicel pH 101 to the cellulase from Trichoderma reesei (grams of cellulose/FPU of enzyme) was greater than 5. Penner and Liaw (1994) reported that the optimum substrate to enzyme ratio was 1.25 g of the microcrystalline substrate Avicel pH 105 per FPU of the cellulase from T. reesei. Glucose yields of 50-80% are generally obtained within the first 24 h and further 72 h incubation is required to obtain final yields of 80-95%. And it has been proposed that a 6-10% (w/v) lignocellulosic substrate concentration is the upper limit of slurry viscosity that can be effectively mixed.

It is thought that the susceptibility of cellulosic substrates to enzymatic hydrolysis depends on a number of substrate structural features, such as cellulose crystallinity, the degree of cellulose polymerization, the lignin content and the surface area accessible to cellulases. The importance of each factor in determining the susceptibility of the substrate has not been fully resolved. However, it has been strongly suggested that the most influential factor is the surface area accessible to cellulases. A strong correlation between accessible surface area and the hydrolysis area has been obtained in several studies (Gregg and Saddler, 1996; Chang *et al.*, 1981; Chmiola *et al.*, 2006), although there is still some debate whether the methods of determining surface area truly reflect the area accessible to cellulase.

Particle size of biomass

Effective hydrolysis requires maximum contacts between cellulase and cellulose. To provide large effective cellulose site, accessible surface area of biomass. For this purpose, reduction of particle size has been considered as a necessary step prior to

pre-treatment. Recent studies (Moreira, 2000; Larson *et al.*, 2001; Reith *et al.*, 2002) have reported that one of the most important variables during cellulose conversion is particle size. Generally, when larger particles are used, heat transfer problems involved in both pre-treatment and hydrolysis process may result in overcooking of the outside of the chip and incomplete autohydrolysis of the interior (Woods and Bauen, 2003). Rivers and Emerts (1987) stated that particle size may not be as important to the yield of enzymatic hydrolysis as once thought. Reduction of particle size, however, requires significant amounts of energy. With the significant energy input, the utilisation of very small particle size would not be desirable to optimise the effectiveness of the process and to improve the economy (Ballesteros *et al.*, 2000). Therefore, there is a need to obtain a suitable particle size which balances the efficiency of hydrolysis and related energy input.

Cellulose structural properties

It has been known that enzymatic hydrolysis of lignocellulosic biomass is affected by the structural properties of cell-wall components (Grethlein, 1984; McMillan, 1994; Chang and Holtzapple, 2000; Mosier *et al.*, 2005). The degree of cellulose crystallinity is a major factor affecting enzymatic hydrolysis of the substrate. It has been reported that a decrease in cellulose crystallinity especially influences the initial rate of cellulose hydrolysis by cellulase (Laureano-Perez *et al.*, 2005). Physical or chemical pre-treatment to disrupt the crystalline structure of cellulose is often used to promote the hydrolysis of biomass. In addition to cellulose crystallinity, lignin affects the enzymatic hydrolysis of lignocellulosic biomass because it forms a physical barrier to attack by enzymes (Fengel and Wegener, 1984). Several researchers have found that delignification treatment of lignocellulosic biomass increases the yield of monosaccharides by enzymatic hydrolysis (Mooney *et al.*, 1998). Hemicellulose is known to coat the cellulose microfibrils in the plant cell wall (Saha, 2003), forming a physical barrier to access by hydrolytic enzymes, and removal of hemicellulose has been reported to increase the enzymatic hydrolysis of cellulose (Yang and Wyman, 2004; Ohgren *et al.*, 2007).

Temperature and pH

Apart from substrate and enzyme activities, reaction condition also affects enzymatic hydrolysis (Sun and Cheng, 2002). Temperature and pH are considered as important factors for reaction conditions because the enzymes are normally actively in certain range of temperature and pH. The optimal temperature is suggested 45-50 °C (Gregg and Saddler, 1996) and this has been used by many researchers. However, considering process integration becoming promising, it is an advantage if the optimal temperature can be lower. Most fermentation operates at a compromised temperature of around 38 °C due to the temperature sensitivity of the fermentation organism, instead of the enzyme optimum temperature of 45°C - 50 °C.

Studies have suggested (Janssen *et al.*, 1999; Nierstrasz *et al.*, 1999; Wilkins *et al.*, 2005) that the optimal pH value is 4.5-5.0. The value of pH affects the washing procedures after pre-treatment as most pre-treatment involves acid additives. On one hand, if the pH is too low, the solution becomes toxic to the enzyme. On the other hand, to receive higher pH value, it requires large amount of water for washing purpose leading to the increase of cost and weight loss.

3.4.2.4 Hydrolysis kinetics

In order to understand the mechanics behind the enzymatic hydrolysis, kinetics and further modelling studies of hydrolysis are necessary in different stages of processing of biomass. They span the entire domain of operations, namely, enzyme characterisation and modification, substrate preparation, reactor design, and optimisation of feeding profiles of substrate in a fed-batch operation. There are two types of modelling approaches, empirical and mechanistic modelling. Empirical models relate the factors using mathematical correlations, without any insight into the underlying mechanisms. These are easy to develop and are useful in enzyme characterisation and substrate preparation. Mechanistic models are developed from the reaction mechanisms, mass transfer considerations and other physical parameters that affect the extent of hydrolysis. As these models address the underlying dynamics of the process, they can be extensively used in every stage.

Mechanistic models vary in their complexity based on the intended use of the models. These models are useful in describing the reaction mechanism between the ligninocellulosic biomass and enzyme. Details are discussed in Chapter 4.

3.4.3 Conclusion

Hydrolysis is a limiting step in the whole bio-ethanol process. The process yield needs to be improved by studying biomass structure, effective pre-treatment methods, cellulase system, process optimisation as well as mechanism understanding. Due to the tough crystalline structure, the enzymes currently available require several days to yield an efficient hydrolysis rate (more than 80%). Since long process times tie up reactor vessels for long periods, these vessels must have large capacity or use many of them. Either option is expensive. Currently the cost of enzymes is also too high and research aiming at bringing down the cost of enzymes continues.

3.5 Fermentation

Fermentation is the process where the hydrolysed products (sugars) are converted into ethanol with a variety of micro-organisms, generally either bacterium, yeast, or fungi under oxygen-free conditions (Lynd, 1996). According to the reactions, the theoretical yield is 0.51 kg ethanol and 0.49 kg carbon dioxide per kg sugar.

$$3C_5H_{10}O_5 \rightarrow 5C_2H_5OH+5CO_2 \quad (3.2)$$

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH+2CO_2 \quad (3.3)$$

Fermentation of glucose to ethanol is a well established process and used widely in the brewing industry. Glucose can be fermented in anaerobic conditions using yeast such as Saccaromyces cerevisiae. Theoretically 100 g of glucose will give 51.4 g of ethanol and 48.8 g carbon dioxide. In practice the actual yield, due to

microorganisms using glucose for their own growth, is less than 100%. However, providing nutrients for the yeast in the fermentation broth can decrease this. The non cellulose fraction of MSW may be of use in this case as they contain many minerals and nutrients required by living organisms. Another reason for less than 100% conversion in this process is inhibition of the fermentation through the presence of lignin (Wooley *et al.*, 1999).

The methods of C6 sugar fermentation have been known for centuries. Attention has also been brought to convert C5 sugar after it became possible to free C6 sugars in lignocellulosic crops (Hamelinck *et al.*, 2005). For the purpose of efficiency and economics of the process, it is important to recover and ferment C5 sugar into ethanol since they represent a high percentage of the available sugars. A number of yeast has been identified to convert xylose to ethanol since 1980s (DOE, 2003. Nowadays, bacteria have drawn more attention from researchers since it can ferment more quickly than yeast. Recently, *Zymomonas mobilis*, a Gram-negative ethanol-producing bacterium, has been of considerable interest for ethanol production because it can produce ethanol with higher specific rates of glucose uptake and ethanol production (Tao *et al.*, 2001). Another bacterium is *Saccharomyces cerevisiae* that has been mentioned with feasible results of ethanol production by Yu (2003).

In the early research stage, different sugars are fermented in different sequential reactors. Therefore, there is a trend towards combining different reaction steps together in fewer reactors. Mid-product inhibition can be avoided and the yield of ethanol is potentially higher when hydrolysis and fermentation reaction are connected directly. Another trend of fermentation is to use genetic engineering and new screening technologies bringing bacteria and yeast capable of fermenting both glucose and xylose (DOE, 2003). It is showed that all five of the major sugars - glucose, xylose, mannose, galactose and arabinose can be utilised together by near-term fermentation using genetically engineered yeast and bacteria. Future technology will improve the fermentation efficiency of the organism by yielding ethanol in less time and its resistance by requiring less detoxification of the hydrolysate (Graf and Koehler, 2000; Wooley *et al.*, 1999b).

Another option in the fermentation step of EFC is to use Simultaneous Saccharification and Fermentation (SSF). Studies are currently taking place into SSF of steam pre-treated wood and have resulted in an increase in yield and production rate. It has also been reported that SSF is less sensitive to infection than Separate Hydrolysis and Fermentation (SHF). However, the method does have a weakness in the fact that yeast may become severely inhibited by presence of inhibitors in the slurry after steam pre-treatment (Novem, 1999).

3.6 Product recovery

The product stream from fermentation is a mixture of ethanol, cell mass and water. In this product flow, ethanol from cellulosic biomass has likely product concentrations < 5 %. According to Hamelinck *et al.* (2005), the maximum concentration of ethanol tolerated by the microorganisms is about 10% at 30 °C. To maximise cellulase activity, the operation at maximum temperature of 37 °C is set (Lynd, 1996). On the processing side, slurries become difficult to handle when containing over 15% solids, which also corresponds to 5% ethanol (Lynd, 1996). In the first step, ethanol is recovered in a distillation or beer column, where most of the water remains with the solids part. The product (with 37% ethanol) is then concentrated to a concentration just below the azeotrope (95%) (Wooley *et al.*, 1999). Eventually 99.9% of the ethanol in the beer can be retained in the dry product by recycling between distillation and dehydration (Wooley *et al.*, 1999).

3.7 Residual solids/power production/wastewater treatment

The main solid residual from the process composed of lignin and ash. Its amount and quality differs with feedstock and the applied process. Production of coproducts from lignin, such as high-octane hydrocarbon fuel additives, may be important to

the competitiveness of the process (DOE, 2003). Lignin can replace phenol in the widely used phenol formaldehyde resins. The amount of ash generated will depend strongly on whether acid is used in pre-treatment/hydrolysis. The solid wastes is not hazardous (Lynd, 1996). Unfermented sugars in the liquid effluent form a non-negligible energy source which can be partly recycled (40% (Wooley *et al.*, 1999)), and partly dried and fired (as syrup) in the boiler. The residual water contains significant amounts of organic compounds such as protein, acetic acid, furfural and HMF, and needs processing before its disposal into the environment. Wooley *et al.* (1999) describes a treatment with two serial digesters, of which the first (anaerobic) produces biogas that is fired in the boiler (8% of the total boiler load in that study). After the second (aerobic) digester, 99% of the organic material is eliminated. Any sludge formed is also processed in the boiler.

3.8 Process integration

When enzymatic hydrolysis is applied, different levels of process integration have been investigated as shown in Fig. 3.2. In the case of process integration, pre-treatment of the biomass is necessary to make the cellulose more accessible to the enzymes. A cellulase enzyme hydrolysis step followed by fermentation, is called Separated Hydrolysis and Fermentation (SHF). In the SHF configuration, the joint liquid flow from both hydrolysis reactors enters the fermentation reactor. The mixture from fermentation reactor is then distilled to remove the ethanol. In a second reactor, xylose is fermented to ethanol, and the ethanol is again distilled (DOE, 2003; Grethlein and Dill, 1993). The cellulose hydrolysis and glucose fermentation may also be parallel to the xylose fermentation. The advantage of SHF is that the configuration with separate reactors allows for better process control (Nedalco, 2003)

Simultaneous Saccharification and Fermentation (SSF) consolidates hydrolyses of cellulose with the direct fermentation of the produced glucose. This reduces the number of reactors involved by eliminating the separate hydrolysis reactor. It avoids the problem of product inhibition associated with enzymes (DOE, 2003). SSF has

been regarded as the major option because it results in higher yields and also shorter residence times for various substrates (Reith *et al.*, 2002; Woods and Bauen, 2003; de Boer and den Uil, 1997). However, Jarboel et al. (2007) reported that lactic acid was formed during SSF process when softwood is utilized as substrate. Despite the advantages, two drawbacks of the SSF process have been identified (Lynd, 1996): (i) the inability to recycle yeast; and (ii) the difficulty of finding optimal temperatures for the action of cellulase (45 °C - 50 °C) and growth of the yeast microorganism (30 °C - 37 °C).

Simultaneous Saccharification and Co-Fermentation (SSCF) has the advantages of SSF but co-ferment hexoses and pentoses sugars (SSCF) in the same reactor. This process is being tested on pilot scale (DOE, 2003). In Consolidated BioProcessing (CBP), ethanol and all required enzymes are produced by a single microorganism community, in a single reactor (Lynd, 1996). Application of CBP leads to no capital or operating costs for dedicated enzyme production (or purchase). It also provides compatible enzyme and fermentation systems (Lynd, 1996). However, there are no organisms or compatible combinations of microorganisms available that not only produce cellulase and other enzymes at the required high levels and but also produce ethanol at the required high yields (Lynd, 1996; Graf and Koehler, 2000).

Figure 3.2 Integration of hydrolysis and fermentation process (*Hamelinck, et al.*, 2005)

3.9 Promising prospects of biomass-to-ethanol

From the literature review above, it can be seen that a large number of research studies were considered and carried out in this process. However, the ethanol yield still needs to be improved with advance technology since the current ethanol price is not competitive enough in the market. To make the cost of biomass ethanol reach the point at which it is competitive with petrol, the process technology must be improved and low price substrates, as feedstock must be investigated. A major challenging of the biomass conversion to ethanol remains to substantially reduce the commercialising risk and cost, and greater emphasis on developing a fundamental understanding of the technology for biomass-to-ethanol process would reduce application costs and accelerate commercialisation. The following presents a list of promising research area of bioethanol conversion process based on the current literature:

1. Investigation of promising biomass feedstock that is available in large quantity and low cost. This has two main purposes: (i) reducing ethanol cost, and (ii) replace primary biomass source that has contributed to food supply security;
2. Development of efficient pre-treatment process for both process performance and economic purposes; it has also been shown that combination of two or several methods to reach the maximum efficiency has potentials;
3. Improve the process performance of enzymatic hydrolysis as it has been considered as the most promising system with the advantages of potentially high yield (70%-85%) and low cost compared with acid hydrolysis. This process is influenced by substrate features, cellulase system, and process conditions;
4. Co-fermentation of hemicellulose sugars to chemical by-products to maximise economic benefits.
5. Integration of hydrolysis, fermentation and possible cellulase production process to reduce reactor volume capacity and intermediate product inhibition. The main challenge is to find solutions for the compatibility problem of enzyme and yeast due to the fact that they operate in different optimal temperature ranges.

3.10 Conclusions

From the overview of the whole process, pre-treatment and hydrolysis are still believed to be the most critical processes for ethanol production, although process integration starts to attract more and more attention. These two processes make significant contribution to the cost reduction if the product yields are improved.

Although there are quite a few types of pre-treatment technologies available in the literature, no single one type proved to be the best. The selection of pre-treatment methods is suggested subject to the properties of biomass. From the review, it is clear that dilute acid treatment and steam explosion, as the traditional technologies, are still among of the most popular options. Research into the combination of these two methods has improved the process efficiency. However, the problems of high temperature resulting high energy input and increase of crystallinity remains the main concerns. Based on the current available literature, this work will continue to explore the possibility of using these typical pre-treatment methods for biomass of municipal solid waste.

The improvement of hydrolysis process is also necessary. Although studies have shown some of the factors that affect the process reaction, there are still debates that which factor(s) are critical. It is necessary to optimise the process by introducing different factors. Furthermore, since most of hydrolysis studies are based on the primary biomass sources especially pure cellulose, it is still unclear how the complex substrate features of lignocellulosic materials affect the process. This work will examine these issues by carrying out laboratorial experiments.

Chapter 4

Cellulase - cellulose adsorption mechanisms, kinetics, and modelling

4.1 Introduction

During enzymatic hydrolysis, cellulase is used to catalyse cellulose. The adsorption of cellulase-cellulose has been considered as a fundamental step for hydrolysis. Extensive research focused on the understanding of cellulase system for primary biomass sources. However, the hydrolysis process of lignocellulosic biomass is more complicated compared with pure cellulose because the action of cellulase enzyme systems is impacted by substrate properties in addition to concentration - such as crystallinity, accessible area, and the presence of lignin - which depend on the particular substrate being investigated and change as the reaction proceeds. In order to seek a fundamental understanding of enzymatic hydrolysis that incorporates information about cellulase components and substrate features in addition to concentration, quantitative models are tremendously valuable. Systematic comparison of models' predicted value to experimental measurements is the most and rigorous means available to test whether our understanding of cellulase components and their interactions is sufficiently accurate to explain the observed phenomenon. In addition, once a quantitative model is validated, it can be used to rapidly formulate new hypotheses of significance in both fundamental and applied contexts.

This chapter is devoted to review existing studies on cellulase-cellulose adsorption occurred during enzymatic hydrolysis process in the literature. A significant portion

of this review concerns the understanding of mechanisms of cellulose hydrolysis and its kinetics. Thereafter, the review focuses on cellulase-cellulose adsorption system, with attention given to features of the widely studied *Trichoderma* cellulase system and substrate structure. The Section 'Quantitative Models' presents a classification scheme and summarises features of models reported in the literature. The following section offers concluding perspectives and outlines outstanding challenges associated with understanding and modelling noncomplex cellulase systems. Finally, a model is chose to simulate the experimental results from this work. The theory, assumptions and equations are also given. Regression model is introduced to model the effects of substrate features on cellulose-cellulase adsorption.

4.2 Mechanisms of adsorption

The process of enzymatic hydrolysis consists of two steps: first, the adsorption of cellulase enzyme on to the surface of cellulose; and second, the breakdown of cellulose to fermentable sugars (Ghose and Bisaria, 1979; Ryu *et al.*, 1984). Many researchers have suggested that the process of adsorption of enzyme onto the substrate is a crucial step during the enzymatic hydrolysis (Mes-Hartree *et al.*, 1987; Vallander and Eriksson, 1987; Klyosov *et al.*, 1986; Dourado *et al.*, 1999; Zhang and Lynd, 2004). Thus, the phenomenon of enzyme adsorption is extremely important in determining the parameters controlling the rate of cellulose hydrolysis and to develop more efficient conditions for the conversion process of cellulose (Singh *et al.*, 1990, Chernoglazov *et al.*, 1988; Hogan *et al.*, 1990; Steiner *et al.*, 1988).

The enzymic mechanism of cellulose degradation was firstly proposed by Reese *et al.* (1950) with the concept of C_1-C_x. In the original hypothesis by Reese *et al.* (1950), "C_1" was believed to be an enzyme disaggregating the anhydroglucose chains in native cellulose, which were then hydrolysed by "C," enzymes to soluble oligosaccharides. However, results from fractionation studies on culture filtrates of the two fungi *Trichoderma viride* (Berghem and Pettersson, 1973) and *Trichoderma*

koningii (Wood and McCrae, 1972; Halliwell and Griffin, 1973) have shown that the "C_I" enzyme is a β-1, 4-glucan cellobiohydrolase. These findings strongly suggest that the C_I-C_X, concept by Reese *et al.* (1950) must be reconsidered and an alternative mechanism for enzymatic cellulose degradation must be investigated.

A study by Lee and Fan (1982) has suggested that the mechanism of cellulose hydrolysis involves physical disruption of insoluble cellulose in addition to endo- and exo-acting enzymes. However, the importance of such disruption, as well as the cellulase components responsible for it, is still not entirely clear (Zhang and Lynd, 2004). It is widely observed that the cellulose structure causes a rapid decrease in reaction rate with increasing hydrolysis (Zhang *et al.*, 1999; Valjamae *et al.*, 1999). Explaining this observation at a mechanistic level has important fundamental and applied implications. However, very little work involving detailed characterisation has been done (Zhang and Lynd, 2004). It may be explained that the declining reactivity of residual cellulose during enzymatic hydrolysis is a result of factors such as less surface area and fewer accessible chain ends and/or adsorption of inactive cellulase on the surface of cellulose (or lignocellulose) particles which block further hydrolysis (Lee *et al.*, 1996).

It has been reported that both the accessible area of cellulose (Fan *et al.*, 1980) and the cellulase adsorptive capacity (Ooshima *et al.*, 1983) per gram cellulose decrease as hydrolysis proceeds. It is speculated that the availability of glucan and chain ends per gram may also decrease with conversion (Zhang and Lynd, 2004). It also has been indicated that the loss of cellulose reactivity at the end of hydrolysis and/or increased reactivity for "new" cellulase/cellulose encounters as compared to "old" encounters, as Carrard *et al.* (2000) reported that fresh addition of substrates can stimulate more soluble sugar release. Zhang and Lynd (2004) concluded that when cellulase enzyme systems act on insoluble cellulosic substrates, three processes occur at the same time: 1) chemical and physical changes in the residual solid-phase cellulose; 2) primary hydrolysis, involving the release of soluble intermediates from the surface of reacting cellulose molecules; and 3) secondary hydrolysis, involving hydrolysis of soluble intermediates to lower molecular to glucose, as shown in 4.1.

Figure 4.1 Mechanistic hypothesis of enzymatic hydrolysis for cellulose (Zhang and Lynd, 2004)

In the literature, most of the available data on cellulose hydrolysis concerns the rate of secondary hydrolysis, often based on release of reducing sugars or soluble glucose equivalent. However, to improve the fundamental understanding of cellulose hydrolysis, better characterisation of chemical and physical changes associated with residual cellulose as well as secondary hydrolysis is necessary (Zhang and Lynd, 2004).

4.3 Kinetics of adsorption

4.3.1 Reaction rate and adsorption limiting factors

Due to the highly complex substrate, enzymatic hydrolysis involves two distinct stages: enzyme-substrate complex formation and cellulose hydrolysis. There are two major steps involved in enzyme-substrate complex formation including mass transfer of enzyme from the bulk aqueous phase to the insoluble cellulose surface and then the enzyme-substrate complex formation. Three major steps composed of cellulose enzymatic hydrolysis include transfer of reactant molecules to the active site of the enzyme-substrate complex, reaction promoted by the enzyme, and then

transfer of soluble products to the bulk aqueous phase. With these complex heterogeneous reaction mechanisms mentioned, it is very difficult to model the hydrolysis process (Movagarnejad *et al.*, 2000; Zhang *et al.*, 1999).

Most of studies described in the literature have observed the nonlinearity when plotting sugar conversion versus hydrolysis time at a given enzyme loading (Lee and Fan, 1982; Holtzapple, 1984, Lynd, 2002; Mtui *et al.*, 2005); this indicates that the rate of cellulose hydrolysis decreases as hydrolysis proceeds (Zhang *et al.*, 1999). Several factors have been reported that lead to a decrease in hydrolysis rates as the reaction progresses. They include product inhibition, lower substrate reactivity (higher crystallinity, higher lignin content, and substrate accessibility), enzyme inactivation, and enzyme loss due to irreversible lignin adsorption. Without the complication of product inhibition or cellulase inactivation, Desai and Converse (1997) concluded that the loss of substrate reactivity is not the principal cause for the long residence time required for complete conversion of biomass. Eriksson *et al.* (2002) concluded that thermal instability of the enzymes and product inhibition were not the main cause of reduced hydrolysis rates; instead the adsorbed enzymes become inactive and that unproductive binding is the main cause of hydrolysis rate reduction.

In spite of the efforts of many investigators, the principles involved in enzymatic hydrolysis of cellulose are still far from being completely understood (Zhang and Lynd, 2004). The difficulties in studying enzymatic hydrolysis are caused by the substrate features and its constituents as well as the multiplicity and the complex cellulase enzyme system (Eriksson *et al.*, 1990; Wong *et al.*, 1988). Moreover, the process is involved with insoluble substrates, which has been seen as one of the most difficult and undeveloped areas in enzyme kinetics. Since cellulosic materials are water-insoluble solid substrates, the cellulose-cellulase system is heterogeneous and the hydrolysis reaction involves several steps. Despite the complex of hydrolysis, the adsorption of cellulase on cellulose has been considered as a crucial step (Lee and Fan, 1982, Dourado *et al.*, 1999): the adsorption of enzyme molecules on the cellulose surface is a prerequisite step for subsequent catalytic reaction.
Since cellulose is an insoluble structured substrate, some of its kinetic characteristics are substantially different from those of the usual enzyme-catalyzed

homogeneous reactions. However, the earlier kinetic studies on the enzymatic hydrolysis of cellulose have often neglected the kinetic characteristics that stem from the heterogeneous nature of the cellulose substrate.

The kinetics of the enzymatic hydrolysis is significantly affected by the structural features of cellulose. Amongst the structural features of cellulose, surface area and crystallinity have been considered the most important (Cowling and Kirk, 1976; Fan *et al.*, 1980; Sewalt *et al.*, 1997; Zhu, 2005; Zhu *et al.*, 2008). It has been postulated that surface area is important because the contact between the enzyme molecules and the surface of cellulose is a prerequisite for hydrolysis to proceed. A high surface area would tend to increase the accessibility of the enzyme molecules to the surface. The crystallinity of cellulose has also been considered important because the cellulolytic enzyme can degrade to a greater degree the more accessible amorphous region of cellulose than the less accessible crystalline region. As the crystallinity increases, cellulose becomes increasingly resistant to further hydrolysis. These two major structural features of cellulose have been shown to profoundly influence the kinetics of the enzymatic hydrolysis of cellulose (Fan *et al.*, 1980; Sewalt *et al.*, 1997; Zhu, 2005).

Another important factor, which also affects the kinetics of cellulose hydrolysis, is the mode of interaction between the enzyme and cellulose molecules (Lee and Fan, 1982, Rahmouni et al., 2001; Zhang and Lynd, 2004). Since cellulose is an insoluble and heterogeneous substrate, its hydrolysis involves the following steps: 1) transfer of enzyme molecules from the bulk aqueous phase to the surface of the cellulose particles, 2) formation of an enzyme-substrate (E-S) complex upon adsorption of the enzyme molecules to the surface of the cellulose particles, 3) transfer of molecules of the reactant, water, to the active sites of the E-S complex, 4) surface reaction between water and cellulose promoted by the E-S complex, 5) transfer of the soluble products, glucose and cellobiose, from the cellulose particles to the bulk aqueous phase, and 6) decomposition of cellobiose into glucose in the aqueous phase by β-glucosidase (Lee and Fan, 1982; Eaton and Hale, 1993). It has been reported that the mass-transfer resistance can be substantially reduced by enhancing mixing and by maintaining the cellulose concentration at a proper level (Van Dyke, 1972). Under the condition of negligible mass-transfer resistance, the

hydrolysis rate should depend mainly on the modes and/or rates of adsorption and surface reaction. Several studies (Gharpuray *et al.*, 1983; Sinitsyn *et al.*, 1991; Lynd *et al.*, 1996; Kim and Holtzapple, 2005) have been published on the adsorption of cellulase to the cellulose particles; however, relatively little attention has been given to interrelate this phenomenon to the kinetics of hydrolysis.

4.3.2 Kinetics models

Despite the unclear principle for enzymatic hydrolysis, it has been widely recognised that the first step of enzymatic hydrolysis is cellulase adsorbed to the surface, or penetrating into the cellulose substrate (Lee *et al.*, 2000). After the adsorption of the cellulase on the surface of cellulose fibers, the cellulase-cellulose complex is formed (as shown in Figure 4.2). The adsorption step reaches steady-state within half an hour (Lynd *et al.*, 2002), which is rapid compared to the time required for hydrolysis (at least 24 h). Currently there are three types of adsorption models on which most variations are based: Michaelis-Menten model, Langmuir isotherm adsorption, dynamic adsorption model.

Figure 4.2 Schematic drawing of an enzyme-catalyzed reaction of an insoluble substrate

Note: this figure is obtained from Holtzapple *et al.* (1984a)

4.3.2.1 Machaelis-Menten model

The Michaelis-Menten kinetic model has been used to describe the enzymatic hydrolysis of cellulose in several studies (Suga *et al.*, 1975; Ohmine *et al.*, 1983;

Caminal *et al.*, 1985). However, this model is only valid for the situations where the concentration of enzyme is the limiting factors (i.e. where enzyme concentration is much lower than the substrate concentration) (Brown and Holtzapple; 1990). To determine the maximum rate of an enzyme for the hydrolysis reaction, experimental work is carried out by increasing the substrate concentration ([S]) until a constant initial rate of product formation is found. This maximum rate determined is the maximum velocity (V_{max}) of the enzyme. Under this experimental condition, enzyme active sites are saturated with substrate.

The reaction rate, V, increases with increasing substrate concentration [S], until the maximum rate V_{max}. However, there is no clearly-defined substrate concentration at which level the enzyme is saturated with substrate (Nelson and Cox, 2000). Another measure to characterise an enzyme is the substrate concentration at which the reaction rate reaches half of its maximum value ($V_{max}/2$). This concentration is shown to be equal to the Michaelis constant (KM).

The following shows the most convenient derivation of the Michaelis-Menten equation, described by Briggs and Haldane (1925):

$$E+S \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} ES \xrightarrow[K_2]{} E+P \qquad (4.1)$$

The enzymatic reaction is assumed to be irreversible, and the product does not bind to the enzyme. The rate of production of the product, d[P]/dt is referred to as the reaction rate, V in enzyme kinetics. It depends on the conversion rate constant, k_2 (kcat) and, the concentration of enzyme bound to substrate [ES].

A key assumption of this derivation is the quasi steady state approximation, that is, the substrate-enzyme concentration change much more slowly than those of the product [P] and substrate [S] (Suga *et al.*, 1975). This allows expressing the relationship between the substrate-enzyme concentration and substrate and enzyme concentrations in terms of the various rate constants:

$$[ES] = \frac{k_1 [E][S]}{k_{-1} + k_2} \qquad (4.2)$$

To simplify the equation, the Michaelis constant is defined as:

$$K_m = \frac{k_{-1} + k_2}{k_1} \quad (4.3)$$

Therefore,

$$[ES] = \frac{[E][S]}{K_m} \quad (4.4)$$

The total enzyme concentration of ($[E_0]$) is the total amount of free enzyme concentration in solution ([E]) plus the substrate-enzyme concentration ([ES]), allowing deriving the free enzyme concentration from (4.4):

$$[E_0] = [E] + [ES] \quad (4.5)$$

$$[E] = [E_0] - [ES] \quad (4.6)$$

Replacing (4.6) in (4.4), it can be written:

$$[ES] = \frac{([E_0] - [ES])[S]}{K_m} \quad (4.7)$$

Rearranging gives:

$$[ES]\frac{K_m}{[S]} = [E_0] - [ES] \quad (4.8)$$

$$[ES]\left(1 + \frac{K_m}{[S]}\right) = [E_0] \quad (4.9)$$

$$[ES] = [E_0]\frac{1}{1 + \frac{K_m}{[S]}} \quad (4.10)$$

The reaction rate is:

$$V = \frac{d[P]}{dt} = k_2 [ES] \quad (4.11)$$

Substituting (4.10) in (4.11) and multiplying the numerator and denominator by [S] gives:

$$\frac{d[P]}{dt} = k_2 [E_0] \frac{[S]}{K_m + [S]} = V_{max} \frac{[S]}{K_m + [S]} \quad (4.12)$$

However, relying on the law of mass action derived from the assumptions of free diffusion and thermodynamically-driven random collision, the Michaelis-Menten kinetics, is only valid for homogeneous reaction systems (Fan and Lee, 1980). As the substrate of enzymatic hydrolysis is insoluble and the reaction is heterogeneous, the Michaelis-Menten equation needs to be further developed.

4.3.2.2 Langmuir isotherm

Langmuir adsorption model has been used to quantify the relationship between adsorbed and free cellulases in solution in some studies (Fan and Lee, 1981; Holtzapple *et al.*, 1984; Bothwell and Walker, 1995; Boussaid and Saddler, 1999). It assumes that that adsorption can be described by single adsorption equilibrium constant and a specified adsorption capacity. Compared to Michaelis-Menten model, the Langmuir isotherm (Eq. 4.13) has become the most common description of cellulase adsorption (Zhang and Lynd, 2004).

The Langmuir isotherm can be represented as:

$$E_a = \frac{W_{max} K_p E_f}{1 + K_p E_f} \quad (4.13)$$

Where E_a is adsorbed cellulase (mg or μmol cellulase/L);

W_{max} is the maximum cellulase adsorption = A_{max}*S (g/L);

A_{max} is the maximum cellulase adsorption per g cellulose (mg or μmol cellulase / g cellulose);

S is cellulose concentration (g cellulose/L);

E_f is free cellulase (mg or μmol cellulase/L);

K_P is the dissociation constant ($K_P=E_a/E_f/S$) in terms of L/g cellulose.

The distribution coefficient or partition coefficient, R, is defined as:

$$R=K_pW_{max} \quad (4.14)$$

The Langmuir equation is widely used because it provides a good fit to the data (Fan and Lee, 1981; Beldman et al., 1987; Kyriacou et al., 1988; Medve et al., 1997 Boussaid and Saddler, 1999), and it represents a simple mechanistic model that can be used to compare kinetic properties of various cellulase–cellulose systems. But it has also been reported (Zhang et al., 2004) that cellulase binding does not comply with assumptions used in the Langmuir model due to one or both of the following: 1) partially irreversible cellulase adsorption; 2) interaction among adsorbing cellulase components, especially at high concentrations.

4.3.2.3 Dynamic adsorption model

In addition to equilibrium adsorption models, a dynamic adsorption model has been used by some investigators (Converse *et al.*, 1988; Converse and Optekar, 1993; Nidetzky and Steiner, 1993; Nidetzky *et al.*, 1994). The hypothesis was firstly raised by Converse *et al.* (1988), that there is a slow deactivation of the adsorbed enzyme. The decline in concentration of the adsorbed enzyme is represented by a modest product inhibition and, more importantly, the assumption that the concentration of the adsorption sites is proportional to the square of the remaining substrate concentration.

The mechanism is assumed to be as follows:

$$E + C \Leftrightarrow E_a \Rightarrow P + E \quad (4.15)$$

$$E_a \Leftrightarrow E_d \Rightarrow E \quad (4.16)$$

$$E + P \Rightarrow E_p \quad (4.17)$$

Free enzyme E combines with adsorption site C to form an active adsorbed enzyme E_a, at a rate k_2EC. This adsorbed enzyme can either promote the reaction of the substrate to product P at a rate K_1, E_a, in which case the enzyme returns to solution and contributes to the pool of free enzyme E, or it can slowly convert to an inactive form E_d at a rate k_4E_a,. This deactivated enzyme can either reactivate at a rate k_5E_d or it can be converted to free enzyme E at a rate proportional to the rate at which the substrate is solubilised; i.e., it is freed when the cellulose cage around it is removed. We therefore take this rate to be k_6 (-dS/dt)*E_d^*, where E_d^* is E_d/S, the deactivated enzyme per unit substrate. The rate of hydrolysis is assumed to be proportional to the concentration of the active adsorbed enzyme E_a:

$$\frac{d[S]}{dt} = -k_1 E_a \tag{4.18}$$

However, so far very little is known of the dynamics of binding during hydrolysis. Since hydrolysis itself changes the substrate, it can be expected that the fraction of bound protein also changes during hydrolysis (Converse and Optekar, 1993).

4.4 Cellulase-cellulose adsorption

4.4.1 Trichoderma cellulase system

Intensive attention has been focused on *Trichoderma* Cellulases due to its significant part to the high levels of cellulase secreted. *Trichoderma viride* is aggregate from all unknown *Trichoderma* species; while *Trichoderma reesei* is developed from a single isolate (QM6a). Most commercial cellulases are produced from *Trichoderma spp.*; a few produced by *Aspergillus niger* (Esterbauer *et al.*, 1991; Nieves *et al.*, 1998).

The *Trichoderma* cellulase mixture consists of many catalytically active proteins. At least two cellobiohydrolases (CBH1-2), five endoglucanases (EG1–5), and β-glucosidases have been identified by 2D electrophoresis (Vinzant *et al.*, 2001).

CBH1, CBH2, and EG1 are the three main components of the *Trichoderma* cellulase system, representing 60 ± 5%, 20 ± 6%, and 12 ± 3% of total cellulase protein, respectively (Goyal *et al.*, 1991; Gritzali and Brown, 1978; Knowles *et al.*, 1987; Kyriacou *et al.*, 1987; Nidetzky *et al.*, 1994). The structure of CBH1, CBH2, and EG2 features a catalytic domain and a cellulose-binding domain connected by a glycolysated peptide linker (Gilkes *et al.*, 1991; Lee and Brown, 1997; Linder and Teeri, 1997). The catalytic domain structures of CBH1 and CBH2 are entirely different but both feature tunnel-shaped structures formed by disulfide bridges. In CBH2, two well-ordered loops form a 20 Å long tunnel adjacent to a/β-barrel structure (Rouvinen *et al.*, 1990). In CBH2, four surface loops form a tunnel of 50 Å adjacent to a β-sandwich structure (Divne *et al.*, 1993, 1994). The tunnel-shaped topology of CBH1 and CBH2 allows for a structural interpretation of the processive action of exoglucanase. The catalytic sites of both cellobiohydrolases are within the tunnel near the outlet; hence β-glucosidic bonds are cleaved by retaining (CBH1) or inverting (CBH2) mechanisms. Structural analyses, as opposed to measurement of hydrolysis products, provides direct evidence that cellobiose is the primary product of hydrolysis mediated by CBH1 and CBH2 (Divne *et al.*, 1993, 1994; Davies *et al.*, 1997). The *Trichoderma* CBH1 and CBH2 can cleave several bonds following a single adsorption event before the dissociation of the enzyme substrate complex (Imai *et al.*, 1998; Teeri *et al.*, 1998a, b; Valjamae *et al.*, 1998). As a result, the action of CBH1 and CBH2 lead to a decrease in the degree of polymerization (DP) of cellulose (Kleman-Leyer *et al.*, 1992, 1996; Srisodsuk *et al.*, 1998). Cellobiohydrolase activity is often measured by reducing sugar release from Avicel, often called "Avicelase" activity. Avicel is a common used substrate for measuring exoglucanase activity, because it has the highest ratio of chain ends to accessible internal β-glucosidic bonds.

EG1 and CBH1 have significant similarity (45% identity, Penttila *et al.*, 1986); they belong to the same family (Cel7) and use a retaining mechanism. The active site of EG1 is a groove rather than a tunnel (Henriksson *et al.*, 1996), allowing glucan chains to be cleaved randomly to two shorter chains resulting in a rapid decrease in DP (Kleman-Leyer *et al.*, 1992, 1994; Srisodsuk *et al.*, 1998; Whitaker, 1957; Selby, 1961; Wood and McCrae, 1978). EG1 activity can be measured based on the rate of change of the viscosity of a soluble cellulose derivative such as

carboxymethylcellulose (CMC) (Miller *et al.*, 1960; Wood and McCrae, 1978). However, CMCase activity has been shown to correlate poorly with the ability to hydrolyse insoluble cellulose even for purified endoglucanases (Himmel *et al.*, 1993; Klyosov, 1988; 1990). Among three purified *T. viride* endoglucanases obtained by Shoemaker and Brown (1978), the one exhibiting the highest rates of Avicel hydrolysis had the lowest CMCase activity. Klyosov (1990) clearly pointed out that the specific endoglucanase activities from many microorganisms measured on CMC do not correlate with activities against insoluble cellulose.

It should be noted that the division into endo- and exoglucanases is not absolute (Barr *et al.*, 1996; Irwin *et al.*, 1993; Henrissat and Davies, 1997; Teeri, 1997; Teeri *et al.*, 1998a, b). A processive endoglucanase in T. fusca E4 has been reported by Iewin *et al.* (1993). It is also suggested that some endoglucanase activity is inhibited by the *T. reesei* CBH2 (Enari and Niku- Paavolar, 1987; Kyriacou *et al.*, 1987) and CBH1 (Schmid and Wandrey, 1990), as well as the CBH2 (Boisset *et al.*, 2000). It has been reported by Stahlberg *et al.* (1993) that all *T. reesei* cellulases had some endo-acting activity. It is also suggested by Warren (1996) and Zhang and Wilson (1997) that exoglucanase could exhibit some endoglucanase activity; this is due to temporary conformational changes of loops on the tunnel structure that expose their active sites. The observation by Kleywegt *et al.* (1997) and Meinke *et al.* (1995) support this hypothesis.

4.4.2 Substrate features

Cellulose in lignocelluloses is composed of crystalline and amorphous components. The amorphous component is digested more easily by enzymes than the crystalline component. The crystalline cellulose exists in the form of microfibrils, which are paracrystalline assemblies of several dozen (1, 4) β-D-glucan chains hydrogen-bonded to one another along their length. The (1, 4) β-D-glucan chains are tightly linked by numerous hydrogen bonds, both side-to-side and top-to-bottom in a lattice like manner. The glucan chains in the core of the microfibril have a precise spacing (Buchanan *et al.*, 2001). Cross-linking glucans are polysaccharides

that can hydrogen-bond to cellulose microfibrils. They may coat microfibrils but are also long enough to span the distance between microfibrils as well as link them together to form a network. Most cross-linking glycans are called "hemicelluloses." Hemicelluloses are largely composed of aldopentoses (arabinose, xylose, galactose and mannose), which are in either pyranose or a furanose form (Wyman, 1996). Hemicelluloses also link the polyphenolic portion of the plant cell, known as lignin–carbohydrate complexes as reported by Buchanan *et al.* (2001). The most distinguishing feature of secondary walls is the incorporation of lignins. These are complex networks of aromatic compounds called phenylpropanoids (Laureano-Perez *et al.*, 2005). Lignins are the most abundant organic natural products known after cellulose; it accounts for as much as 20–30% of all vascular plant tissue. According to Buchanan *et al.* (2001), the phenylpropanoids, hydroxycinnamoyl alcohol and "monolignols" (p-coumaryl, coniferyl, and sinapyl alcohols), represent most of the lignin networks. Lignin, covalently linked to cellulose and xylan, indicates that the orientations of polysaccharides may serve as a template for the lignin patterning. A range of cross-linking possibilities exist which includes hydrogen bonding, ionic bonding with Ca^{+} ions, covalent ester linkages, ether linkages, and van der Waals interactions (Buchanan *et al.*, 2001). A great influence on digestibility of forage crops by animals is exerted by lignin–carbohydrate interactions (Laureano-Perez *et al.*, 2005). As a result of the location of the cellulose fraction within the cell wall, enzymatic access is restricted by the lignin and hemicellulose interference. Hence, pre-treatment of the biomass is necessary.

Adsorption of cellulase on cellulosic materials has been extensively studied. However, only a few studies have concerned with the influence of the physicochemical properties of cellulose adsorbent. Mandels (1971) studied the adsorption of cellulase on three different preparations of pure cellulose, and reported that the adsorption of protein and enzyme appear to be dependent on temperature, where enzyme is active in the temperature range of 37 °C - 50 °C; whilst actively decrease outside this temperature range, and type of cellulose used. It has been reported that the adsorption of cellulase on spent bagasse" and newspaper (Castanon and Wilke, 1980) resulted in a decreased hydrolysis rate and the extent of adsorption was found to be mainly due to the change of cellulose

structure by the hydrolysis reaction. A report by Lee and Fan (1982) showed that the adsorption of soluble protein at initial reaction time was related to the specific surface area of cellulose. Some of the results reported are inconclusive and somewhat confusing due to the fact that lots of studies are carried out with different methods and analytic techniques.

Ohmine *et al.* (1983) concluded that the falloff in hydrolysis rate could not be fully explained by the changes in substrate crystallinity and product inhibition. They concluded that there must be some other "rate-retarding factor." Matsuno (1984) has suggested that such a factor might be the reversible inactivation of the adsorbed enzyme due to diffusion into the cellulose fibrils. It is hypothesized that the arrangement of the enzyme components needed for their synergistic effect' is sterically hindered in small pores. Converse and Optekar (1993) supported such a slow deactivation of the adsorbed enzyme.

4.4.3 Quantitative Models

According to Zhang and Lynd (2004), quantitative models of enzymatic hydrolysis of cellulose can be classified as nonmechanistic model, semimechanistic model, functionally based model and structurally based model. The term 'nonmechanistic model' is used for models that are based on data correlation without any explicit calculation of adsorbed cellulase concentration. Although such model is useful for correlating data, they are not likely to be reliable under conditions different from those for which the correlation was developed. Moreover, they are limited utility for testing and developing understanding. Semimechanistic models feature a defensible adsorption model but based on concentration as the only variable that describe the state of the substrate, and/or are based on a single cellulose hydrolyzing activity. In particular, models with concentration as the only substrate variable are called as "semimechanistic with respect to substrate," whereas models with a single cellulose hydrolysing activity are calld as "semimechanistic with respect to enzyme" (Zhang and Lynd, 2004). Most of the hydrolysis models proposed in the leterature are in the category of semimechanistic models. Semimechanistic models

can be useful in the context where including the minimal information necessary for descriptive purposes. However, it cannot describe or give insight into behaviours determined by substrate features. Similarly, semimechanistic models with respect to enzyme cannot describe or give insight into behaviours determined by multiple hydrolysing activities. Models featuring an adsorption model, substrate state variables in addition to concentration, and multiple enzyme activities are referred to "functionally based models." Functionally based models are particularly useful for developing understanding at the level of substrate features and multiple enzyme activities, as well as identifing rate-limiting factors. Functionally based models could conceivably be used for bioreactor design, however, the application in this context to date is limited (Zhang and Lynd, 2004). A further limitation of functionally based models is that they provide little information relative to design of cellulases at the molecular level. Models based on structural features of cellulase and their interaction with their substrates are called "structurally based models."Structurally based models are useful for molecular design as well as developing understanding of the relationship between cellulase structure and function. However, structurally based models are not currently available in the literature; it requires major advances in the general field of inferring protein function from structure (Zhang and Lynd, 2004).

Table 4.1 Classification scheme for models of enzymatic cellulose hydrolysis (Zhang and Lynd, 2004)

4.3.3.1 Nonmechanistic model

Nonmechanistic models provide correlations for either fractional conversion or the rate of reaction as a function of various factors (Zhang and Lynd, 2004). Factors, such as enzyme loading and substrate concentration (Sattler *et al.*, 1989), as well as pre-treated biomass properties (Chang and Holtzapple, 2000; Gharpuray *et al.*, 1983; Koullas *et al.*, 1992) are rincorporated into models with conversion as the output. Factors including hydrolysis time (Karrer *et al.*, 1925; Miyamoto and Nisozawa, 1945), enzyme loading (Miyamoto and Nisozawa, 1945), and cellulose conversion (Ooshima *et al.*, 1982) are incorporated into models with rate as the output. Table 4.2 presents a summary of nonmechanistic models existing in the literature

4.3.3.2 Semimechanistic model

Semimechanistic models with respect to substrate and enzyme are based on an adsorption model; however, it uses a single variable to describe the state of the substrate and describe the action of cellulase in terms of a single solubilising activity (Zhang and Lynd, 2004).

A representative model in this category is the HCH-1 model developed by Holtzapple *et al.* (1984a, b), which describes the initial rate of hydrolysis by:

$$V = \frac{k\,[s]\,[E]\,i}{(\alpha + [s] + \varepsilon\,[E])} \qquad (4.19)$$

Where k is a rate constant; α is a lumped affinity constant; q, the number of cellulose sites covered by an adsorbed enzyme molecule, and i, the fraction of total enzyme which is not inhibited by product. The quantity i represent inhibition by glucose (G) and cellobiose (G_2) according to:

$$i = \frac{1}{1 + \frac{[G]}{K_{I1}} + \frac{[G_2]}{K_{I2}}} \qquad (4.20)$$

Where, K_{I1} and K_{I2} are inhibition constants. This model was used to simulate a total of 50 different hydrolysis conditions with a ten-fold range in enzyme concentration and a thirty-fold range in cellulose concentration. Agreement with experimental data was rather good, and appeared better than some other models (Howell and Stuck, 1975; Huang, 1975).

Semimechanistic models with respect to enzyme have variables in addition to concentration to describe the state of the substrate. The motivation for many models in this category contributes to the widely observed trend of declining rate with increasing conversion (Zhang and Lynd, 2004). Some stdudies (Converse and Grethlein, 1987; Converse *et al.*, 1988; Luo *et al.*, 1999; Movagarnjad *et al.*, 2000; Oh *et al.*, 2001; Philippidis *et al.*, 1992, 1993) have reported an assumed change in shape and surface area over the course of hydrolysis. However, these models need to be tested against experimental data (Zhang and Lynd, 2004). Others (Fan and Lee, 1983; Gonzalez *et al.*, 1989; Gusakov *et al.*, 1985a, b; Nidetzky and Steiner, 1993; Peitersen and Ross, 1979; Ryu and Lee, 1982; Scheiding *et al.*, 1984) proposed several "two-substrate" models that partition cellulose into a less reactive highly crystalline fraction, and a more reactive amorphous fraction.These models have met with some success in terms of correlating data, the trend of increasing CrI with increasing conversion, however, they requires experiental data has to confirm.

Semimechanistic models with respect to substrate (only), involve concentration as the only substrate state variable and two or more solubilising activities. Examples of models in this category in the literature to date are based on endoglucanase and exoglucanase.

4.3.3.3 Functionally Based Models

There are a few functionally based models, involving multiple substrate variables and solubilising activities in the literature. Moo-Young and co-workers (Okazaki and Moo-Young, 1978; Suga *et al.*, 1975) developed models based on the Michaelis-Menten model and assuming that all β-glucosidic bonds are accessible that incorporated two solubilising activities (endoglucanase and exoglucanase) as

well as β-glucosidase. In addition, these investigators used concentration and DP as substrate variables. The model developed by Suga *et al.* (1975) predicts that substrate DP changes as a function of time in the presence of endoglucanase, and that exoglucanase and endoglucanase synergism occurs for the degradation of longer chain cellulose molecules. The model reported by Okazaki and Moo-Young (1978) predicted that the degree of endo-exo synergism is strongly impacted by DP. Converse and Optekar (1993) considered competitive adsorption of exoglucanase and endoglucanase for a limited number of sites, and predicted a lower DS under oversaturating conditions. The model developed by Fenske *et al.* (1999) incorporated with the observation of a decline in hydrolysis rate with increasing cellulose concentration (Huang and Penner, 1991; Valjamae *et al.*, 2001).

Table 4.2 Nonmechanistic models in the literature

Nonmechanistic model	Independent variable	Dependent variables			>1 substrate variable
		Time	Enzyme	Substrate	
Miyamoto and Nisozawa, 1942	Conversion	Variable	Variable	Fixed	No
Holtzapple *et al.*, 1984a -model 1	Conversion	Variable	Fixed	Fixed	No
Gharphuray *et al.*, 1983	Conversion	Fixed	Fixed	Fixed	Yes
Sattler *et al.*, 1989	Conversion (max)	Fixed	Fixed	Variable	No
Adney *et al.*, 1994	Conversion (max)	Fixed	Fixed	Variable	No
Koulas *et al.*, 1992	Conversion (max)	Fixed	Fixed	Fixed	Yes
Chang and Holtzapple, 2000	Conversion	Fixed	Fixed	Fixed	Yes
Karrer *et al.*, 1925	Hydrolysis rare	Variable	Fixed	Fixed	No
Ooshima *et al.*, 1982	Hydrolysis rare	Fixed	Fixed	Fixed	No
Holtzapple *et al.*, 1984b -model2	Hydrolysis rare	Fixed	Fixed	Fixed	No

Table 4.3 Semimechanistic models in the literature

Semimechanistic models	Substrate features	Enzyme features	E-S interaction	
			Adsorption/MM	Inhibition
1. Semimechanistic models with respect to substrate and enzyme (1 substrate state variable, 1 solubilising activity)				
Huang, 1975			M-M	Competitive
Beltrame *et al.*, 1982			Langmuir	
Holtzapple *et al.*, 1984a and b, 1991			Langmuir	Non-competitive
Nakasaki *et al.*, 1988			M-M	
Steiner *et al.*, 1988			Langmuir	
Howell and Stuck, 1975		Plus BG	M-M	
Ghosh *et al.*, 1982		Plus BG	M-M	Competitive
Asenjo, 1984		Plus BG	M-M	Competitive
Caminal *et al.*, 1985		Plus BG	M-M	Competitive
Borchert and Buchholz, 1987		Plus BG	Langmuir	Competitive
Guaskov and Sinitsyn, 1992		Plus BG	M-M	Non-competitive
Moldes *et al.*, 1999		Plus BG	M-M	Competitive
Belkacemi and Hamoudi, 2003		Plus BG	M-M	
2. Semimechanistic models with respect to enzyme only (>2 substrate state variables, 1 solubilising activity)				
Converse and Grethlein, 1987	AS+[S]		M-M	
Converse *et al.*, 1988	AS+[S]		Langmuir	Competitive
Movagarnejad *et al.*, 2000	AS+[S]		Langmuir	
South *et al.*, 1995	AS+[S]		Langmuir	
Ryu and Lee, 1982	[S]+X		Langmuir	
Philippidis *et al.*, 1992 and 1993	A+C	Plus BG	Langmuir	Non-competitive
Luo *et al.*, 1999	AS+[S]	Plus BG	Langmuir	Non-competitive
Oh *et al.*, 2001	AS+[S]	Plus BG	Langmuir	Non-competitive
Peitersen and Ross, 1979	AS+[S]	Plus BG	M-M	
Fan and Lee, 1983	A+C	Plus BG	Langmuir	Non-competitive
Scheiding *et al.*, 1984	A+C	Plus BG	Langmuir	Non-competitive

Table 4.3 (continued) Semimechanistic models in the literature

Semimechanistic models	Substrate features	Enzyme features	E-S interaction	
			Adsorption/MM	Inhibition
Guaskov *et al.*, 1985a and b	A+C	Plus BG	Langmuir	Competitive
Gonzalez *et al.*, 1989	A+C	Plus BG	M-M	Competitive
Nidetzky *et al.*, 1993	A+C	Plus BG	Langmuir	
Wald *et al.*, 1984	AS+(A+C)	Plus BG	Langmuir	Competitive
Gan *et al.*, 2003	AS+(A+C)	Plus BG	Langmuir	Competitive
3. Semi mechanistic models with respect to substrate only (1 substrate state variable and 2 solubilising activities				
Beltrame *et al.*, 1984	Only [S]	Endo+Exo+BG	M-M	Non-competitive
Nidezky *et al.*, 1994	Only [S]	Endo+Exo	Langmuir	

Note: AS=surface area; [S] substrate concentration; X=cellulose conversion; A, amorphous cellulose; C, crystalline cellulose

Table 4.4 Functionally based models in the literature

Functionally based models	Substrate features	Enzyme features	E-S interaction	
			Adsorption/MM	Inhibition
Suga *et al.*, 1975	[S], DP	Endo+Exo	M-M	
Okazaki and Moo-Young, 1978	[S], DP	Endo+Exo	M-M	Non-competitive
Converse and Optekar, 1993	[S], AS	Endo+Exo	Dynamic adsorption	
Fenske *et al.*, 1999	[S], AS, DP	Endo+Exo	Langmuir	

4.5 Selection of adsorption models

Various theoretical, empirical, and hybrid models have been developed to predict the enzymatic hydrolysis of primary biomass (Holtzapple *et al.*, 1984a, b; Medve *et al.*, 1998; Movagarnejad *et al.*, 2000). Following the classification scheme, functionally bases and structurally based models have advantages of studying cellulase components and molecules. However, for the time being, structurally based models require more advances on protein structure, and the activities on functionally based model are very limited. Despite the advantage of semimechanistic models using only substrate viable (as shown in Table 4.3), by considering that the main focus of this work is substrate and its features, semimechanistic models are introduced in this study.

Among the existing semimechanistic models listed in Table 4.3, half of them are based on the Michaelis-Menten model. The Michaelis-Menten model is only valid for the limiting case of substrate being in excess relative to enzyme (Lynd *et al.*, 2002). In light of the small fraction of β-glucosidic bonds accessible to enzymatic attack, this condition is particularly limiting for cellulosic substrates. Models based on a Langmuir adsorption model do not implicitly assume excess in either enzyme or substrate, and thus have a considerably broader range of potential application. Although dynamic adsorption models have been proposed, few have been incorporated into kinetic models that lead to a prediction of hydrolysis rate. Hence, Langmuir adsorption semimechanistic models are desirable for this work.

The HCH-1 Model with Langmuir adsorption developed by Holtzapple *et al.* (1984a) has been suggested to have good data correlation according to Zhang and Lynd (2004). This model was developed for pure cellulose (Solka Bloc BW200) based on non-competitive inhibition of the products. Since 1984, the HCH-1 model has been used to test experimental data (Brown and Holtzapple, 1990, O'Dwyer *et al.*, 2006). O'Dwyer *et al.* (2006) reported that the HCH-1 Model can predict biomass digestibility for lime-pre-treated corn stover over an enzyme loading range of 0.25 - 50 FPU/g dry biomass and substrate concentration range of 10 - 100 g/L and prove the non competitive inhibition cellulase-cellulose reaction system.

However, like most of cellulase adsorption model, this HCH-1 model has not been tested on other cellulosic materials except corn stover.

However, as HCH-1 model only considers the effect of substrate and enzyme concentration. It does not take into account of substrate properties such as lignin content and cellulose crystallinity. In fact, few models has been incorporated such substrate features information. Zhu (2005) developed a regression model which studied the relationship between biomass digestibility and substrate features by using regression models. This work will inhibit the use of regression model but explore the relationship between cellulose-cellulase adsorption and substrate features, as enzyme adsorption has been recognised as an essential and crucial step for enzymatic hydrolysis. The purpose of introducing the regression model is to provide some fundamental information of effects of substrate features on cellulase adsorption. This should provide significant information to develop a theoretical quantitative model for the second generation biomass in the future.

4.5.1 HCH-1 model

An enzyme-catalysed reaction of an insoluble substrate is illustrated in Figure 4.2. The HCH-1 mechanism' (without inhibition) is given as:

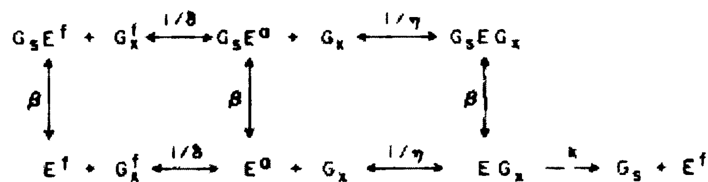


Figure 4.3Reaction mechanism for the HCH-1 model

The assumption made for HCH-1 model is that enzymes adsorbs onto the cellulose surface via Langmuir adsorption equilibrium. The model also assumes the pseudo-steady-state:

$$\frac{d[E^a]}{dt} = \frac{d[EG_x]}{dt} = 0 \tag{4.21}$$

Figure 4.3 shows the proposed reaction mechanism. Free enzyme (E^f) adsorbs onto a free cellulose site (G_x^f) to become adsorbed enzyme (E^a). The adsorbed enzyme complexes with the cellulose become an enzyme-substrate complex (EG_x). The complex catalyzes the hydrolysis of the cellulose site with reaction rate k to yield soluble product (Gs). All enzyme species can complex with product to become inhibited enzyme ($G_s E^f$, $G_s E^a$, and $G_s EG_x$). For simplicity, it is assumed that the product binding constant that the adsorption constant (δ) and the complexing constant (η) are not affected by the binding of product to the enzyme.

According to Holtzapple *et al.* (1984a), the reaction velocity is proportional to the concentration of uninhibited enzyme-substrate complex:

$$V = k\,[E\,G_x] \tag{4.22}$$

The constants in the model are defined as follows:

$$\frac{1}{\delta} = \frac{[E^a]}{[E^f][G_x^f]} \tag{4.23}$$

$$\frac{1}{\eta} = \frac{[EG_x]}{[E^a]} \tag{4.24}$$

$$\beta = \frac{[G_s\,E^f]}{[G_s][E^f]} = \frac{[G_s\,E^a]}{[G_s\,E^a]} = \frac{[G_s\,EG_x]}{[G_s][EG_x]} \tag{4.25}$$

A material balance of substrate species yields the following expression

$$[G_x^f] = \frac{[G_x]}{1 + \tau\,(1 + \beta\,[G_s])\,[\,1/\delta + (1/\delta)\,(1/\eta)][E^f]} \tag{4.26}$$

Where, τ is the number of cellulose sites covered by an adsorbed or complexed

enzyme. A material balance of enzyme species yields the following expression:

$$\left[E^f\right] = \frac{[E]}{\left(1+\beta\,[G_s]\right)\left(1+\left[1/\delta+(1/\delta)\,(1/\eta)\right]\left[G_x^f\right]\right)} \tag{4.27}$$

Using equation (4.18)-(4.23), substitutions can be made for $[EG_x]$ so that the reaction velocity can be expressed in terms of known variables:

$$V = \frac{\kappa\,[G_x][E]\left(\frac{1}{1+\beta\,[G_s]}\right)}{\alpha+\phi\,[G_x]+\tau\,[E]} \tag{4.28}$$

Where

$$\kappa = \frac{K}{\eta+1} \tag{4.29}$$

$$\alpha = \frac{\eta\delta}{\eta+1} \tag{4.30}$$

$$\phi = \frac{\left[G_x^f\right]}{[G_x]} \tag{4.31}$$

Where assumption is made that the concentration of adsorbed enzyme is the maximum which simplify $\phi = 1$

By combining equation (4.26) and (4.27), an expression for $[G_x^f]$ in equation (4.31) was:

$$0 = -\,[G_x^f]^2 + ([G_x] - \alpha - \varepsilon[E])[G_x^f] + \alpha[G_x] \tag{4.32}$$

By using quadratic formula, $[G_x^f]$ can be obtained from equation (4.32). Hence, ϕ can be expressed as follows:

$$\phi = \frac{\left[G_x - \alpha - \varepsilon[E]\right] + \sqrt{\left([G_x] - \alpha - \varepsilon[E]\right)^2 + 4\alpha[G_x]}}{2[G_x]} \tag{4.33}$$

The final models and the integrated forms are shown in Table 4.5.

Table 4.5 presents HCH-1 model and simplified HCH-1 model

Model	Equation
Simplified HCH-1 model	$\frac{d[G_s]}{dt}=\frac{k\,[G_x][E]\left(\frac{1}{1+\beta\,[G_s]}\right)}{\alpha+[G_x]+\varepsilon\,[E]}$
HCH-1 model	$\frac{d[G_s]}{dt}=\frac{k\,[G_x][E]\left(\frac{1}{1+\beta\,[G_s]}\right)}{\alpha+\phi\,[G_x]+\varepsilon\,[E]}$ where $\phi=\frac{[G_x]-\alpha-\varepsilon\,[E]+\sqrt{([G_x]-\alpha-\varepsilon\,[E])^2+4\,\alpha\,[G_x]}}{2\,[G_x]}$

4.5.2 Regression model

Zhu (2005) introduced the regression model investigating the relationship between substrate features and biomass digestibility. However, effects of substrate features on cellulase-cellulose adsorption have not been revealed at mechanism level. This work will introduce regression model to study the role of substrate features on cellase-cellulose adsorption.

Regression model is a form of regression analysis in which the relationship between one or more independent variables and another variable, called dependent variable, is modelled by a least squares function. A simple example of parametric regression is a linear regression model with a single independent variable. The linear relationship between the dependent variable and independent variable x can be expressed by the following form:

$$y = a_0 + a_1\, x + \varepsilon \qquad (4.34)$$

Where a_0 and a_1 are the parameters of the model and ε is random error. The regression parameters a_0 and a_1 can be determined using the observations of the dependent variable $\{y_1, y_2,\ldots, y_n\}$ and the independent variable $\{x_1, x_2, \ldots, x_n\}$ based on least squares error. The parameters are estimated so as to give a "best fit" of the data. Most commonly the best fit is evaluated by using the least squares

method, but other criteria have also been used. Once a_0 and a_1 are quantified, this model can be used to predict y within a certain range of a given x.

Multiple linear regressions are extensions of simple linear regression with more than one dependent variables. It attempts to model the relationship between two or more explanatory variables and a response variable by fitting a linear equation to observed data. In this case, multiple independent variables x_1, x_2... x_k are involved. Similar to Equation (4.34), the multiple (parametric) linear regression model can be written as:

$$y = a_0 + a_1 x_1 + a_2 x_2 + \ldots + a_k x_k + \varepsilon \tag{4.35}$$

Where k is the number of independent variables. a_0, a_1, a_2... a_k are regression parameters. Models that are more complex in structure than Equation (4.35) may still be analysed by multiple linear regression techniques. Such multiple linear regression models can be written as:

$$g(y) = a_1 f_1(x_1) + a_2 f_2(x_2) + \ldots + a_k f_k(x_k) + \varepsilon \tag{4.36}$$

Where g and $f_1, f_2, \ldots, f_p$ are various functions or transformations assigned *a* priori to the dependent and independent variables. For example, models that include second-order polynomial and interaction effect with three independent variables (x_1, x_2, x_3) may take the following form:

$$\begin{aligned} y &= a_0 + a_1 x_1 + a_2 x_2 + a_3 x_3 \\ &+ a_{11} x_1^2 + a_{22} x_2^2 + a_{33} x_3^2 \\ &+ a_{12} x_1 x_2 + a_{13} x_1 x_3 + a_{23} x_2 x_3 + \varepsilon \end{aligned} \tag{4.37}$$

A more general form of multiple linear regression model can be written as

$$Y = X \beta + \varepsilon \tag{4.38}$$

where $Y = n \times 1$ vector of the dependent variables

$X = n \times p$ matrix of the independent variables

β = p × 1 vector of the regression parameters

ε = n × 1 vector of random error.

The least squares criterion leads to normal equations

$$X'X\,\acute{\alpha} = X'Y \quad (4.39)$$

Solving Equation III-9 for the least-squares estimator of β (provided X'X is non singular)

$$\acute{\alpha} = (X'X)^{-1}X'Y \quad (4.40)$$

Multiple linear regression models are often used as empirical models or approximating functions. That is, the true functional relationship between y and x1, x2... xk is unknown, but utilizing the complex forms of independent variables, the multiple linear regression model adequately approximates the true unknown functions.

4.6 Conclusions

Although cellulase-cellulose adsorption has been extensively studied, the mechanisms of the hydrolysis are far from being completely understood. Nevertheless, it is widely accepted that cellulase adsorption is an essential and crucial step controlling hydrolysis reaction rate. Kinetics models have been proposed including Michaelis-Menten model, Langmuir adsorption and dynamic adsorption. Although some studies have concerned the effects of substrate features on cellulose-cellulase adsorption, very few models have incorporated such information. Particularly, most models available in the literature are initially developed for pure cellulose and primary biomass. However, cellulase-cellulose adsorption system is far more complicated for the second generation biomass as it is not only affected by the complex enzyme system but also insoluble cellulose with the structure features.

This work adapts the HCH-1 model developed to consider substrate conditions based on Langmiur adsorption to correlate the experimental data. This should provide information whether the same hydrolysis mechanisms applied to the MSW-biomass. However, this model does not provide information on the effects of substrate features. As this work investigate the use of MSW-feedtock with a complex substrate structures, it is necessary to study how the substrate features affect the hydrolysis process especially in cellulase-cellulose adsorption. Hence, regression model is introduced to provide answers to such important issues.

Chapter 5

Materials and methods (experimental and analytical)

5.1 Introduction

This work is based on experimental investigation to convert MSW to glucose. This chapter aims to describe the experimental work undertaking in this research, including the experimental setup and the monitored data. This work includes sample preparation, waste characterisation, pre-hydrolysis and enzymatic hydrolysis. Analytical methods such as UV - spectrophotometer, and high performance liquid chromatography (HPLC) are also explained. Finally the optimisation of hydrolysis with fractional experiments and cellulase-cellulose adsorption investigation are described.

The waste characterised in this report consists of five common lignocellulosic material wastes; these are potato peelings (PP), carrot peelings (CP), grass (GR), newspaper (NP) and scrap paper (SP), combination of 50% carrot peelings and 50% potato peelings as representative of kitchen organic waste (KOW), combination of 50% newspaper and 50% scrap paper as representative of paper and card waste (PCW), and 20% each of the five selected wastes (CP, PP, GR, NP and SP) as mixed MSW.

5.2 Sample preparation

Carrots and potatoes (basic type) were obtained from Sainsbury supermarket in Totenham Court Road, London. They were peeled; the peelings were chopped into small pieces and placed on paper plate covered in aluminium foil. Grass was collected from Gordon Square (London) directly. Newspaper used was London Metro, and scrap paper was used white office paper. The plates with the samples were then placed in a Memmert oven at 105 ºC overnight. The sample was milled using a food processor (Magimix Le Micro mini chopper) as shown in Figure 5.1. The dried sample was placed in the processor container, the section with blade was screwed into the container and the unit placed on the processor base. The processor was then switched on at the plug and set to speed three until the sample was milled to the appropriate size.

Figure 5.1 Magimix Le Micro mini chopper

Note: this figure is obtained from the website of John Lewis where this equipment was purchased.

A 2 mm and 12 mm (size 20) screen was used to obtain the sample that falls in the right size range. A sample bag, labelled with the name and size of the sample was used to collect the samples after screen. Goggles and dusk mask were worn while the milling was in operation. When the entire sample had been milled, the food processor was switched off at the mains and the plug removed. Pressurised air and

brushes were used to clean the processor. The screen was removed and cleaned using brushes and pressurised air and placed back with the rest of the screens.

5.3 Waste characterisation

5.3.1 Introduction

Waste Composition Determination Municipal Solid Waste (biomass) contains structural or non structural carbohydrates. The term 'structural carbohydrates' refers to cellulose and hemicellulose while the term 'non structural carbohydrates' refers to protein, ash, sucrose, chlorophyll, nitrate/nitrites and waxes. On one hand, non structural carbohydrates can be removed from the biomass by using washing or extracting ways. On the other hand structural carbohydrates are parts of the biomass composition. Municipal solid waste contains also lignin that is a complex phenolic polymer (NREL, 2007). The description of the reagents, materials, apparatus, procedure and calculations follow the NREL document. The composition determination will be for raw materials from the selected wastes (CP, PP, GR, NP and SP) prepared following Section 5.2.

5.3.2 Moisture content

A crucible was weighed using an analytical balance and the weight was recorded (w1). About 2 grams of the prepared sample were put into the crucible and the weight recorded accurately (w2). The sample was placed in a Memmert oven at 105 °C overnight and left in a desiccator until cooled. The sample was then weighed and the weight recorded (w3). Throughout all experiments, before a crucible was used, it was washed, dried in an oven and left to cool in a desiccator. A Sartorius analytical balance was used throughout which weighed samples to 4 decimal places.

The moisture content was calculated using equations 5.1, 5.2 and 5.3.

The weight of the sample before drying (w4) = w2 – w1 (5.1)

The weight of the sample after drying (w5) = w3 – w1 (5.2)

$$\text{Moisture content (\%)} = \frac{(w4 - w5)}{w4} \quad (5.3)$$

5.3.3 Preparation for cellulose, hemicellulose, and lignin determination

Cellulose, hemicellulose and lignin make up a major portion of biomass samples. These constituents must be measured as part of a comprehensive biomass analysis; Carbohydrates can be structural or non-structural. Structural carbohydrates are bound in the matrix of the biomass, while non-structural carbohydrates can be removed using extraction or washing steps. Lignin is a complex phenolic polymer. The determination of cellulose content, hemicellulose content and lignin content are followed the procedures of "determination of structural carbohydrates and lignin in biomass provided by National Renewable Energy Laboratory (NREL).

This procedure uses a two-step acid hydrolysis to fractionate the biomass into forms that are more easily quantified. 300.0 ± 10.0 mg of the sample were weighed and placed into a glass tube. Record the weight to the nearest 0.1 mg. Label the glass tube with a permanent marker. Each sample is analysed in triplicate. 3.00 ± 0.01 mL (or 4.92 ± 0.01 g) of 72% sulphuric acid were added to each tube. A Teflon stir rod was used to mix for one minute, or until the sample was thoroughly mixed. The tubes were then placed in a water bath set at 30 ± 3°C and the sample was incubated for 60 ± 5 min. The sample was stired every five to ten min by using the stir rod without removing the sample from the bath. Stirring is essential to ensure even acid to particle contact and uniform hydrolysis. Upon completion of the 60-min hydrolysis, the tubes were removed from the water bath. The acid was diluted to a 4% concentration by adding 84.00 ± 0.04 mL deionised water using an automatic burette. Dilution can also be done by adding 84.00 ± 0.04 g of DI water using a balance accurate to 0.01 g. The Teflon caps were screwed on securely. The sample was mixed by inverting the tube several times to eliminate phase separation

between high and low concentration acid layers. The autoclaved hydrolysis solution was vacumm filtered through one of the previously weighed filtering crucibles. The filtrate was captured in a filtering flask.

The lignin fractionates into acid insoluble material and acid soluble material. The acid insoluble material may also include ash and protein, which must be accounted for during gravimetric analysis. The acid soluble lignin is measured by UV-Vis spectroscopy. During hydrolysis the polymeric carbohydrates are hydrolysed into the monomeric forms, which are soluble in the hydrolysis liquid. They are then measured by HPLC. A set of standard sugar is prepared for calibration purposes with the sugar determined by HPLC.

The samples for HPLC analysis are done by passing a small aliquot of the liquor collected through a 0.45 μm membrane filter into an autosampler vial. If it is suspected that the sample concentrations may exceed the calibration range, the samples was diluted and the dilution rate recorded as needed. The concentrations should be corrected for dilution after running.

Table 5.1 Suggested concentrations ranges for 10.4.1 calibration standards

Component	Suggested concentration range (mg/ml)
D-cellobiose	0.1-0.4
D-glucose	0.1-0.4
D-xylose	0.1-0.4
D-galactose	0.1-0.4
L-arabinose	0.1-0.4
D-mannose	0.1-0.4
CVS	Middle of linear range, concentration not equal to a calibration point

The acid insoluble residues that contain both acid insoluble lignin and ash were dried in the oven at 105 °C over night. The oven-dried residues then were ashed by using a muffle funace set at 575 ± 25 °C. An appropriate number of crucibles with identifiers were placed in the muffle furnace for a minimum of four hours, and then cooled in a desiccator. The crucibles were weighed to the nearest 0.1 mg and recorded. 2.0 g sample of the oven-dried residues were weighed to the nearest 0.1 mg. An ashing burner and clay triangle with stand was used to burn the residues placed in the crucible over the flame until smoke appears. The smoke was immediately ignited and the samples were allowed to burn until no more smoke or

flame appears. The crucible was cooled before being placed in the muffle furnace. The crucibles with the samples were then placed in the muffle furnace for 24 hours and then cooled in a dessicator. The weight of both crucibles and the ash were recorded for further calculation.

5.3.4 Calculation

5.3.4.1 Calculation for Lignin

Lignin consists of acid soluble lignin and acid insoluble lignin. In this study, both types of lignin are measured. The calculation for acid soluble lignin is based on the results obtained from UV – spectrophotometer.

Acid Soluble Lignin (ASL)

Acid soluble lignin is calculated by using Equation 5.4 after the measurement with UV – spectrophotomer mentioned in Section 5.3.3:

$$\%ASL = UVabs * Volumefiltrate * Dilution * 100 / (\varepsilon * ODWsample) \quad (5.4)$$

Where:

UVabs = average UV-Vis absorbance for the sample at 250 nm

Volume hydrolysis liquor = volume of filtrate, 87 mL

Dilution = (Volume sample + Volume diluting solvent)/ Volume sample

ε = Absorptivity of biomass at specific wavelength, 55 L/(g*cm)

ODW = oven dry sample = Weight pan plus sample – Weight dry pan plus dry sample

Acid Insoluble Lignin (AIL)

Acid insoluble lignin and ash content are portions of the solid residue. Acid insoluble residue (AIR) is calculated by Equation 5.5:

$$\%AIR = (Weight\ dry\ sample - Weight\ crucible) * 100 / ODWsample \quad (5.5)$$

Following acid insoluble lignin (AIL) is calculated by Equation 5.6:

$$\%AIL = \%AIR - \%Ash \quad (5.6)$$

5.3.4.2 Calculation for cellulose and hemicellulose

In order to calculate cellulose and hemicellulose, the calculations from NREL (2007a) are used. Cellulose contains cellubiose and glucose while hemicellulose contains xylose, galactose, arabinose and mannose. By using the HPLC, the quantities of the above mentioned sugars after the end of process mentioned in 5.3.3 were measured. The percentage of each sugar recovered is calculated after dilute acid hydrolysis by using Equation 5.7:

$$\%R\ sugar = (C\ detected\ /\ C\ known)*100 \quad (5.7)$$

Where:

C detected = concentration detected by HPLC

C known = known concentration of sugar before hydrolysis=10 g/L

In order to correct the corresponding sugar concentration values obtained by HPLC, it is used %R sugar and it is calculated by Equation 5.8:

$$C_x = (CHPLC * dilution\ factor)/ (\%R\ sugar/100) \quad (5.8)$$

Where:

C_{HPLC} = concentration of sugar as determined by HPLC, mg/ml

%R sugar = recovery of specific SRS component

C_x = concentration in mg/mL of a sugar in the hydrolysed sample after correction for loss.

Dilution factor = 200

The concentration of polymeric sugars can be calculated from the concentration of monomeric sugars by using an anhydro correction of 0.88 for C-5 sugars (xylose

and arabinose) and a correction of 0.90 for C-6 sugars (glucose, cellobiose, galactose and mannose). Equation 5.9 is used for the calculation:

$$Canhydro = C_x * \text{Anhydro correction} \quad (5.9)$$

So the percentage of each sugar is calculated by formula 5.10:

$$\%Sugar = Canhydro * V\ filtrate * (1g/1000mg) * 100 / ODW \quad (5.10)$$

Where:

V filtrate = volume of filtrate = 87ml

5.3.4.3 Calculation for ash content

Ash content is calculated by using Equation 5.11:

$$\%Ash = (\text{Weight crucible plus ash} - \text{Weight crucible}) * 100 / ODWsample \quad (5.11)$$

5.4 Pre-treatment

Pre-treatment methods involved in this work include acid treatment (sulphuric acid, nitric acid and hydrochloric acid), steam treatment, microwave treatment, combination of two steps treatment and acid-impregnated steam or microwave treatment. The detailed procedures are mentioned in the following sub-sections. Following every treatment, the samples were washed with deionised water until the pH of the filtrate remained constant at an approximate value of 4.5. The pre-treated samples were drained and air-dried overnight to final 45% moisture content.

After the biomass has been treated with one or a combination of above mentioned methods, enzymatic hydrolysis with the obtained biomass (0.5 g dry weight basis) was carried out. The cellulase used for each reaction was 60 FPU/g. Samples were taken after 24 h of hydrolysis. Sample liquid was analyzed using the HPLC for hemicllulose sugars and UV - spectrophotometer for soluble lignin concentration.

The residues were analysis following the NERL standard procedure in order to detect the content of cellulose, lignin, hemicellose and ash content. The liquid product was hydrolysed with enzyme for further analysis of glucose yield. Design Expert was used for experiment setup design and data analysis.

5.4.1 Acid treatment

Sulphuric acid was obtained from 98% concentration solution, nitric acid from 72% concentration, and chloric acid from 70% concentration. The concentrated acid was mixed with DI water in order to obtained 4% solution. Then biomass and dilute acid (4%) were mixed at a solid/liquid ratio of 0.5 g in 50 mL. The mixed solution was place in a water bath at 60 °C for 180 min. After 180 min, the mixed biomass-acid solution was filtered with a vacuum filter. The treated biomass was washed until the pH was higher than 4.5. All the liquor was collected and well mixed to determine released sugars. When the concentration was too high, liquor was diluted for the best performance of analysis.

5.4.2 Steam treatment/ microwave treatment

Steam treatment at 121 °C (or 134 °C) and microwave treatment at 700 W are also carried out without any added chemicals. Biomass and DI water were mixed at a solid/liquid ratio of 0.5 g in 50 mL in an open beaker and placed in the centre of autoclave or a rotating circular glass plate in the microwave. Steam treatment was undertaken in an autoclave at a constant temperature (121 °C or 134 °C). The operation of autoclave follows the standard methods indicated in the instruction from the supplier. Microwave treatment was carried out in a domestic microwave oven (Matsui TS106WH) at full power output of 700 W for 2 min (Zhu *et al.*, 2006). The idea was to speed the reaction at high temperature inside the microwave oven and to save time in comparison with the steam treatment. A few bubbles started to appear on the surface of the material indicating the onset of boiling.

5.4.3Two-step of acid treatment followed by steam treatment

In addition to one step treatment as mentioned above, two different pre-treatment methods were combined by treating the BMSW fraction with 4% H_2SO_4 for 180 min at 60 °C first followed by steam treatment at 121 °C for 15 min and microwave treatment at 700 W for 2 min. After 3 h acid adsorption, the biomass was drained in order to remove the excess acid. After the steam and microwave treatment, the sample was washed with deionised water until the pH of the filtrate remained constant at an approximate value of 4.5. The pre-treated samples were drained and air-dried overnight to final 45% moisture content.

5.4.4 Acid-impregnated steam treatment

5.4.4.1 Procedures

Two different pre-treatment called acid impregnated steam treatment or microwave treatment were also carried out in this work. For these two acid impregnated treatment methods, 4% sulphuric acid is added into the reaction glass with biomass sample, and then the contained is put into autoclave or microwave for reaction directly. After the steam and microwave treatment, the sample was washed with deionised water until the pH of the filtrate remained constant at an approximate value of 4.5. The pre-treated samples were drained and air-dried overnight to final 45% moisture content.

5.4.4.2 Optimisation

A number of factors that may affect the process are studied, including residential time, acid concentration, and temperature. 'Design expert' software package is introduced in this work in order to study the effects and combined effects from these factors. Level of factors is carefully selected based on the literature review and experimental results and input to the software programme, in order to generate an experimental setup. Then, eight runs of experimental work are carried out according to the setup shown in Table 5.2 (below).

After the experiments, samples are obtained and processed following the biomass composition analytic procedures mentioned in Section 5.2. The amount of lignin and hemicellulose that have been removed are calculated by using HPLC and UV - spectrophotometer. The experimental results are imported to the software package to analyse the results of the experiments by using the analysis of variance (ANOVA). ANOVA is a technique that uses p-value in order to examine the significance of the effect of the main factors or the interactions of them. P-value is the probability that shows the significance of a factor that means the rejection of the null hypothesis that is that all the factors have the same effect in the experiments. If the p-value is smaller than 0.05 then the effect of the factor or the interaction of the factors should be considered as significant. Certainly if the p-value is smaller than the limit of 0.0001 then the factor or the interaction of the factors is considered very significant. On the other hand when the p-value is higher than 0.10 then the factor itself or the interaction between factors should not be considered significant (Montgomery and Runger, 2004).

Table 5.2 Experimental setup according to design expert package programme

Run	**Factor A** **Acid concentration (%)**	**Factor B** **Residential time (min)**	**Factor C** **Temperature (°C)**
1	4.0	60	134
2	0.4	10	121
3	4.0	10	121
4	0.4	60	134
5	0.4	10	134
6	0.4	60	121
7	4.0	60	121
8	4.0	10	134

5.5 Hydrolysis

5.5.1 Enzymatic hydrolysis

Following pre-treatment of the selected BMSW fractions, enzymatic hydrolysis is carried out to obtain glucose. The procedures are modified from NREL standard procedures according to substrate concentration and enzyme loading when a

specific condition is required. Enzymatic hydrolysis is carried out at an incubator at 50 °C.

The first procedure is to prepare the reagents required for the experiment. The reagents include biomass sample, sodium citrate buffer, cellulase, Beta-glucosidase, puromycin and water. Most of the reagents are obtained from SIGMA. The solution for sodium citrate buffer with pH 4.8 and 0.1 M, was made from citric acid and sodium citrate. For example, for 500 mL solution, 10.505 g citrate acid and 14.705 g sodium citrate was weighted, and then water was diluted till 500 mL The pH of the solution was adjusted to 4.8 using sodium citrate.

To each vial is added an appropriate volume of the cellulase enzyme preparation to equal approximately 60 FPU/g cellulose and the appropriate volume of β-glucosidase enzyme to equal 64p NPGU/g cellulose. Puromycin is also added to prevent the microorganism growth during the course of reaction. The amount of puromycin is 10 mg/mL.

During the experimental work, enzymatic hydrolysis of 0.5 g of pre-hydrolysed substrate on a 105 °C dry weight basis was carried out at 50 °C in vials (50 ml) placed in an incubator at 68 rpm for 96 h. The liquor was kept at pH = 4.8 using 0.1 M citrate acid-sodium citrate buffer.

Samples are taken at certain time defined according to every experimental plan. Glucose is determined by using HPLC. Before the samples are injected into the detector, samples are filtered through 0.45 μm membrane film and diluted 100 times.

The method to calculate the glucose yield is presented by NREL (1995). The chemical reaction $(C_6H_{10}O_5)n + nH_2O \rightarrow (C_6H_{12}O_6)n$ presents the conversion of cellulose to glucose. Glucose yield as a percentage of the theoretical yield was calculated by using equation 5.12:

$$\text{Glucose yield\%} = [\text{Glucose}] * 100 / (1.11 * f * [\text{Biomass}]) \quad (5.12)$$

Where:

[Glucose] = glucose concentration (g/L)

[Biomass] = dry biomass conc. following the enzymatic hydrolysis = 5 or 15 g/L

f = cellulose quantity in dry biomass (g/g)

1.11 = factor corresponds to mass balance of the conversion of cellulose to sugar.

5.5.2 Enzyme selection

Two enzymes, *T. viride* and *T. reesei* (Cellulase from *T. viride* and cellulase from *T. reesei* ATCC26921, respectively; Sigma, UK) are separately used as received in combination with the enzyme beta-glucosidase (Sigma, UK). Possible differences in the cellulase activity of the enzymes (Melander et al., 2006) were measured by the Filter Paper assay developed by Mandels et al. (Mandels *et al.*, 1971), and the activity was expressed in terms of Filter Paper Units (FPU). The β-glucosidase activity was measured by the PNPG assay (Paquot and Thonart, 1982), and reported as PNPG units (PNPGU). All the experiments were performed at enzyme to substrate ratios of 60 FPU g^{-1} substrate (Nguyen and Saddler, 1991) and 64 PNPGU g^{-1} substrate, and 10 mg*ml^{-1} puromycin for 96 h. Samples were withdrawn from the reaction media after 24 h for pre-hydrolysis treatment comparison and at 2, 5, 9, 24, 48, 72, and 96 h for kinetic studies. Sugar concentrations were determined routinely from centrifuged samples (13,000 rpm, 5 min) using HPLC. Glucose yield as percentage of the theoretical yield [percentage digestibility, obtained from the equation which involves the transfer of cellulose to sugar $(C_6H_{10}O_5)n + nH_2O = (C_6H_{12}O_6)n$ was computed by using the formula given by the National Renewable Energy Laboratory (Standard Biomass Analytical Procedures).

5.5.3 Hydrolysis optimisation

Individual factors that affect the hydrolysis process were studied. Experimental work was carried out by changing one factor's variation following the enzymatic

hydrolysis process mentioned in Section 5.5.1. These factors were selected according to literature that suggested the importance, including particle size, substrate concentration, enzyme loading, β-glucosidase loading, temperature, and pH value.

5.5.3.1 Experimental design

Design Expert 7.1 software package was selected for the design of the experiments aimed at identifying the key parameter for hydrolysis process. It is a highly efficient package that uses factorial methods to minimise the number of experiments required. It is used to perform statistical analysis of the experiment data and plot 2D and 3D graphics that show the interaction between factors. The software is especially useful to optimise of the design process. (Design Expert 7.1, 2007)

The reason for using this package for this work is that there are 6 factors and each factor has 2 levels, i.e. each factor can take two values: (1) particle size 0.2 and 1.2 mm, (2) substrate concentration 5 and 15 g/L, (3) cellulase concentration 10 and 100 FPU/g substrate, (4) β-glucosidase concentration 10 and 100 PNPGU/g substrate, (5) pH 3.7 and 5.0 and (6) temperature 37 °C and 50 °C. In general, runs of an experiment are conducted at all combinations of factors and levels. If the reaction temperature and the reaction time are to be considered in order to obtain a better yield of the process, reaction time and reaction temperature are the factors of the experiment. If the reaction temperature has a range of values between 80 and 100 °C and the reaction time between 45 and 60 min, those values are the levels of the factors, in this case, two levels for each factor. Therefore, this means that the experiment will have four different combinations in order to cover all the cases (Montgomery and Runger, 2004). Thus in this study 6 factors with each having 2 levels to be considered would require 2^6, that is 64 runs. However, in order to minimise the number of runs to reduce time and cost, Design Expert 7.1 software was introduced. Instead of choosing 64 runs, half fractional design gave 32 runs. This choice works on the main effects that are identified as the most important. This design is as good as the full factorial design and it has the advantage of saving a large number of runs (Design Expert 7.1, 2007). Following, Table 4.1 shows the

details of the runs that were carried out.

5.5.3.2 Analysis of results

The experimental results are imported to the software package to analyze the results of the experiments by using the analysis of variance (ANOVA), which has detailed in Section 5.5.2.

Table 5.3: Design of experiments

Run	Particle size (mm)	Substrate concentration (g/L)	Cellulase concentration (FPU/g substrate)	β-glucosidase concentration (PNPGU/g substrate)	pH	Temperature (°C)
1	0.2	5	100	100	3.7	37
2	1.2	15	100	100	5.0	50
3	0.2	5	100	100	5.0	50
4	1.2	5	100	10	5.0	50
5	1.2	5	100	100	3.7	50
6	1.2	15	10	10	5.0	50
7	0.2	5	10	10	3.7	37
8	0.2	5	100	10	5.0	37
9	1.2	5	10	100	3.7	37
10	0.2	5	10	100	3.7	37
11	0.2	15	100	10	3.7	37
12	0.2	15	10	100	3.7	37
13	1.2	5	10	100	5.0	50
14	1.2	15	100	100	3.7	37
15	0.2	15	10	10	3.7	50
16	1.2	15	100	10	5.0	37
17	0.2	15	10	100	5.0	50
18	0.2	15	100	10	5.0	50
19	1.2	15	10	100	3.7	50
20	1.2	15	10	100	5.0	37
21	1.2	5	10	10	5.0	37
22	0.2	15	100	100	5.0	37
23	1.2	15	100	10	3.7	50
24	0.2	5	10	10	5.0	50
25	1.2	5	100	10	3.7	37
26	0.2	5	100	10	3.7	50
27	0.2	5	10	100	5.0	37
28	1.2	5	100	100	5.0	37
29	1.2	15	10	10	3.7	37
30	1.2	5	10	10	3.7	50
31	0.2	15	10	10	5.0	37
32	0.2	15	100	100	3.7	50

5.5.4 Cellulase-Cellulose adsorption

The hydrolysis experimental work follows the procedures mentioned in Section 5.5.3. For each run, samples are taken at time 0, 0.5 min, 1 min, 1.5 min, 2 min, 3 min, 5 min, 10 min, 30 min, 60 min, 2 h, 5 h, 9 h, 24 h, 48 h, 72 h and 96 h. For this study, the main focus is to measure the adsorbed protein content. In this work, free protein is measured by using UV spectrophotometer and the adsorbed protein is obtained by using the total protein substitute to the amount of free protein measured.

5.6 Analytic methods

5.6.1 UV-visible spectrophotometer

In this work, the model used was Model: UV - spectrophotometer and the UV length were 250 nm.

In UV-Visible spectroscopy, the molecules of the diluted substances absorb electromagnetic radiation. Following, Figure 5.2 shows the UV-visible absorption spectrophotometer dual beam.

Figure 5.2: Conceptual diagrams of absorption spectrometer dual beam. L = light source(s), M = monochromator, C = chopper, B = beam splitter, R = reference sample, S = test sample and D = detector. (Tranter, 1999)

The polychromatic radiation passes through the monochromator and only a particular wavelength gets out. Then the light is separated into equivalent beams by using choppers or rotating mirrors. One beam goes to the sample and the other to the reference. Sample and reference are in cuvettes because they are transparent to the light. Generally in most instruments the two beams are remerged in a single optical path to the same detector where the concentration of the compounds of the sample are measured and then transferred in a computer (Tranter, 1999).

Standard protein solution was prepared and measured for calibration purpose with different concentrations (0.0001 g/ml, 0.0002 g/ml, 0.0003 g/ml and 0.0004 g/ml). Calibration curves were produced according to the results from the standard solutions.

The results on the computer screen connected to UV spectrophotometer are manually recorded and compared with the calibration curve, giving the concentration of free protein in each solution. The amounts of adsorbed protein are obtained by substituting the total amount of protein before reaction to the amount of free protein measured.

5.6.2 HPLC Analysis

In this work, the analysis of sugars were carried out using an High Performance Liquid Chromatograph (HPLC) (model: PerkinElmer series 200) with a refractive index detector. The column used was the C18 type. A 100% pure HPLC water was selected as solvent according to the requirement of column. The flow rate was set at 1.0 ml/min.

Standard sugar solutions including glucose, xylose, mannose, glactose and arabinose were prepared with different concentration (0.1 g/L, 0.2 g/L, 0.5g/L and 1.0 g/L). The solutions were then placed in the autosampler for HPLC analysis. Different peaks were appeared in chromatographic files that were saved in the computer automatically. The results were read according to the data quantitative process and drawn in a calibration curve. This curve was then used for reading the experimental data through the whole work.

The components of a basic HPLC system were shown in the simple diagram 5.3. The following describes the way HPLC is performed based on Figure 5.3. The Mobile Phase solvent contained in the solvent reservoir (left) is delivered by a high pressure pump (also called solvent manager) at controlled flow rate, usually in mm/min, to the HPLC column. At the left-end of the column, an autosampler injects the sample in the solvent. Solvent and sample travel through the HPLC column that contains a chromatographic packing material. The separation of the compounds included in the sample is achieved at this stage. At the right end of the column lies a detector that recognises the separated compounds as they come out. There exists a range of detectors that can be used depending on the characteristics of the

compounds, including UV detector, refractive index detector, fluorescent detector and lightscattering detector. Solvent and sample exiting the detector are disposed to waste. At the end of the process, a computer connected to the detector displays the chromatogram that is used to recognise and calculate the concentration of the compounds.

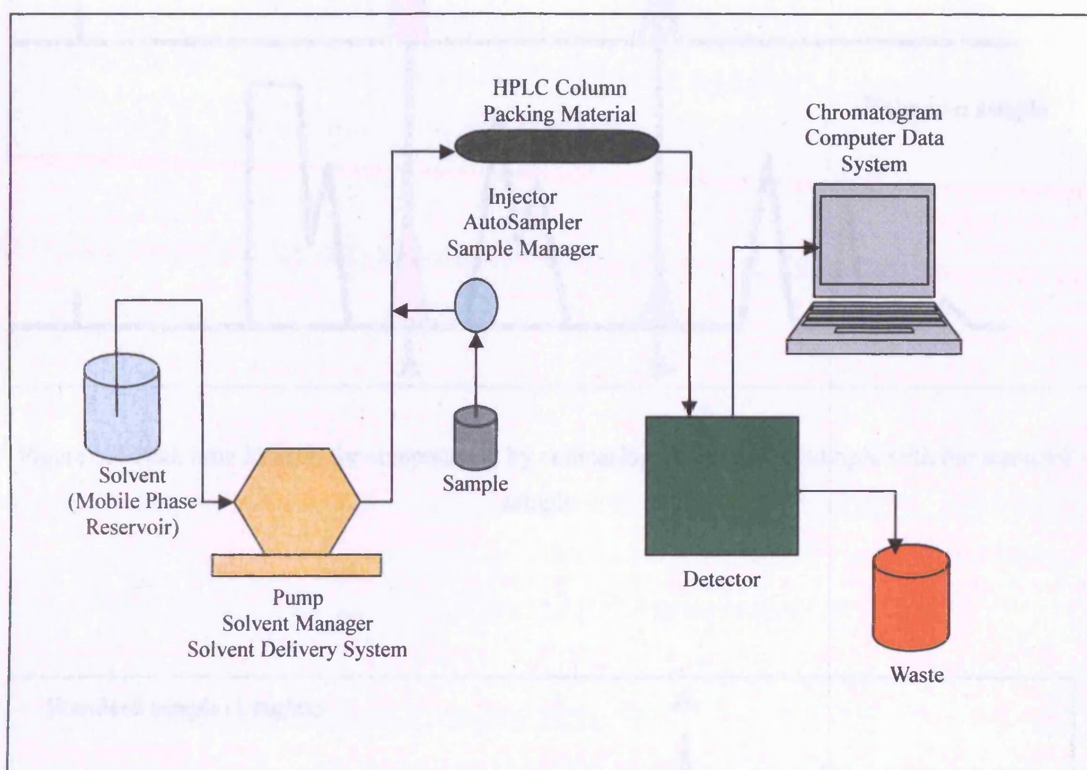


Figure 5.3: HPLC diagram

In order to identify the components of a given sample, at a certain retention time, qualitative analysis were carried out. As the following Figure 5.4 shows, the component A in the unknown sample will elute the same time as the standard sample. In this work, standard solution of each single sugar is used to determine the peak time.

Quantitative analysis are used to determine the concentration of the components in a sample, according to the peak area (or height) is proportional to the concentration (or amount) of the component. In this work, the concentration of each sugar is determined by comparing the peak height with that of standard sample as shown in Figure 5.5.

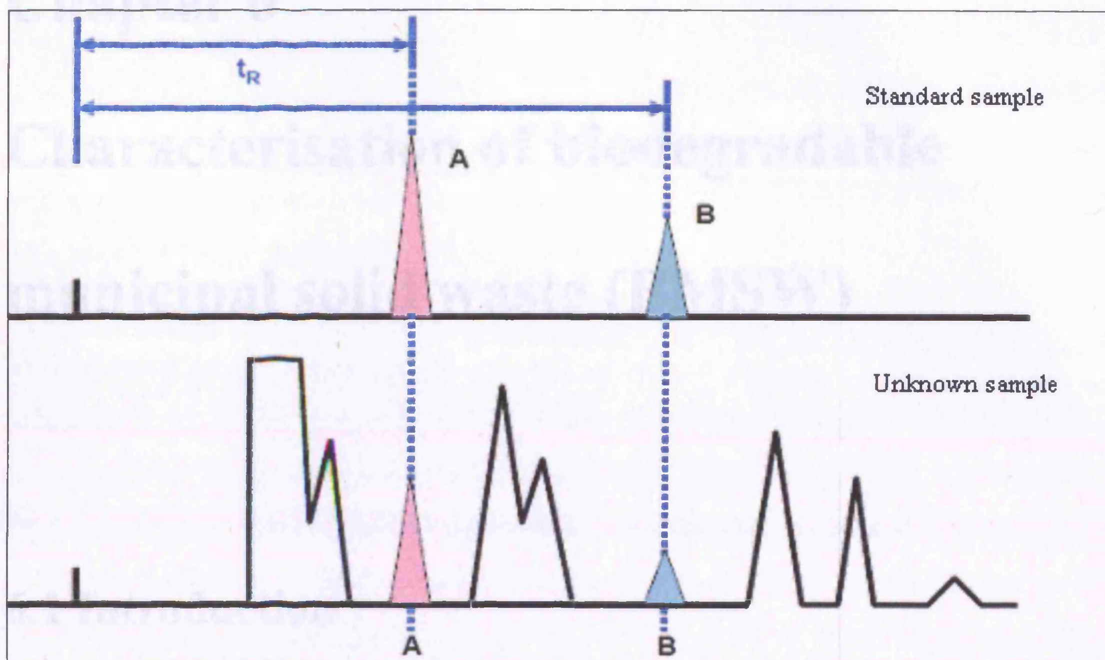


Figure 5.4 Peak time identify for compound A by comparing the unknown sample with the standard sample

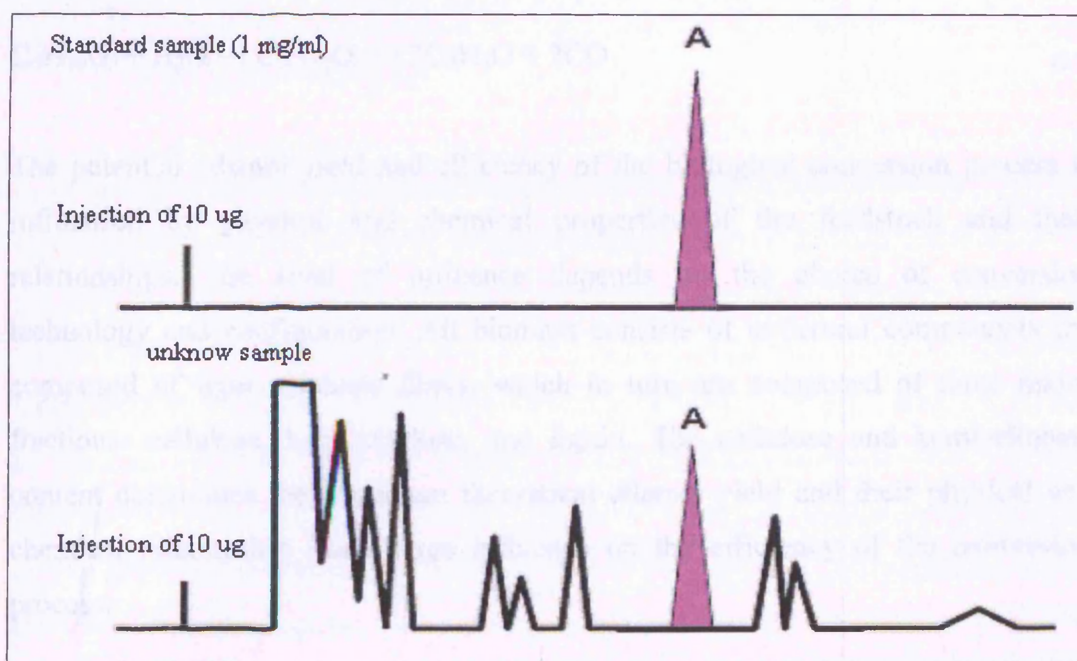


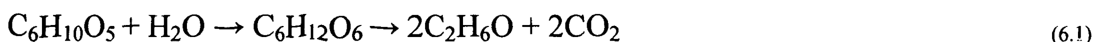
Figure 5.5 Comparing the peak height of unknown sample with the standard sample for compound A.

Chapter 6

Characterisation of biodegradable municipal solid waste (BMSW)

6.1 Introduction

As mentioned in Chapter 2, biomass is defined as renewable organic materials, such as wood, agricultural crops or wastes, and municipal wastes, especially when used as a source of fuel or energy. Biomass contains cellulose, hemi-cellulose and lignin. It converts to ethanol via glucose and has the following reaction formula:



$$C_6H_{10}O_5 + H_2O \rightarrow C_6H_{12}O_6 \rightarrow 2C_2H_6O + 2CO_2 \quad (6.1)$$

The potential ethanol yield and efficiency of the biological conversion process is influenced by physical and chemical properties of the feedstock and their relationships. The level of influence depends on the choice of conversion technology and configuration. All biomass consists of structural components are composed of lignocellulosic fibers, which in turn are composed of three major fractions: cellulose, hemicellulose, and lignin. The cellulose and hemicellulose content determines the maximum theoretical ethanol yield and their physical and chemical relationship has a large influence on the efficiency of the conversion process.

Most research on characterisation focus on the primary biomass such as agricultural crops or paper sludge. However, little information can be obtained on waste biomass especially on organic waste. In this chapter, the characterisation of waste as

biomass sources and chemical compositions of the selected samples is studied with the aim of understanding the possibility of using MSW as biomass feedstock. It is also concerned with understanding how different types of biomass affect the downstream chemical processes (pre-hydrolysis and enzymatic hydrolysis). This analytical methods used for the study were modified from the US National Renewable Energy Laboratory (NREL).

In this work, the composition of selected wastes is analysed in three different categories: (i) moisture content, (ii) chemical composition and (iii) cellulose structure. The compositions of each category are shown in the three sub-sections that follow with relevant tables and figures. For each type of model waste, the chemical composition analysis includes cellulose content, acid soluble lignin, acid insoluble lignin, hemicellulose, and ash content; the analysis of cellulose structure includes crystallinity, bulk density and particle size. All the measurements are on dry basis, i.e. after 24 h drying in oven at 105 °C, except for the moisture content studies. Detailed methodologies on each of the analysis are described in Chapter 5.

6.2 Moisture content

High moisture content is responsible for increased shipping and handling costs and can accelerate degradation. High moisture content biomass has significantly lower net energy density than the one with low moisture content by mass due to the weight of the water, but also by volume due to the energy required to evaporate the water. Indeed, transport of biomass is not very efficient as a significant proportion of the load is water. Storage of high moisture content biomass is also poorly efficient, with less net energy available, but also it brings additional problems such as a greater risk of composting, causing loss of biomass and potentially a fire risk from elevated temperatures and mould formation. Moreover, moisture content also influences the need for dewatering and drying prior to the bioethanol production, which is one of the most energy intensive processes in the full production cycle.

The optimum moisture content depends on the chosen conversion technology and the initial moisture content of the feedstock. In this section, moisture contents were measured for each type of waste before any further processing as described in details in chapter 3. The moisture percentage results are presented in Table 6.1.

Table 6.1 Moisture percentage of raw waste materials at 105 °C

Raw Material	Moisture (%)
CP	15
PP	9
GR	24
NP	10
SP	14

The moisture contents shown in Table 6.1 represent the average value of a large number of data points taken to account for seasonal variations. Looking at the results in Table 6.1 it becomes obvious that grass (GR), as the representative of green organic waste, has the highest moisture content of 24%, followed by carrot peelings (CP, 15%), scrap paper (SP, 14%), newspaper (NP, 10%) and potato peelings (PP, 9%). The highest moisture content in grass can be due to excess water as the grass was directly collected from a garden where water is adsorbed from roots. Green waste, such as grass which often generated from gardens directly, has different moisture content at various seasons. For instance, due to the rain, the moisture content is often higher in spring and summer compared with autumn and winter, which should be considered when transporting and storing the biomass. The moisture content for newspaper and scrap paper are generally lower than grass, because these papers have been processed in the factory. Fresh vegetables and fruit are also high in water content, generally greater than 70%, and frequently greater than 85% (Dimambro *et al.*, 2007). This is consistent with results reported for the total moisture content for mixed KOW waste which is estimated to be about 70%.

The higher moisture content of organic waste, especially for KOW, is a disadvantage for thermochemical processes, which are more efficient when processes dehydrated feedstocks (CPA, 2005). The high moisture content in such waste results in a lower fermentable content. It also results in low substrate concentrations and thus diluted product streams which even after successful

pretreatment and hydrolysis (Claassen *et al.*, 2000). This situation could be improved by either increasing the concentration with a penalty from inhibitor increase, or to mix the waste with other more dehydrated feedstocks.

In this work, for the purposes of experimental replicate and storage, all the samples were dried overnight in oven at 105 °C. Since all the experimental works are based on dry basis, moisture content is not considered as a limiting factor for the further process (pre-treatment, hydrolysis and fermentation). However, it must be noted that in the large scale plant the process always starts from the wet biomass, where the drying process is energy intensive as mentioned above. It has been suggested that high initial moisture content may result in a reduction in enzyme yield due to static hindrance of the growth of the organisms through reduction in interparticle spaces and impaired O_2 transfer (Nigam, 1990; Ramesh and Lonsane, 1990; Xavier and Lonsane, 1994). However, Lonsane *et al.* (1985) suggests that lower moisture content also results in a decline in enzyme yield, which could be due to suboptimal growth, less substrate swelling and high water tension.

As mentioned at the beginning of this section, higher moisture content is a disadvantage for transportation and storage, but lower moisture content means more water required in the chemical process such as pretreatment. Therefore, in order to assess the pros and cons of moisture content, it is important to consider the whole process as a system quantifying how much moisture content affects on each step or process.

6.3 Chemical composition

The feedstock composition is key factor of the yields of ethanol, and thorough data are needed in order to convince financial institutions that the feedstock quality will be as forecasted throughout the economic life of the plant. For bioethanol, maintaining cellulose and hemicellulose content is critical to achieving target yields, whereas changes in lignin and ash content can impact downstream operations such as the boiler/generator.

The following chemical composition analyses are based on 10 g dry weight waste biomass. These include cellulose, hemicellulose, lignin (i.e. acid insoluble lignin (AIL) and acid soluble lignin (ASL)) and ash content. The analyses are carried out following the detailed methodologies in Section 5.2. The results are shown in Table 6.2.

Table 6.2 Raw material composition on dry weight basis (105 °C)

Biomass	Cellulose (%)	AIL (%)	ASL (%)	Hemicellulose (%)	Ash content (%)	Total (%)
CP	42.41	11.69	14.09	23.30	9.97	100±1.46
PP	21.84	8.79	11.93	42.78	9.04	100±3.62
GR	22.50	5.40	18.45	39.59	16.57	100±2.51
NP	44.21	11.49	14.25	24.36	6.78	100±1.09
SP	63.76	2.74	11.34	6.83	16.76	100±1.43

Note: *CP- carrot peelings, PP-potato peelings, GR-grass, NP-newspaper, SP-scrap paper.*

Cellulose is a linear polymer of glucose. The orientation of the linkages and additional hydrogen bonding make the polymer rigid and difficult to break. In hydrolysis the polysaccharide is broken down to free sugar molecules by the addition of water. This is also called saccharification. The product, glucose, is a six-carbon sugar or hexose.

Hemicellulose consists of short highly branched chains of various sugars: mainly xylose (five-carbon), and further arabinose (five-carbon), galactose, glucose and mannose (both six-carbon). It also contains smaller amounts of non-sugars such as acetyl groups. Hemicellulose, because of its branched, amorphous nature, is relatively easy to hydrolyse (Hamelinck *et al.*, 2005).

Lignin is present in all lignocellulosic biomass. Any ethanol production process will have lignin as a residue. It is a large complex polymer of phenylpropane and methoxy groups, a non-carbohydrate polyphenolic substance that encrusts the cell walls and cements the cells together. It is degradable by only few organisms, into higher value products such as organic acids, phenols and vanillin. Via chemical processes valuable fuel additives may be produced. Although these by-products can significantly enhance the competitiveness of ethanol technology, the present study deploys lignin

only for power generation (Hamelinck *et al.*, 2005).

From Table 6.2, it is obvious that highest cellulose content is found in scrap paper (63.76%), followed by newspaper (44.21%), carrot peelings (42.41%), grass (22.50%), and potato peelings (21.84%). It can be deduced that scrap paper has the largest amount of cellulose while potato peelings has the smallest. But there are the opposite results regarding the largest amount of hemicellulose which is in potato peelings biomass (42.78%) and the smallest which is in scrap paper (6.83%). The hemicellulose content indicates how much by-product (xylose, galactose, mannose and arabionose) will be produced after hydrolysis process. It is known that the combination of hemicellulose and lignin provides a protective sheath around the cellulose, which must be modified or removed before efficient hydrolysis of cellulose can occur, and the crystalline structure of cellulose makes it highly insoluble and resistant to attack. To economically hydrolyse (hemi) cellulose, more advanced pre-treatment technologies are required than in processing sugar or starch crops. After the cellulose and hemicellulose have been saccharified, the remainder of the ethanol production process is similar to grain-ethanol. The highest lignin content was found in newspaper samples (AIL 11.49% plus ASL 14.25%), indicating that the newspaper will be most difficult to pre-treat, as the main purpose of pre-treatment is to remove lignin in the biomass. Scrap paper, with the lowest lignin content (AIL 2.74% plus ASL 11.34%), is expected to provide easier access to the cellulose content in the material. The acid soluble lignin, ASL, is higher than the acid insoluble lignin, AIL, in each type of the waste biomass considered in this study. This indicates that the acid pre-treatment method can not break down the lignin structure completely. 50% reduction might be the maximum expected.

Fruit and vegetables contain lower levels of cellulose and lignin than wood; however there is a significant difference in composition between the pulp and the peelings of fruit or vegetables. The peelings contain higher proportions of lignin and lower proportions of carbohydrates. New research by WRAP (Waste and Resources Action Programme) has revealed that about half of the food thrown away by households in the UK is edible food, with the rest comprising of peelings, meat bones etc (WRAP, 2007). Therefore, since KOW waste may consist of either the peelings or whole fruit or vegetable and this has to be considered.

As a biomass sources, kitchen waste provides about 86% biodegradable matters. As kitchen waste accounts for about a quarter of total waste, it provides a very good biodegradable source. However, the high portion of lignin existing in the biomass requires some particular pre-treatment methods. With more than half of lignin are acid soluble, additives of acid in the pre-treatment is an alternative. With 42.4% cellulose from carrot peelings and 21.8% from potato peelings as shown in Table 4.2, 1000 g of KOW is expected to produce about 330 g glucose at maximum.

The higher lignin content of GOW waste implies the need for greater pre-treatment to separate the cellulose and hemicellulose components. However the lignin in grass is not as restrictive to microorganisms as the lignin in other components such as branches (EPA, 1999). Moisture content is estimated to be 17.7%; however, this must be treated with caution as other studies have estimated it to be 30% (CEC, 1999). The ethanol theoretical yield of about 420 L/dry tonne, derived using (DOE, 2007), is also much higher than that estimated by NREL of 192 L/dry tonne (OOE, 2000). This might be expained due to the variance of cellulose and moisture contents of GOW from different regions or seasons.

Table 6.5 presents the comparison of the results from research findings and from the literature into the biochemical composition of different PCW materials (newspaper and scrap paper). Most PCW materials are chemically processed to remove lignin from mixtures of hardwoods and softwoods. There are exceptions to this, most notably newsprint that is primarily mechanically processed spruce and pine, and therefore has the same composition on a dry-weight basis as native wood (Wyman, 1996). The pulping process alters the biomass structure of paper and card and a number of sources indicate that this could improve the conversion process and reduce the need for pre-treatment (Rivers and Emert, 1988; Clanet *et al.,* 1998). Enzymatic hydrolysis of separated paper has been shown to convert quickly and nearly completely to bioethanol and the lower levels of five carbon sugars reduce the dependence on higher hemicellulose hydrolysis efficiency (Dale and Musgrove, 2004). Different pre-treatment methods have been shown to improve the conversion process for different PCW materials and an optimal process would have to be developed based on site-specific samples (Rivers and Emert, 1988).

Taking into consideration the quality of paper for recycling, three PCW waste feedstocks can be broadly identified and their biochemical composition and theoretical ethanol yield estimated from table 6.5. Optimum PCW is assumed to be segregated high quality paper such as white office paper which has a higher proportion of polysaccharides and low proportion of lignin. Low demand PCW is assumed to be segregated corrugated cardboard which is towards the bottom of the hierarchy of recovered paper (CPI, 2007). The targeting of low-grade waste paper for ethanol production is a strategy currently being pursued by Universal Entech and Bio-Process Innovation (Dale and Musgrove, 2004). Mixed PCW is assumed to be at the bottom of the hierarchy of recovered paper (CPI, 2007). The prospects for recycling mixed paper are more limited, making it potentially available for other recovery options such as biomass source; however consideration of contamination issues would be required.

Table 6.3 Biochemical composition of kitchen organic waste materials (percentage wet weight)

Kitchen Organic waste materials	Cellulose	Hemicellulose	Lignin	Ash	Moisture Content	KOW waste proportion (% wet weight)
Carrot, raw [a]	3.9	1.3	23.9	8.3	88.3	0.3
Carrot peel [b]	42.4	23.3	25.8	10	15	0.9
Potato, raw, skin [b]	21.8	42.8	20.7	9	9	1.5
Potatoes, white, flesh and skin, raw [a]	0	0	13	5.1	81.6	4.5
Potatoes, white, flesh and skin, baked [a]	0	0	8.5	5.1	75.4	0.7
Potatoes, french fried, all types, salt not added in processing, frozen, oven-heated [a]	11.1	3.7	6.8	4.9	63.1	0.3
Potato chips, plain, salted [a]	17.2	5.7	4.5	4	2.3	0.4
Carrots, cooked, boiled, drained, with salt [a]	12.2	4.1	30.5	6.8	90.2	0.9

a Figures obtained from (USDA, 2007). Where the breakdown of values between mon- di- and poly- saccharide components is not know it is assumed to be in the ratio of 33/33/33, Where the breakdown of values between disaccharide and polysaccharide components is not know it is assumed to be in the ratio of 50/50. Where breakdown of values between polysaccharide components is not known, they have been assumed to be in the ratio of 75% cellulose and 25% hemicellulose.

b Figures obtained from the laboratory at UCL.

Table 6.4 Biochemical compositions of green organics waste materials (percentage wet weight)

GOW	Cellulose	Hemicellulose	Lignin	Ash	% Moisture Content
Leaf [b]	16.7	11.5	47.7	unknown	8.3
Leaf [c]	17.8	23.1	26.1	33.6	8.3
Grass [a]	22.5	39.59	23.95	16.57	24
Grass [b]	34.9	13.3	37.5	unknown	24
Branch [b]	39.3	20.4	36.2	unknown	10
Woody Yard Waste [d]	35.4	19.6	31.5	0.1	30
Hardwood [c]	28.4	17.9	14.5	0.4	5.4
Softwood [c]	35.1	17.1	18.6	0.8	6.5

a. *Figures from laboratory at UCL.*

b. *Figures from (EPA, 1999) Table 1, assumed to be stated in dry weight basis. Figures for Leaf, Grass and Branch adjusted for wet weight basis using assumed moisture content of 8.3%, 24%, and 10% respectively.*

c. *Figures from (Eklind and Kirchmann, 2000) Table 4, adjusted for ash content then for moisture stated in Table 1.*

d. *Figures from (Lissens et al., 2004) adjusted for an assumed moisture content of 30%.*

Table 6.5 Biochemical composition of paper & card waste materials (percentage mean dry weight)

No.	Material Fraction	Cellulose	Hemicellulose				Lignin	Ash
		Glucan	Galactan	Mannan	Xylan	Arabinan		
1	Newspaper[2]	44.2	24.4				25.7	6.8
2	Newsprint[4]	54.7	30.1				14.2	1.0
3	Newsprint[5]	48.5	9.0				23.9	-
4	Newsprint[8]	64.4	0.0	16.6	4.6	0.5	21.0	0.4
5	Newspaper[6]	44.3	0.6	4.9	5.2	0.6	29.3	3.5
6	Newspaper[7]	35.1	2.3	10.7	5.0	3.9	39.1	1.0
7	Newsprint[3]	63.8	0.6	5.0	5.3	0.6	21.3	3.5
8	Office Paper[5]	87.4	8.4				2.3	-
9	White Office Paper[7]	65.4	0.0	0.0	14.4	0.7	19	24.1
10	White Office Paper[1]	71.7	16.4				-	-
11	Scrap Office Paper[2]	63.8	6.8				14.1	16.8

Note: 1. Capek-Menard *et al.*, 1992; 2. UCL; 3. Kemppainen and Shonnardm, 2005; 4., Rivers and Emert, 1988; 5. EPA, 2005; 6. Eklind and Kirchman, 2000; 7. Foyle *et al.*, 2007

Mixed organic waste and BMSW

As calculated in Section 2.3, mixed Organic waste (MOW) accounts for approximately 34% of all MSW arisings in London. The combined moisture of KOW and GOW wastes is estimated to be 43.8%, i.e. close to the 45% reported in other studies (Compact Power, 2007). Tables 5.6 presents the consolidated estimate for mixed organic feedstocks, assumed to consist of an equal ratio of KOW and GOW, and mixed BMSW feedstocks, assumed to consist of an equal ratio of KOW, GOW, and mixed PCW.

Table 6.6 Biochemical composition of mixed waste feedstocks

Organic waste feedstocks	Cellulose (%)	Hemi-cellulose (%)	Lignin (%)	Ash (%)	Moisture Content (%)
Mixed Organics	19.6	11.7	18.9	11.6	43.8
Mixed BMSW	32.7	12.6	18.9	10.9	30.8

6.4 Other Quality Considerations

Apart from chemical composition, other factors affecting the conversion of lignocellulosic materials include non structural components, cellulose fiber crystallinity, and bulk density of materials, particle size, moisture content, and contamination. Nonstructural components, such as extractives and proteins can cause problems with biochemical conversion because of the presence of inhibitors or the raw materials for their production during pretreatment.

Experimental work has been carried out to determine some of these factors (i.e., crystallinity, bulk density and particle size). The results are summarised in Table 6.7.

Table 6.7 Cellulose properties: PP-potato peelings, CP- carrot peelings, GR-grass, NP-newspaper, SP-scrap paper

Biomass	Crystallinity (% of total cellulose)	Bulk density (g/cm^3)	Particle size (mm)
PP	21.2	0.72	0.2-1.2
CP	16.8	0.69	0.2-1.2
GR	37.3	0.35	0.2-1.2
NP	70.8	0.13	0.2-1.2
SP	67.4	0.10	0.2-1.2

6.4.1 Crystallinity

The crystallinity (CrI) of cellulose as determined by X-ray diffraction is considered to be a major deterrent to enzymatic hydrolysis by limiting cellulase accessibility to the beta-1, 4-glucosidic bonds. Assuming that the X-ray CrI is an accurate measure of cellulose order, substrates with higher CrI values may be expected to possess greater resistance to enzymatic hydrolysis.

Among of the biodegradable waste, the highest portion of crystallinity has been determined in paper and card waste, 70.8% for newspaper and 67.4% for scrap paper. This observation may indicate that kitchen waste with least crystallinity is expected to be converted more easily, followed by green waste. Cellulose content gives the amount of potential glucose/ethanol. The amount of Crystallinity content indicates how difficult to convert the cellulose. To achieve maximum product yield, it is not only necessary to remove lignin providing access to cellulose, but also to reduce crystalline regions to amorphous structure. As the main purpose of pre-treatment is to provide maximum access to cellulose during hydrolysis process, it is very important to select the methods that can meet the requirement of both lignin removal and crystallinity reduction.

Puri (1984) suggested that the crystallinity is not changed by chemical or biological methods. But Bhuiyan (2000) stated that crystallinity is increased by heat treatment of wood cellulose and almost twice as much crystallization as original was observed after heat treatment of spruce and buna under a highly moist condition than under the oven-dried condition. Although it has been suggested that the more accessible (amorphous) portion of cellulose degrades more easily than the less accessible

crystalline regions, it is still not clear whether the crystalline structure can be changed by treatment. Moreover, as suggested by Negro (Negro, 2003), during enzymatic hydrolysis the crystallinity of cellulose increases and subsequently becomes more resistant with the increase of hydrolysis reaction time. But the reasons response to this change, are still not clear if because of structure change, due to the removal of amorphous material resulting in accumulation of crystalline structures, or was attributed to the degradation and dissolution of easily accessible components of lignocellulose, namely, hemicellulose and lignin, thus resulting in a product with increased concentration of crystallites.

Although the enzyme preparations from various fungi have been shown to hydrolyse all forms of cellulose, it is suggested that the crystalline nature of cellulose should not be a deterrent to enzymatic hydrolysis. .This work will examine both chemical and heat treatment to identify how the crystallinity is changed in the following chapters by analysing both the residue chemical composition and liquid product after pre-treatment and hydrolysis process.

6.4.2 Bulk density

In its natural form, most biomass is difficult to utilise as a fuel because it is bulky, wet and dispersed (Balatinecz, 1986). Disadvantages of biomass as an energy source include inefficient transportation (high moisture content) and storage (large volumes required). Bulk density is defined as the mass of a unit volume of the product. Lower bulk density means larger volume for a given mass. It is affected by two factors: moisture content and molecule weight. In this work where all the samples are oven dried, the main focus is placed on molecule weight. Apart from the inefficient transportation and storage, bulk density also affects further chemical process. Taking enzymatic hydrolysis as an example, because the biomass with lower density has greater volume, it is unlikely that the enzyme can attack as large areas of cellulose as for biomass with higher density.

Table 6.7 lists the range of bulk densities for the selected model waste. However,

the data does not indicate the nature and composition, but shows a gain in density as the biomass is ground into smaller pieces and oven dried. The values in Table 6.7 are given as the average of three tests for each sample, which are 0.72 g/cm^3 for potato peelings, 0.69 g/cm^3 for carrot peelings, 0.35 g/cm^3 for grass, 0.13 g/cm^3 for newspaper, and 0.10 g/cm^3 for scrap paper. Highest bulk density is found in potato peelings, and lowest in newspaper, which is opposite to crystalline. For the same weight of biomass, the volume of total PCW waste is much larger than for kitchen waste. The relatively low bulk density of paper and card waste may be responsible for less contact between enzyme and cellulose as well as higher costs of transportation and storage. Some studies (Sokhansanja *et al.*, 2002; Suarez and Beaton, 2003) concerning the effect of bulk density on biomass storage and transportation exists in the literature. However, little information can be found regarding the influences on chemical process.

6.4.3 Particle size

The majority of domestic organic waste is found within the 6 mm – 80 mm size range and, where it is not source segregated, recovery equipment can be designed to separate out material of this size range from MSW, removing the majority of ferrous and non-ferrous metals and inert materials, such as glass, stones, batteries etc. (Compact Power, 2007). In this work, all the biomass feedstock are milled with the value ranging from 0.2 mm to 1.2 mm as shown in Table 6.7 (above).

Particle size is indicative of the macro-accessibility of the substrate and may be somewhat representative of substrate surface readily available for enzymatic hydrolysis. Particle size has also been considered to be a major deterrent to enzymatic hydrolysis by limiting accessibility to beta-1, 4-glucosidic bonds through restrictions on the surface area of the substrate. Therefore, as particle size decreased, a concomitant increase in conversion to either glucose or ethanol was expected as the surface area for growth is greater. On the other hand, the interparticle porosity is less, while with larger size, the porosity is greater (e.g. from 13.3% to 18.9% when particle size increases from 0/5mm to 1.0mm for biomass of corn (Chang, 1988), but the

saturated surface area is less. Moreover, small particle size will result in more weigh loss during pre-treatment process. Therefore, this work suggests that it may not be the case that smaller particle size results in higher product yield as stated in some literature (Lynd, 1996; Jones *et al.*, 2007). Further analysis in the following chapter will be conducted on the effects of particle size on the hydrolysis process.

6.4.4 Contamination

Toxins and other substances present in the feedstock can inhibit enzymatic hydrolysis. The contaminants can be broadly split into two types; physical and chemical. Physical contaminants are often present at levels that pose a significant risk to the process. Plastic, metal, glass and dirt can be present even from sorted materials in the form of staples, adhesives, tape, plastic liners, waxes, and polystyrene. Chemical contaminants include: colouring agents, bonding agents, heavy metals, printing ink, and glucose-consuming microorganisms.

It is usually considered as unfeasible to remove the chemical contaminants because the technology does not exist or it is uneconomical. One study claims that printer's ink however has no effect on either saccharification or fermentation (Rivers and Emert, 1988). Some heavy metals are necessary for fermentation but at high concentrations can inhibit yeast fermentation, particularly copper and chromium (Wooley *et al.*, 1999) as shown in table 6.8. However, tests have shown that acid treatment allows for the dissolution of heavy metals and chlorides from the feedstock (Wooley *et al.*, 1999), significantly reducing the level of heavy metals present in the subsequent liquid hydrolysate well below the concentrations that adversely affect the later fermentation step (Wooley *et al.*, 1999). Consideration would need to be made to the separation of the solids and liquids after pre-treatment, and the impact of high heavy metal content in the waste residues.

Microbial contamination of MSW is mainly of faecal origin such as nappies, pet litter and food. About 2.5% contamination microbial contamination is common with eggs and raw meat, the main pathogen sources (USGL, 2007). Microbes are heat sensitive

and are normally killed by temperatures in excess of 60 °C applied for 20 min (Dimambro, 2007).

The contaminations from the BMSW inhibit the microbes during enzymatic hydrolysis and fermentation, it is necessary to minimize it as much as possible before enzymatic hydrolysis. Therefore, during the selection of appropriate pre-treatment methods, consideration should be taken into account if the pre-treatment can remove or will bring the contaminations and how the contamination can be minimized before entering further process.

Table 6.8 Effects of heavy metals on fermentation by yeast (Wooley et al., 1999)

6.5 Conclusions

In this chapter, three major BMSW components: kitchen organic waste, green organic waste and paper and card waste have been classified and characterised according to (i) substrate composition, (ii) cellulose crystallinity, (iii) bulk density and (iv) particle size. The substrate composition indicates how much cellulose, lignin, hemicellulose each model waste has. The cellulose content shows the potential glucose/ethanol product yield. Based on the best available information on each waste category, the potential of biomass sources from BMSW are analysed. Other chemical composition such as lignin and hemicellulose indicates how easy of each model waste can be converted. These contents are also important information when selecting pre-treatment methods for the following chapter on pre-treatment methods.

Chapter 7
Pre-hydrolysis treatment process

7.1 Introduction

Pre-hydrolysis treatment, also termed pre-hydrolysis or pre-treatment, is used to alter the structure of cellulosic biomass to make it more accessible to the enzymatic conversion (Mosier *et al.*, 2005). As mentioned in Section 2.4), cellulose is difficult to hydrolyse for two main reasons (Chang and Tsao, 1983): (i) the linear homopolymer of anhydroglucose has a strong crystalline structure, (ii) cellulose fibres are usually surrounded by lignin which reduces the accessibility to hydrolytic enzymes. Efficient bioconversion of lignocellulosic materials to ethanol, therefore, requires some form of pre-treatment to disrupt the lignin barrier and to open the structure. An illustration is shown in Figure 7.1.

Pre-treatments usually have the following three purposes: (i) to disrupt the crystalline structure of cellulose; (ii) break the lignin seal; (iii), to increase the exposure of the substrate to the hydrolytic enzymes and thus increase glucose yield. Pre-treatment can also affect the structure of the biomass by solubilising or otherwise altering hemicelluloses, altering lignin structure, reducing cellulose crystallinity and increasing the available surface area and pore volume of the substrate. During pre-treatment, hemicelluloses may be hydrolysed to their monomeric constituents and lignin – hemicellulose - cellulose interactions partially disrupted (Ladisch, 1989; Day, 1989)

In this work, various promising pre-treatment methods (mentioned in Section 3.3) are tested on the selected biodegradable waste fraction. The main purpose is to investigate the effective method for waste biomass. The appropriate method, in this

case, acid-impregnated steam treatment, was further analysed by introducing ANOVA analysis. More details about ANOVA analysis were mentioned in Chapter 5 (Section 5.3). The optimal conditions of this pre-treatment method are given and used for further study.

Figure 7.1 Schematic of goals of pre-treatment on lignocellulosic material *(adapted from Hsu et al., 1980).*

7.2 Pre-hydrolysis techniques

Different pre-hydrolysis treatments were investigated, including dilute-acid pre-hydrolysis, steam treatment and microwave treatment or various combinations of two of them applied consecutively. A summary of the experimental variables and combinations of treatments are shown in Table 7.1. In the dilute acid treatment, the effect of sulphuric acid (H_2SO_4), nitric acid (HNO_3), and hydrochloric acid (HCl) was investigated. The results of glucose yield are shown in Figures 7.2 to 7.6. By comparing the different glucose yield produced from different pre-treated biomass samples, the most effective method is then selected for further analysis.

Table 7.1 Experimental conditions in pre-hydrolysis treatment of BMSW fraction selected in this study

Experiment	Dilute acid						Steam treatment		Microwave treatment		Enzymatic hydrolysis Cellulase (FPU/g)
	HNO_3 (%)	time (min)	HCl (%)	time (min)	H_2SO_4 (%)	time (min)	T (°C)	time (min)	Power (w)	Time (min)	
1	—	—	—	—	—	—	—	—	—	—	60
2	4	180	—	—	—	—	—	—	—	—	60
3	—	—	4	180	—	—	—	—	—	—	60
4	—	—	—	—	4	180	—	—	—	—	60
5	—	—	—	—	—	—	121	15	—	—	60
6	—	—	—	—	—	—	—	—	700	2	60
7	—	—	—	—	4	180	121	15	—	—	60
8	—	—	—	—	4	180	—	—	700	2	60
9	—	—	—	—	4	—	121	15	—	—	60
10	—	—	—	—	4	—	—	—	700	2	60

From Figure 7.2 to Figure 7.6, it is obvious that without any pre-treatment, potato peelings give the highest glucose yield (20%), followed by carrot peelings (20%), grass (18%), scrap paper (16%) and newspaper (20%). The conclusion that no pre-treatment of biomass results in a maximum of (20%) glucose yield agrees with other reported findings (such as those of Hamelinck *et al.* (2005)). However, a figure of 20% is not high enough for effective industrial processing. For this reason, this work is concerned with applying further pre-treatment to improve the glucose yield.

In order to assess the suitability of each pre-hydrolysis pre-treatment, for maximum solubilisation of hemicelluloses and subsequent enzymatic hydrolysis, the effect of pre-hydrolysis treatment of BMSW with the acid was investigated using H_2SO_4, HNO_3 and HCl (4%). Grass resulted in the highest glucose yield (42–49%), while CP, PP and SP, all resulted in lower, although similar, glucose yields (15–29%) for all dilute acid pre-hydrolysis treatments. NP resulted in the lowest glucose yields (5–10%). As the main purpose of pre-treatment is to improve glucose yield during hydrolysis process, it is expected that pre-treated biomass should give higher glucose comparing to the case without any pre-treatment. However, with 5-10% glucose yields only, the results from acid treated newspaper are contrary to the expectation. This may be explained by three reasons: (i) the chemical such as ink present in newspaper inhibits both pre-hydrolysis and hydrolysis process (Kim and Dale, 2004); (ii) as pre-treatment involves one washing step, part of cellulose might be lost during the washing; (iii) part of cellulose has been converted into glucose during acid treatment.

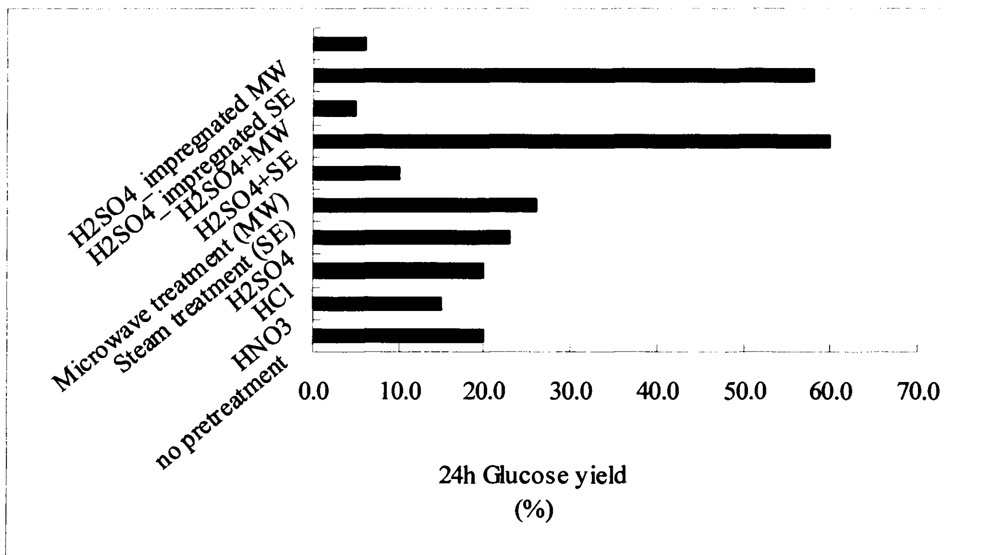


Figure 7.2 Effects of different pre-hydrolysis treatments of selected BMSW fractions in glucose yield after 24 h hydrolysis. Biomass: carrot peelings

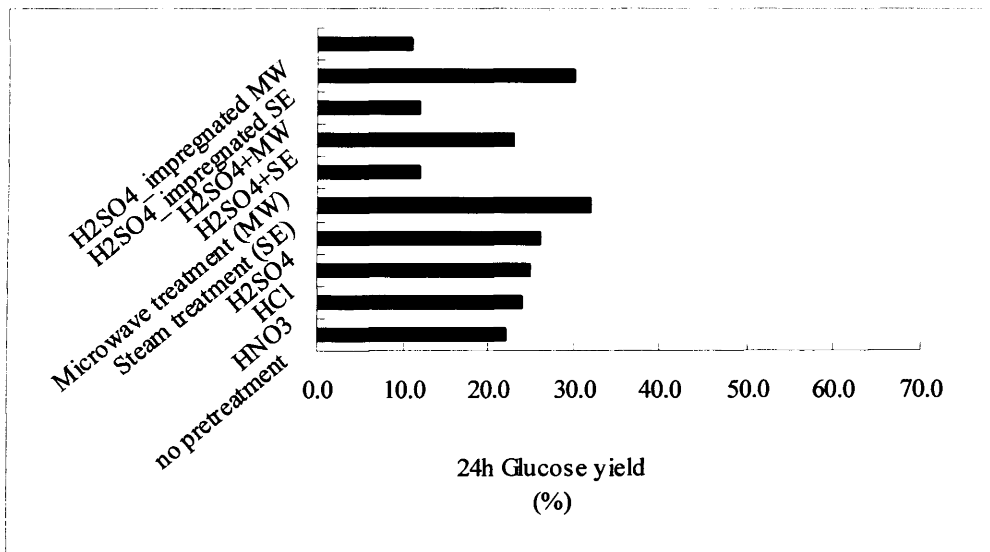


Figure 7.3 Effects of different pre-hydrolysis treatments of selected BMSW fractions in glucose yield after 24 h hydrolysis. Biomass: potato peelings

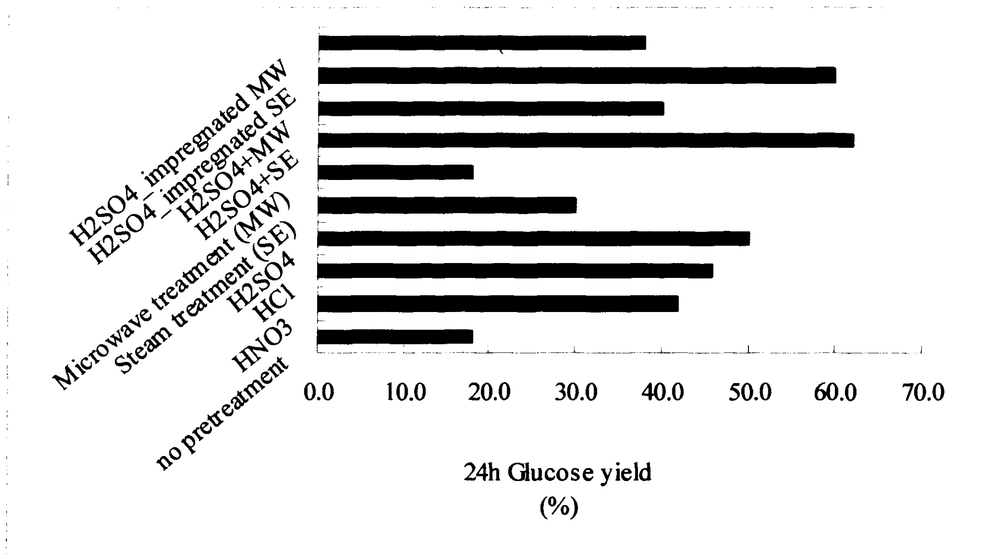


Figure 7.4 Effects of different pre-hydrolysis treatments of selected BMSW fractions in glucose yield after 24 h hydrolysis. Biomass: grass

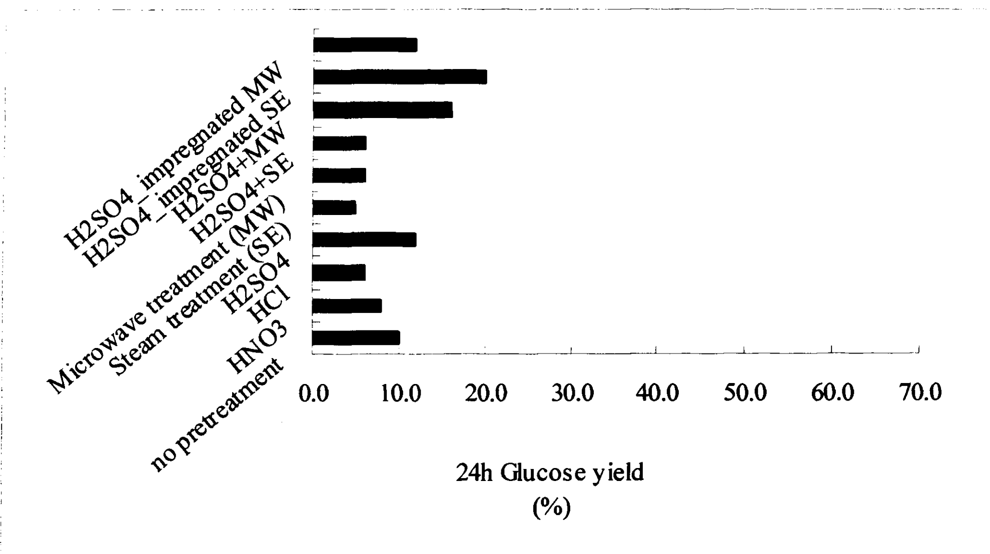


Figure 7.5 Effects of different pre-hydrolysis treatments of selected BMSW fractions in glucose yield after 24 h hydrolysis. Biomass: newspaper

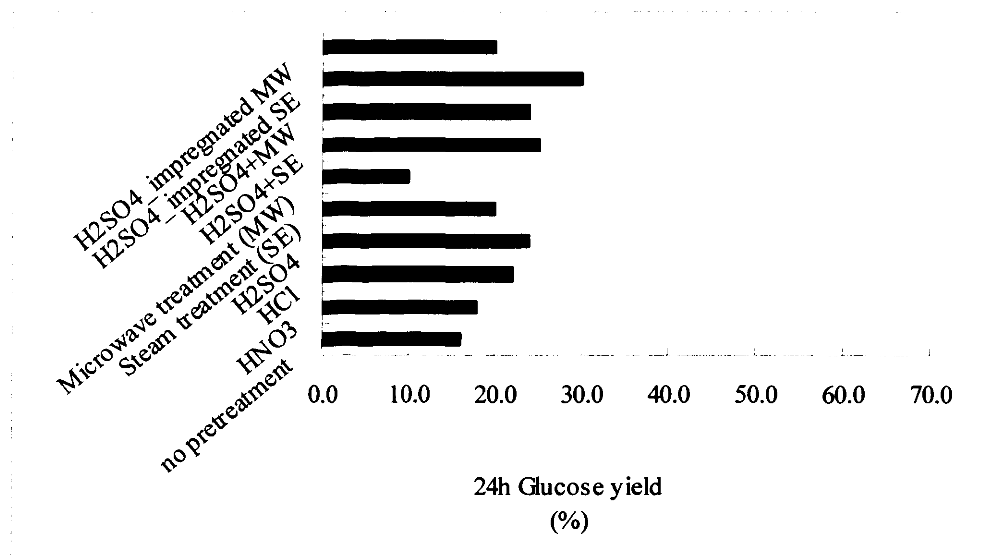


Figure 7.6 Effects of different pre-hydrolysis treatments of selected BMSW fractions in glucose yield after 24 h hydrolysis. Biomass: scrap paper

As one of the functions for pre-treatment is to break down the lignin, acid can easily removes acid soluble lignin. As shown in previous Section 4.4, grass has highest quantity of acid soluble lignin (18.45%), but much less acid insoluble lignin (5.40%). The results agree with the proportion of acid soluble lignin content, that is, biomass with higher acid soluble lignin but less acid insoluble lignin tends to give higher glucose yield. This is because the soluble acid lignin is easier to be removed by acid treatment. Although newspaper has as many lignin (11.49% AIL and 14.25% ASL) content as carrot peeling, the latter have higher cellulose content (42.41%); this results in more glucose being produced. Moreover, the crystallinity (CrI) of newspaper is much higher than that of carrot peelings (70.8 % CrI for newspaper and 21.2% CrI for carrot peelings); this may explain why the glucose yield produced from newspaper is lowest among the selected BMSW fractions. In general, the pre-hydrolysis treatment for the selected BMSW fractions with H_2SO_4 resulted in similar or higher glucose yields than with HNO_3 or HCl (as shown in Figure 7.7). This might be explained due to the fact that H_2SO_4 is a type of polyprotic acid which allows to give more than one proton per acid molecule during the reaction (two protons in this case), compared to HCl and HNO_3 (known as monoprotic acids).

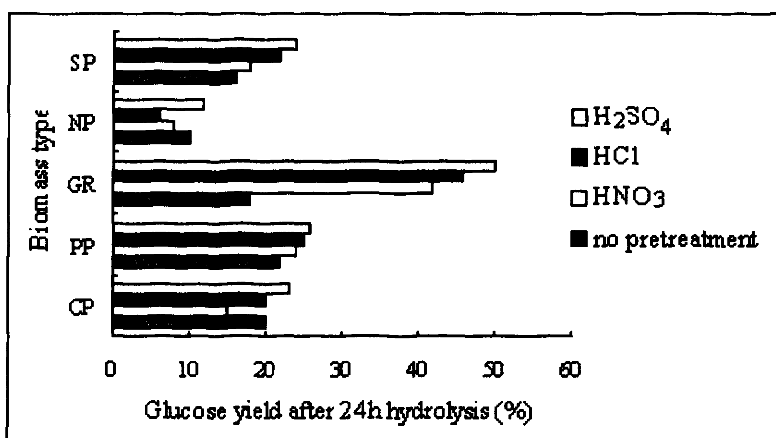


Figure 7.7 comparisons of the pre-treatment effects on the selected biomass. CP-carrot peelings, PP-potato peelings, GR-grass, NP-newspaper, SP-scrap paper

Although dilute acid pre-hydrolysis treatment can improve the cellulose hydrolysis, it needs recovery of acid to be cost effective but this currently requires expensive facility. For this reason, it is necessary to investigate more effective methods. Moreover, this method requires an additional step to neutralise the pH. Steam treatment, on the other hand, causes hemicellulose degradation and lignin transformation due to high temperature, increasing the potential of cellulose hydrolysis. Low temperature and longer residence time are favourable for optimal hemicellulose solubilisation and hydrolysis (Sun and Cheng, 2002). In this work, steam treatment results in glucose yield greater than 20% for most of the biomass feedstock except for newspaper (8%). Although, steam explosion has been recognised as one of the most cost effective pre-treatment processes for hardwoods and agricultural residues, it is less effective for softwoods and organic waste. Limitations of steam explosion include destruction of a portion of the xylan fraction, incomplete disruption of the lignin - carbohydrate matrix, and generation of compounds that may be inhibitory to microorganisms used in downstream processes (Mackie *et al.*, 1985). Due to the formation of degradation products that are inhibitory to microbial growth (enzymatic hydrolysis and fermentation), pre-treated biomass needs to be washed by water to remove the inhibitory materials along with water-soluble hemicellulose (McMillan, 1996). The water wash decreases the overall saccharification yields due to the removal of soluble sugars, such as those generated by hydrolysis of hemicellulose. According to Mes-Hartree *et al.* (1988), typically 20 - 25% of the initial dry matter is removed by water wash. This may be

the reason why relatively low glucose yields are obtained in this work.

The glucose yield produced from selected BMSW fraction with microwave treatment was smaller than 20%. From these observations it may be concluded that heat treatment cause acid insoluble lignin and hemicellulose degradation but does not remove the acid soluble lignin content. Furthermore, heat treatment may cause the crystallinity index to increase (as detailed in Section 7.3.5), resulting in the production of lower glucose yield produced.

The addition of dilute acid in the steam and microwave treatments can effectively improve enzymatic hydrolysis, decrease the production of inhibitory compounds and lead to more complete removal of hemicellulose (Martin *et al.*, 2002). The microwave method can also be applied to improve acid hydrolysis (Li, 1998). Thus in this study the pre-hydrolysis treatment of dilute acid (H_2SO_4) followed by steam treatment to effectively maximize enzymatic hydrolysis was investigated. Subsequently, the results obtained from the best experimental conditions were compared with those from dilute acid treatment followed by microwave treatment. The effective pre-hydrolysis treatment for the selected BMSW fractions in this study occurred when combining dilute H_2SO_4 (4%) followed by steam treatment (Fig. 7.2-7.6). The highest glucose yield after 24 h pre-hydrolysis treatment was obtained from grass and carrot peelings (62% and 60%, respectively), followed by potato peelings (32%). The glucose yield from waste paper was relatively lower; the scrap paper and newspaper resulted in 25 and 6% glucose yield, respectively. Previous studies have reported that softwood newspaper is the most difficult component to digest in a lignocellulosic MSW mixture, due to its high lignin content (Holtzapple *et al.*, 1992)

In this study, acid-impregnated steam treatment was found to give almost as much glucose as two steps of dilute acid treatment followed by steam treatment. But acid impregnated treatment greatly reduces the residence time by 2 h in total. The highest glucose yields were also observed from scrap paper and newspaper (32–46% scrap paper and 18% newspaper) as compared to other pre-hydrolysis treatments. Comparing the combined treatments of H_2SO_4 with steam treatment and H_2SO_4 with microwave, generally higher glucose yields were obtained using the

former treatment, but the glucose yield from newspaper was doubled using H_2SO_4 followed by microwave pre-hydrolysis treatment.

Glucose yields following the combined treatment of dilute acid with microwave and the glucose yield following the same dilute acid treatment alone were compared (Fig. 7.8). Thus the effect of microwave treatment following dilute acid treatment could be revealed. It was observed that the glucose yield was generally lower after microwave treatment. It was suspected that although microwave treatment may be more efficient in removing hemicellulose and lignin present in the selected BMSW fractions and therefore enabling the accessibility of enzymes to the cellulosic chains, it also may have facilitated glucose production at concentrations, which may have been inhibitory during enzymatic hydrolysis. However, more research is required to provide with a rigorous explanation to this observation, especially on the effects on crystallization and cellulose degradation under high temperature and high pressure conditions.

The pre-hydrolysis treatment methods investigated in this study indicated that in general lower glucose yield resulted from newspaper and scrap paper BMSW fractions than from kitchen waste BMSW fractions, i.e., carrot peelings and potato peelings. Nguyen and Saddler (1991) reported a maximum glucose yield approximately 60% after two-stage dilute-acid pre-treatment of softwood, which is the source for newspaper and scrap paper. A lower glucose yield obtained from newspaper and scrap paper in this study than by Nguyen and Saddler (1991) may have occurred due to the dense structure and presence of chemicals, such as fillers, ink and other additives in the newspaper and scrap paper (biosurfactants and antimicrobials to block pulp degradation), which may have inhibited the enzymatic hydrolysis (Kim and Dale, 2004).

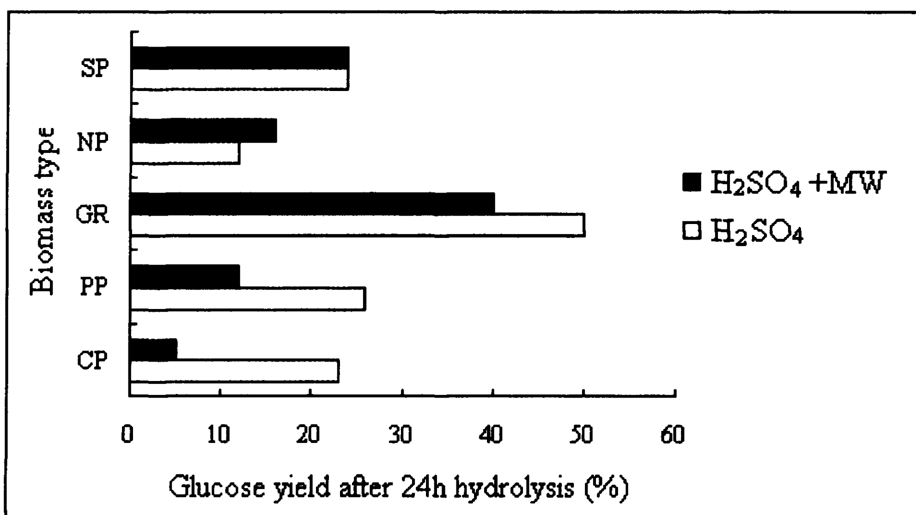


Figure 7.8 Comparison of glucose yields from dilute sulphuric acid treated biomass and the same treatment followed by microwave treatment (MW). CP-carrot peelings, PP-potato peelings, GR-grass, NP-newspaper, SP-scrap paper

7.3 Acid-impregnated steam treatment

7.3.1 Introduction

The previous section highlighted that combination of two-step acid and steam treatment is the most efficient way for most selected waste or combined waste. The glucose yields from biomass with acid impregnated steam treatment are almost as high as the combination of two-step treatment, but it greatly reduces the residence time from 3 h to 1 h. The two-step combination of acid and steam treatment has been reported by a few studies; however, it requires longer residence time and involves one more step for washing which increase the weight loss. This section continues to investigate this acid-impregnated steam treatment method with the aim of finding the best conditions to give the highest glucose yield. In this method, dilute acid is added to selected biomass and then the whole container including the dilute acid and biomass is put into an autoclave for a controlled period of time. The advantages of this method are that it reduce the total residence time for pre-hydrolysis and remove most of lignin and hemicellulose which hence to improve the enzymatic digestibility.

In this study, the key parameters are residence time, acid concentration and temperature range. As most studies of dilute acid hydrolysis, the common acid concentration ranges from 0.4% - 4%. Residence time varies from 10 to 60 min. As explained in the previous section, the temperature range considered in this investigation is 121 °C and 134 °C that was varied in the autoclave.

7.3.2 Analysis of parameters

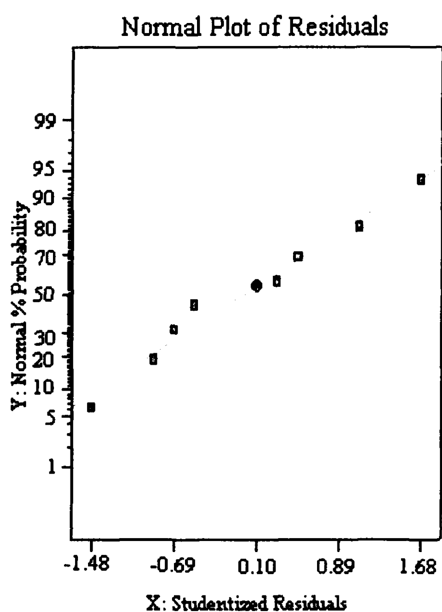
As stated in the previous section, the experimental results from controlling the three main parameters (acid concentration, temperature, and residence time) are analysed. Eight-sample biomass are used; these are carrot peeling, potato peeling, grass, newspaper, scrap paper, combination of carrot and potato peelings (50% each), combination of newspaper and scrap paper (50% each), and combination of all five types of waste (20% each). The analysis results are shown in the following eight figures (Fig 7.9, - 7.16). Each figure contains information for normal plot and cube graph for both lignin and hemicellulose that has been removed. The normal plot shows how the experimental data are presented, and cube graph shows how much lignin/hemicellulose can be removed within each combination of three parameters within the study range.

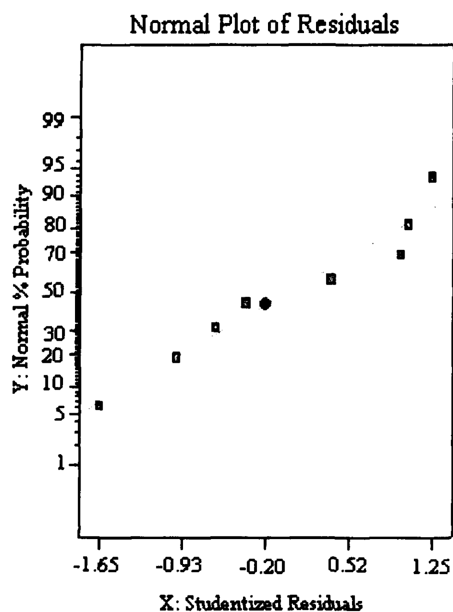
Normal plot is introduced to examine the accuracy of data collected from experimental work. If the all the data points fall in one straight line, this means the data are statistically correct and can be used for further analysis. The reason of using cube graph is because there are three factors in this process. Each axis represent one factor; for instance, x axis represents factor A which is acid concentration; y axis represents factor c (temperature), and z axis represents factor B (residence time). For each factor, as mentioned in Chapter 5, there are two levels (e.g. A_+ and A_-). In the case of factor A, A_+ means the highest level (4%) and A_- means the lowest level (0.4%). Within the cube graph, the data mean the percentage of removal lignin or hemicellulose during this acid-impregnated process. For example, in Figure 7.9c, 93.75 is the amount (in percentage) of lignin can be removed that during this pre-treatment process, at the setting conditions of A_+ (4%

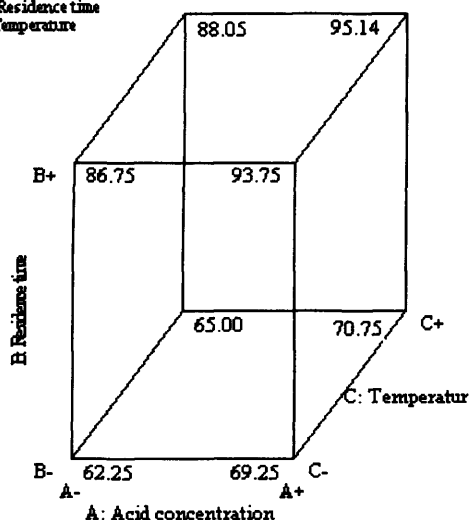
acid concentration), B_+ (residence time 1h) and C_- (temperature 121 °C).

The distributions of the normal plot of residuals (as shown in Figure 7.9 -7.16) form nearly linear patterns for each type of biomass. This indicates the normal distribution is a good model for these data sets as mentioned in Section 5.6). From the analysis results, it can be seen that both highest lignin and hemicellulose removal happen when the temperature is 134 °C, residence time is 60 min, and the acid concentration is 4%. However, the major contribution comes from residence time. Take carrot peeling as an example, the value of lignin removal varies from 86.75% to 93.75% after 60 min reaction, and 62.25% to 70.75% after 15 min; the value of hemicellulose removal ranges from 80.37% to 89.25% after 60 min reaction and 58.62% to 67.44%. The same phenomena have been observed for other feedstock. Though the other two factors contribute to the efficiency of lignin and hemicellulose removal, they are not as significant as the residence time within the study range.

According to the observations, to remove lignin and hemicellulose at a maximum level, it requires longer residence time, higher acid concentration and higher temperature. For instance in Figure 7.9c, 95.14% of lignin when using carrot peelings as biomass is removed when the acid concentration is 4%, temperature at 134 °C and residence time is 60 min. Same can be said for hemicellulose removal (89.25% for carrot peelings from Figure 7.9d). However, longer residence time means greater energy consumption; higher acid concentration requires more resistance materials for the reactor and more water for the neutralisation; higher temperature implies greater energy input and may cause cellulose crystallinity increase. As one of the main purposes of pre-treatment is to reduce the process cost, there is a need to introduce economic consideration along side to obtaining the higher product yield. Moreover, although higher acid concentration increases the removal of both lignin and hemicellulose, as the further step – enzymatic hydrolysis requires neutralizing the reaction, higher acid concentration results in large amount of water washing for detoxification purpose and thus leads to more weight loss.







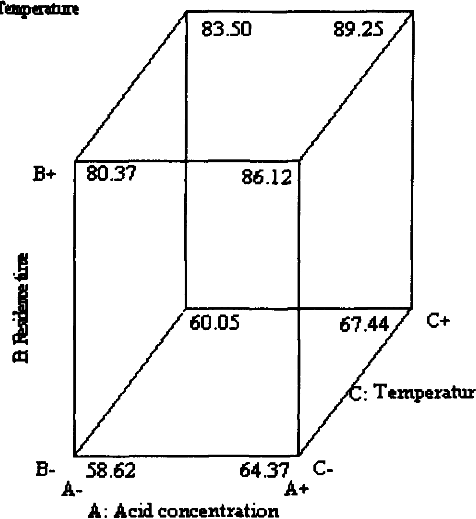
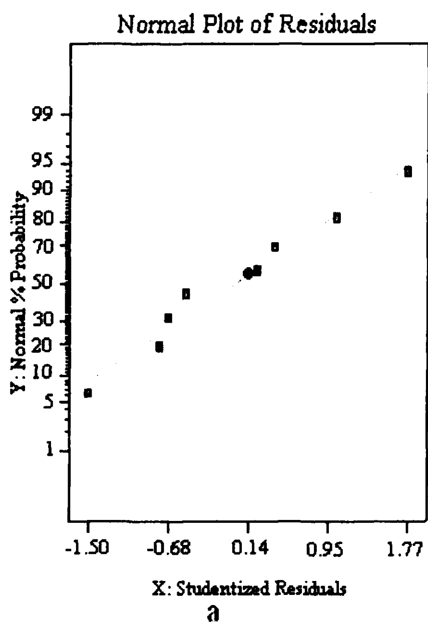
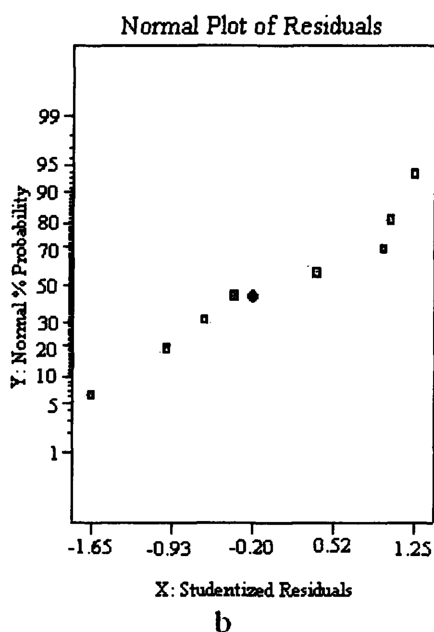
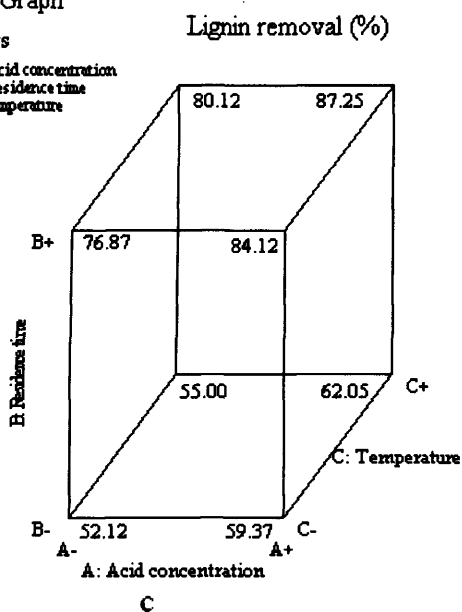


Figure 7.9 Pre-hydrolysis results for the biomass of carrot peelings; a, normal plot of residuals for lignin removal; b, normal plot of residuals for hemicellulose removal; c, cube graph of lignin removals within each combination of all three parameters; d cube graph of hemicellulose removals within each combination of all three parameters.







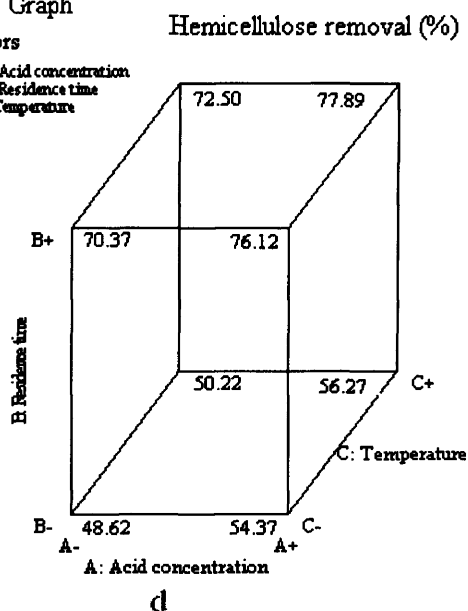
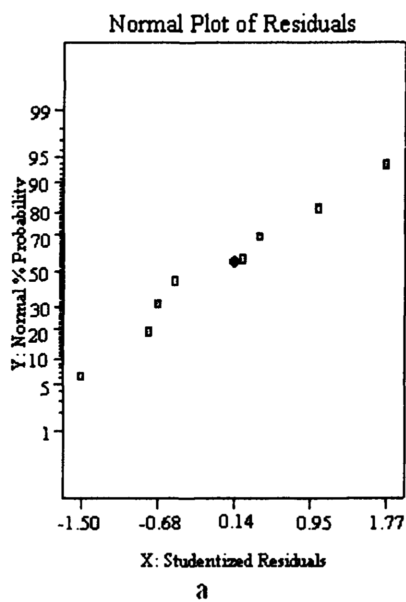
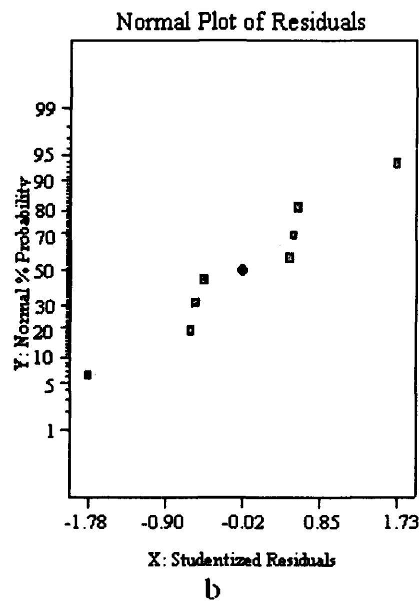
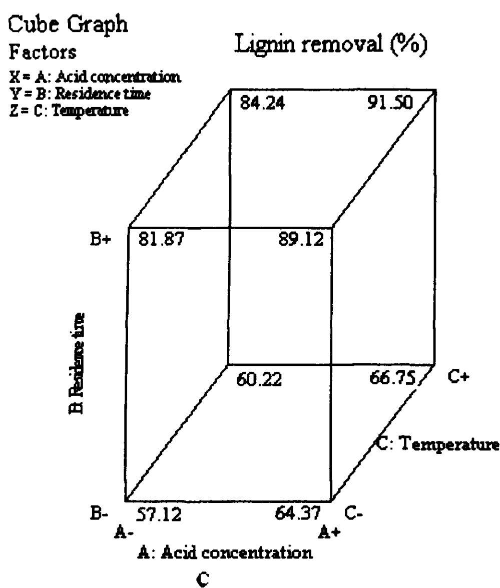


Figure 7.10 Pre-hydrolysis results for the biomass of potato peelings; a, normal plot of residuals for lignin removal; b, normal plot of residuals for hemicellulose removal; c, cube graph of lignin removals within each combination of all three parameters; d cube graph of hemicellulose removals within each combination of all three parameters.







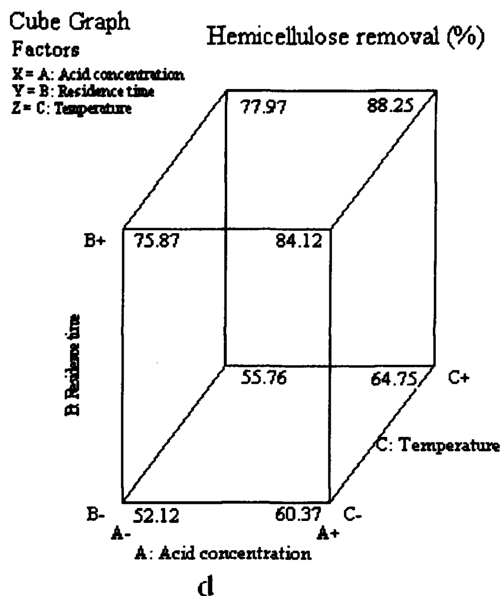
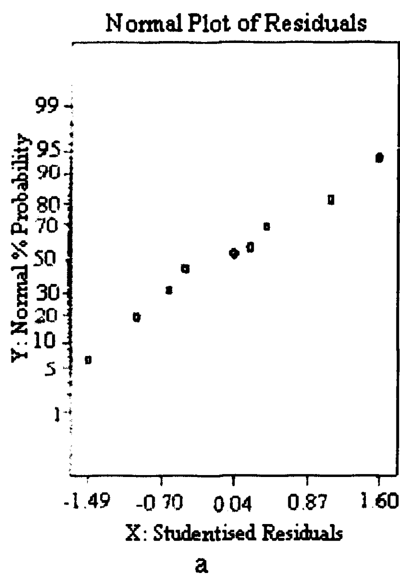
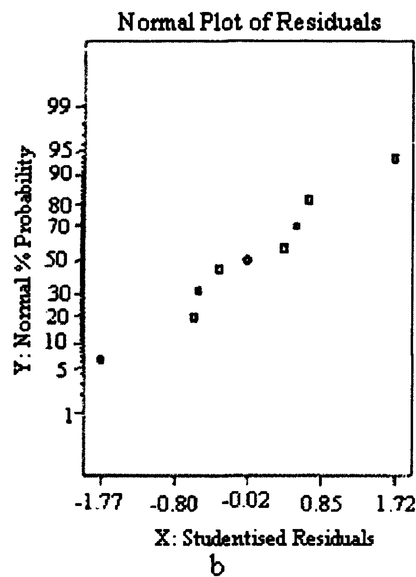
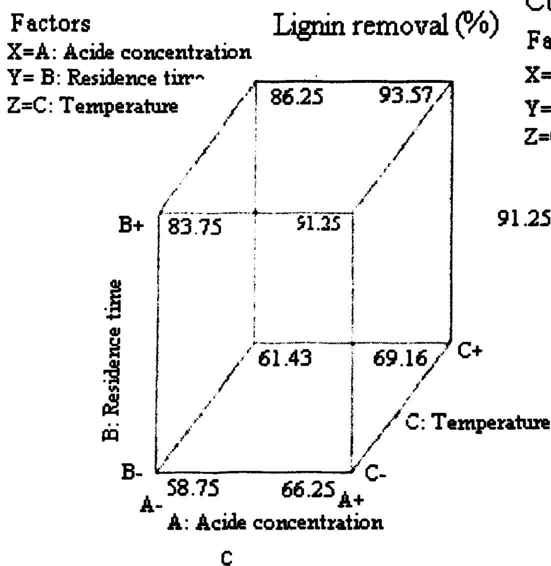


Figure 7.11 Pre-hydrolysis results for the biomass of grass; a, normal plot of residuals for lignin removal; b, normal plot of residuals for hemicellulose removal; c, cube graph of lignin removals within each combination of all three parameters; d cube graph of hemicellulose removals within each combination of all three parameters.







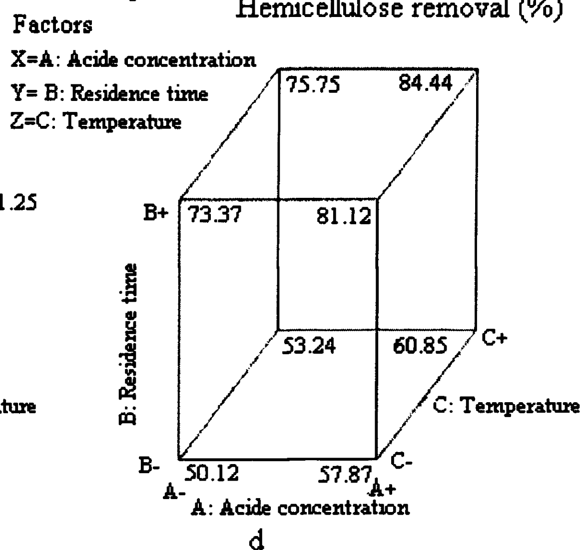
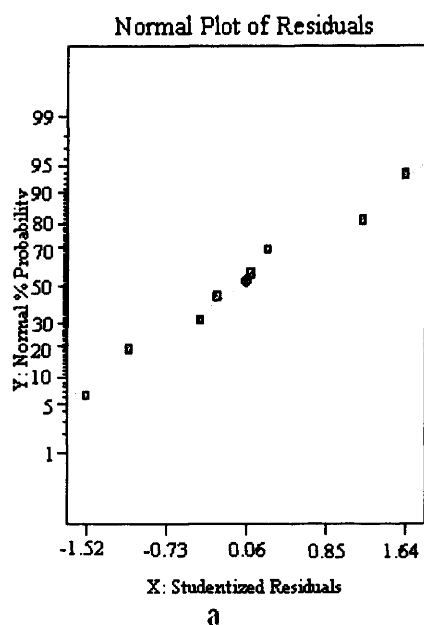


Figure 7.12 Pre-hydrolysis results for the biomass of newspaper; a, normal plot of residuals for lignin removal; b, normal plot of residuals for hemicellulose removal; c, cube graph of lignin removals within each combination of all three parameters; d cube graph of hemicellulose removals within each combination of all three parameters.



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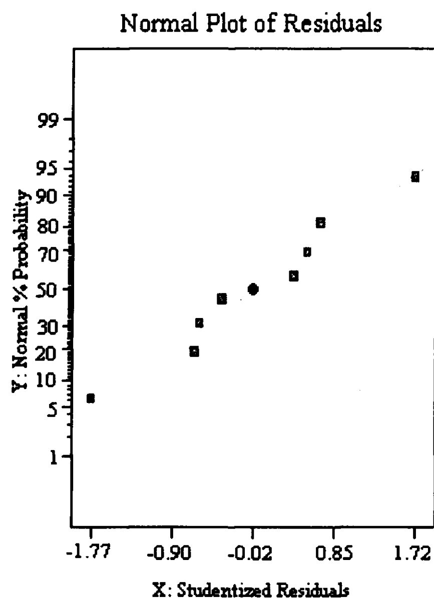

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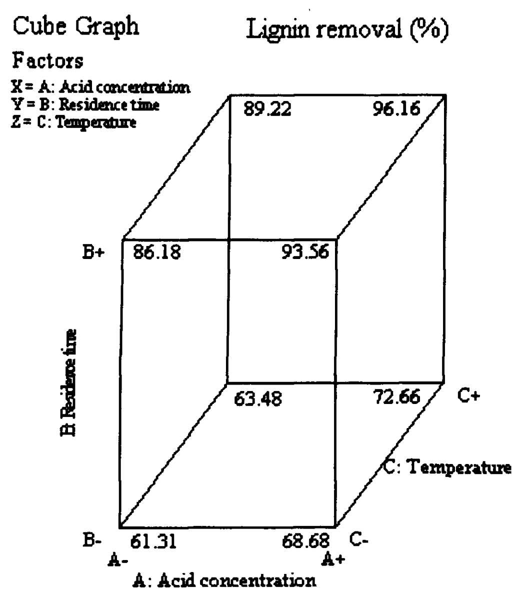

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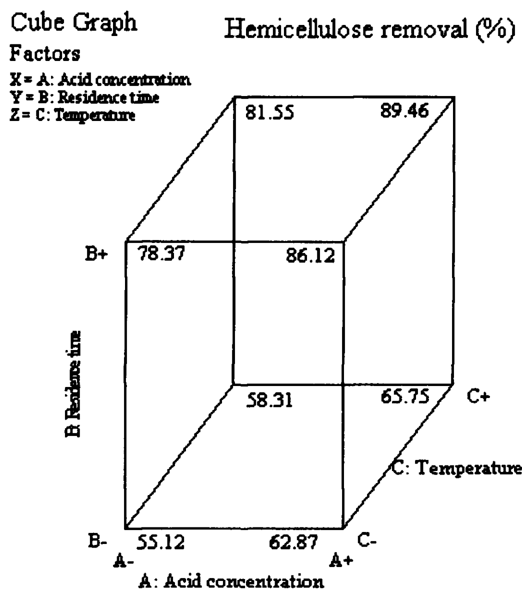

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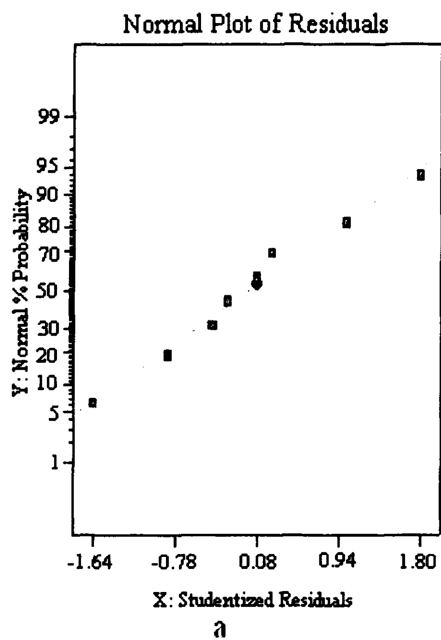
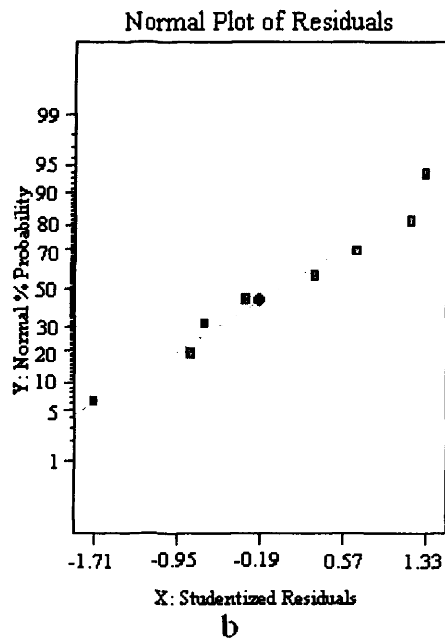
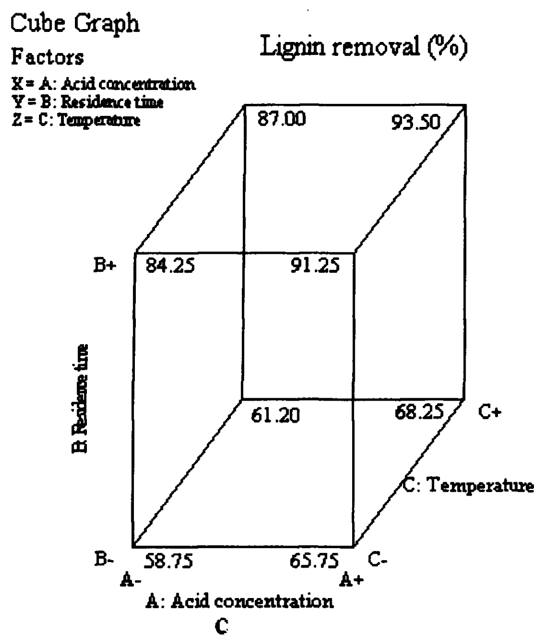
Figure 7.13 Pre-hydrolysis results for the biomass of scrap paper; a, normal plot of residuals for lignin removal; b, normal plot of residuals for hemicellulose removal; c, cube graph of lignin removals within each combination of all three parameters; d cube graph of hemicellulose removals within each combination of all three parameters.







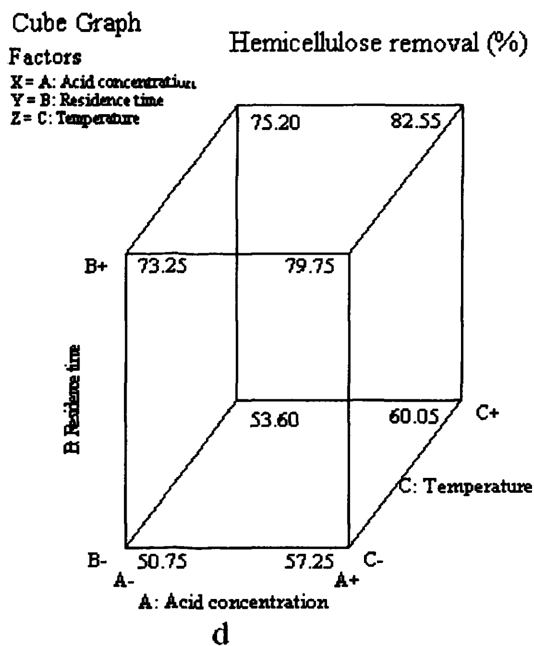
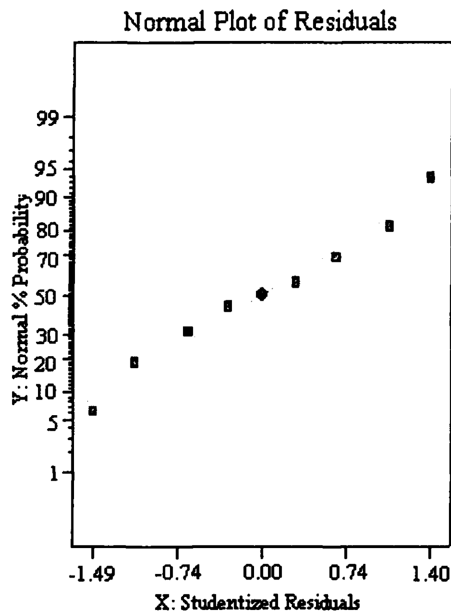


Figure 7.14 Pre-hydrolysis results for the biomass with combination of carrot peelings and potato peelings; a, normal plot of residuals for lignin removal; b, normal plot of residuals for hemicellulose removal; c, cube graph of lignin removals within each combination of all three parameters; d cube graph of hemicelluloses removals within each combination of all three parameters.



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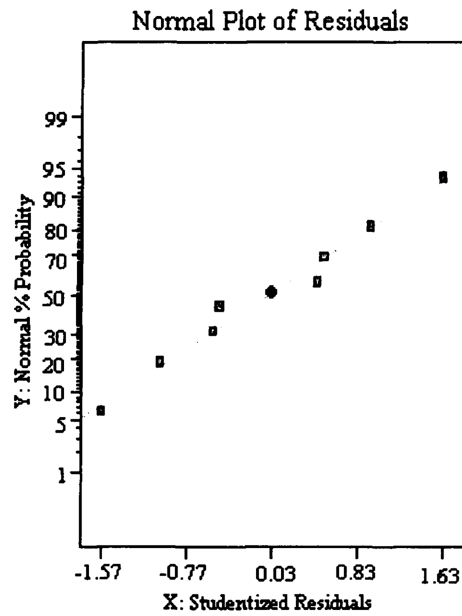

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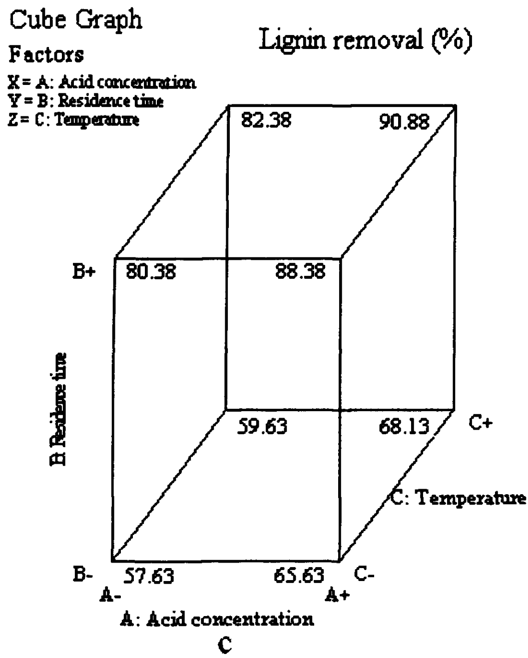

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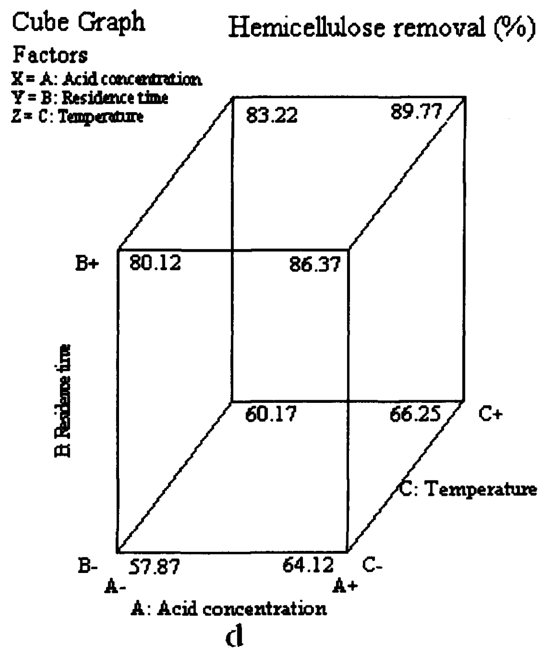

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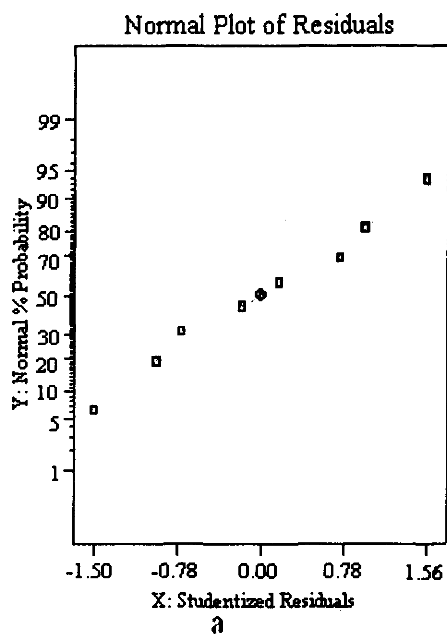
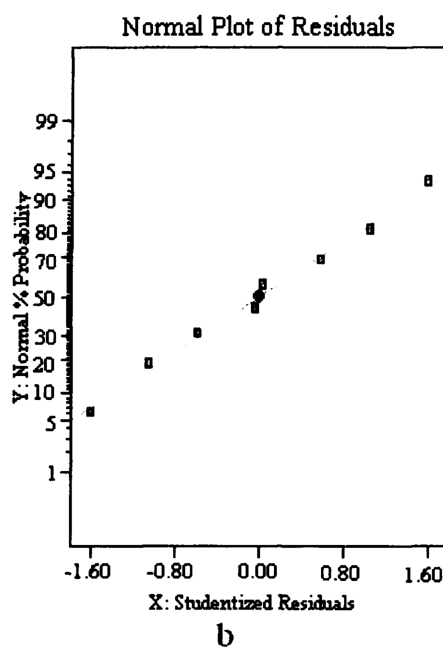
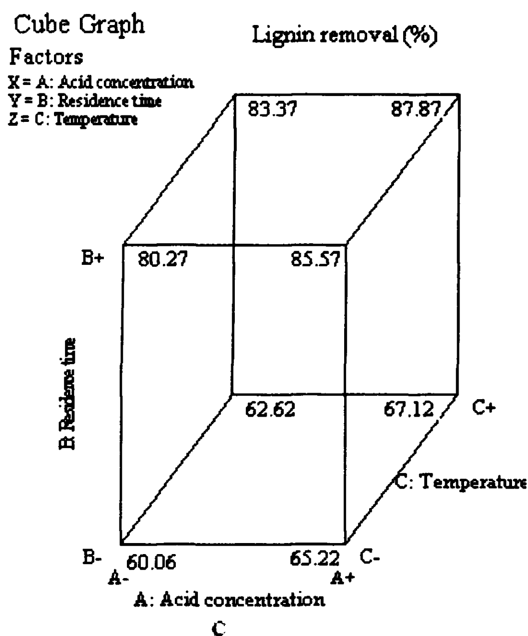
Figure 7.15 Pre-hydrolysis results for the biomass with combination of newspaper and scrap paper; a, normal plot of residuals for lignin removal; b, normal plot of residuals for hemicellulose removal; c, cube graph of lignin removals within each combination of all three parameters; d cube graph of hemicelluloses removals within each combination of all three parameters.







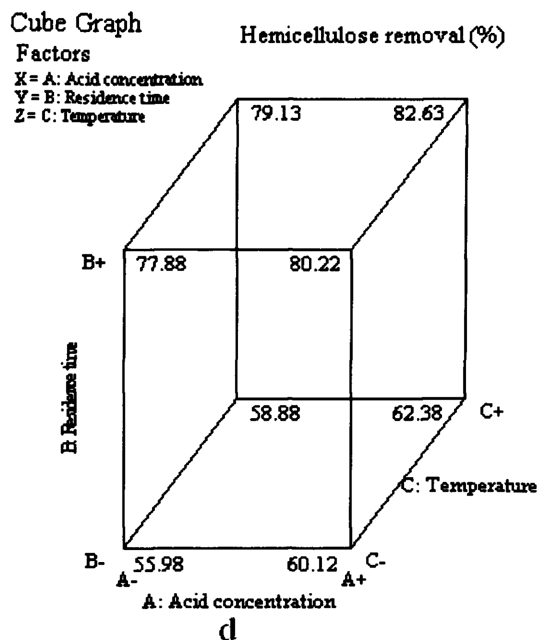


Figure 7.16 Pre-hydrolysis results for the biomass with combination of carrot peelings, potato peelings, grass, newspaper and scrap paper *(20% each)*; a, normal plot of residuals for lignin removal; b, normal plot of residuals for hemicellulose removal; c, cube graph of lignin removals within each combination of all three parameters; d cube graph of hemicelluloses removals within each combination of all three parameters.

McMillan (1996) has reported that the residence time of 30 min was not long enough for the solubilisation of hemicellulose in the biomass. Many researchers (Grohmann *et al.*, 1985; Torget *et al.*, 1990; Torget and Hsu, 1994; Nguyen *et al.*, 2000) reported that it is necessary to have a residence time as long as 60 min. It is also necessary to use different pre-treatment methods that often can reduce more than 80% of lignin/hemicellulose. In this work, considering residence time as 60 min, but using 1% acid concentration and lowest temperature, the lignin and hemicellulose removal can besought to more than 80% for all the selected and combined BMSW fractions (as shown in Table 7.2).

Table 7.2 Amount of hemicelluloses and lignin removed (in percentage) from each type of biomass under the conditions of residence time 60min, 1% acid concentration and 121 °C

Biomass type	Lignin removed (%)	Hemicellulose removed (%)
CP	93.75	86.12
PP	84.12	76.12
Gr	89.12	84.12
NP	91.25	81.12
SP	93.56	93.56
CP+PP	91.25	79.75
NP+SP	88.38	86.37
CP+PP+Gr+NP+SP	85.37	80.22

The Lignin/hemicellulose removal increased with the increase in acid concentration when the pre-treatment time was 60 min, while the galactan is completely hydrolysed when sulphuric acid concentration increased to 1% and residence time to 60 min. Hemicellulose and lignin are significantly influenced by pre-treatment time, thought the acid concentration and temperature have slight effect on removing lignin and hemicellulose. The hemicellulose hydrolysate contained monomeric sugars such as arabinose, galactose, glucose, and xylose with xylose as the major carbohydrate component (McMillan, 1996). The yields of these monomeric sugars in the filtrate have indicated the hemicellulose degradation after acid pre-treatment. The results indicated that residence time of 30 min was not enough for the solubilisation of hemicellulose in the biomass. The increased severity of the pre-treatment conditions resulted in more solubilisation of hemicellulose. More than 80% of the hemicellulose is solubilised into monomeric sugars when pre-treated with 1% sulphuric acid for 60 min. The effect of dilute sulphuric acid concentration and reaction time on the hydrolysis and solubilisation of the biomass can be summarised as follows:

Compared with other studies (Grohmann *et al.*, 1985; Torget *et al.*, 1990; Torget and Hsu, 1994; Nguyen *et al.*, 2000), the experimental results shows this pre-hydrolysis method can achieve to same or even higher results but in a relatively lower temperature (121 °C). This is very important, because the high temperature (such as 170 °C, 200 - 230 °C) not only produced solubilised hemicellulose, but also degrade a large portion of the glucose to hydroxymethyl furfural (HMF) and xylose was degraded to furfural that was inhibitory to xylose fermentation for ethanol production by microorganisms and microbial growth (Grohmann *et al.*, 1984; Lee, 1997). Moreover, higher temperature process (160 °C -230 °C) causes hemicellulose degradation and lignin transformation. Dien *et al.* (2006) pointed out that Dilute acid pre-treatment at the higher temperature (>150 °C) had an unfavourable effect on non-glucose sugar conversion efficiency and yield. From this study, it is suspected that the lower yield could be accounted for by rapid degradation of fructose during dilute acid pre-treatment at elevated temperatures.

Grous *et al.* (1986) reported that 90% efficiency of enzymatic hydrolysis has been achieved in 24 h for poplar chips pre-treated by steam explosion, compared to only 15% hydrolysis of untreated chips. Duff and Murray (1996) showed the factors that affect steam explosion pre-treatment are residence time, temperature, chip size and moisture content. Optimal hemicellulose solubilisation and hydrolysis can be achieved by either high temperature and short residence time or lower temperature and longer residence time (Duff and Murray, 1996). Recent study by Wright (1998) indicate that lower temperature and longer residence time are more favourable, which agrees with the research findings from this work.

Impregnation with sulphuric dilute acid in the steam-explosion process improves the solubilisation of pentosan, permits the use of lower temperatures, results in less destruction of pentosan, and gives a better substrate for enzymatic hydrolysis. Compared to other pre-treatment methods, it is especially useful for the conversion of xylan in hemicellulose to xylose that can be further fermented to ethanol by many microorganisms (McMillan, 1996). Grohmann *et al.* (1985) reported the sulphuric acid pre-treatment of wheat straw and aspen wood. About 80% of xylan was removed at 140 °C for 1 h of reaction time and enzymatic digestibility of cellulose was nearly 80%. Torget *et al.* (1990) investigated the dilute sulphuric acid

pre-treatment of short rotation hardwoods and herbaceous crops. In this work, similar results were achieved with lower temperature (121 °C) within the same residential time.

However, as the temperature of 121 °C causes the increase of cellulose crystallites, (i.e. in the pre-treated biomass), there is more crystallinity cellulose than raw materials; this may affect the use of enzyme in the following step. More details on how the crystallinity affects enzyme adsorption are further discussed in Chapter 9.

From the above discussion, it is clear that the factor residence time is more important for the pre-treatment process compared to the other two factors (temperature and acid concentration). The analysis results are hence confirmed with ANOVA technique (mentioned in Section 5.6). ANOVA is introduced to analysis the effect from each factor. It is a technique that uses p-value in order to examine the significance of the effect of the main factors or the interactions of them. The p-value is the probability of obtaining a result at least as extreme as a given data point, under the null hypothesis. Values of the probability (p-value) less than 0.0500 indicate that model terms (i.e. factors) are significant. Values greater than 0.1000 indicates the model terms are not significant. For example, in Table 7.3, for CP, the p-value of factor temperature is 0.1090, acid concentration 0.0273, and residence time 0.0001. This implies that residence time with p-value 0.0001, has a significant effect on the pre-hydrolysis process. Hence, this factor must be taken into account when seeking an improvement of the process efficiency. From Tables 7.3 and 7.4, it is obvious that the factor of residence time makes significant contribution to the pre-treatment process for both lignin and hemicellulose removal. The contribution of acid concentration is slightly higher than the factor of temperature, but both contributions can be considered as being not significant.

Table 7.3 P-value showing factors contribution to the pre-hydrolysis process (lignin)

Biomass/factors	Temperature (°C)	Acid concentration (%)	Residence time (min)
CP	0.1090	0.0273	0.0001
PP	0.0976	0.0321	0.0002
Gr	0.1021	0.0301	0.0002
NP	0.1011	0.0326	0.0004
SP	0.2250	0.0408	0.0004
CP+PP	0.3500	0.1147	0.0006
NP+SP	0.5781	0.3127	0.0088
CP+PP+Gr+NP+SP	0.1046	0.1046	0.0036

Note: CP-carrot peelings, PP-potato peelings, GR-grass, NP-newspaper, SP-scrap paper

Table 7.4 P-value show factors contribution to the pre-hydrolysis process (hemicellulose)

Biomass	Temperature (°C)	Acid concentration (%)	Residence time (min)
CP	0.2190	0.1430	0.0012
PP	0.2679	0.1566	0.0012
Gr	0.2562	0.1322	0.0035
NP	0.2513	0.1536	0.0040
SP	0.1523	0.1062	0.0030
CP+PP	0.1788	0.3517	0.0060
NP+SP	0.1566	0.2143	0.0180
CP+PP+Gr+NP+SP	0.1453	0.2515	0.0120

Note: CP-carrot peelings, PP-potato peelings, GR-grass, NP-newspaper, SP-scrap paper.

7.3.3 Glucose yield

As discussed in the previous section, sulphuric acid-impregnated steam treatment under the conditions of 1% H_2SO_4, temperature 121 °C, and residence time 60 min reduces more than 80% of lignin and hemicellulose for all the elected BMSW fractions. Enzymatic hydrolysis with 60 FPU cellulase (*T. reesei*) was carried out to show how much glucose can be produced under these conditions. Table 7.6 shows the results of glucose yield from each type of biomass after 24 h reaction.

The highest glucose yield, 86.63%, has been observed from carrot peeling after 24 h reaction. Newspaper produced lowest glucose with 76.46%. The observation agrees with the lignin and hemicellulose removal shown in Figures 7.9 - 7.16. As more lignin and hemicellulose have been removed with the acid-impregnated steam

treatment, enzyme is easier to access the cellulose. It also can be inferred that for newspaper there is some amount of hemicellulose that was not removed. It is clear from Table 7.5 that the cellulose content of some BMSW is lower while lignin and hemicellulose (which are hinderers for the access of enzymes during the hydrolysis) content is higher than other lignocellulosic biomass. So acid and steam pre-treatment contributes in removing these compounds. Dilute acid hydrolysis is an effective method for improving the cellulose hydrolysis and in combination with steam explosion can reduce the compounds that inhibit the enzymatic hydrolysis.

The results of glucose yield also reveals that the combination of 1% H_2SO_4 and steam explosion in autoclave at 121 °C is effective for lignocellulosic biomass such as municipal solid waste in order to obtain better glucose yields during the enzymatic hydrolysis

Table 7.5 Glucose yield after 24 h hydrolysis

Biomass	Glucose yield (%)
CP	86.63
PP	80.89
Gr	81.95
NP	76.46
SP	85.55
CP+PP	82.29
NP+SP	80.55
CP+PP+Gr+NP+SP	82.14

Note: CP-carrot peelings, PP-potato peelings, GR-grass, NP-newspaper, SP-scrap paper

7.3.4 Compositional analysis of solid residuals

The solid residuals (i.e. the pre-treated biomasses) are obtained at 45% moisture content. The solid residuals obtained were analysed in order to understand the composition changes after pre-treatment.

In this section, the selected waste (carrot peelings, potato peelings, grass, newspaper and scrap paper) are pre-treated with 1% dilute sulphuric acid in autoclave with the temperature of 121 °C. This section presents the composition of each type of substrate after pre-treatment process in order to compare them with raw materials.

The calculations of the results have been based on the methodologies detailed in Section 5.3. Table 7.6 gives the results.

Table 7.6: Pre-treated biomass composition (1% H2SO4, 121 °C, 1h)

Biomass	ASL %	Cellulose %	Hemicellulose %	AIL + ash	Total %
CP	1.08	50.46	3.59	44.02	99.15
PP	1.38	65.26	14.27	22.78	103.69
GR	0.91	43.12	4.39	52.07	100.49
NP	1.33	60.07	13.90	29.10	104.40
SP	1.26	59.49	5.52	32.14	98.41
CP + PP	1.20	53.65	8.61	36.64	100.10
NP + SP	1.31	60.71	10.77	32.48	104.27
CP + PP + GR + NP + SP	1.17	53.39	8.18	36.07	98.81

Note: CP-carrot peelings, PP-potato peelings, GR-grass, NP-newspaper, SP-scrap paper.

From Table 7.6 it can be seen that ASL and hemicellulose have been reduced by the process of acid and steam pre-treatment (from 14.09% to 1.08% for carrot peelings, from 11.93% to 1.38% for potato peelings, from 18.45% to 0.91% for grass, from 14.25% to 1.33% for newspaper, and from 11.34% to 1.26% for scrap paper). More than 50% of reduction of hemicellulose contents has been observed (f from 23.30% to 3.59% or carrot peelings, from 42.78% to 14.27% for potato peelings, from 39.59% to 4.39% for grass, from 24.36% to 13.90% for newspaper). An exception was found for scrap paper that only shows a reduction of hemicellulose contents about of 19%, from 6.82% to 5.52%. This is due to the fact that the hemicellulose content is already very low in raw scrap paper (6.82%). Hence, it is difficult to achieve a higher efficiency. Certainly reductions of both lignin and hemicellulose are the purpose of the pre-treatment. Another point is that ASL is very low for every biomass after the pre-treatment; but the hemicellulose contents for potato peelings and newspaper the percentage are still relatively high, (about 14%). The relative high contents of hemicellulose explain the lower glucose yields produced from both substrates (80.89% for potato peelings and 76.46% for newspaper) compared to others as shown in Table 7.6. Wu *et al.* (1999) reported that steam explosion under optimal conditions can reduce hemicellulose content of wood chips from 65% to 37% (about 43% of reduction). Compared to the optimal pure steam explosion, the acid-impregnated treatment in this work has the advantage of more hemicellulose reduction and less hemicellulose content (less than 15%) presented in each type of pre-treated biomass waste.

7.3.5 Cellulose Crystallinity

Cellulose crystallinity was also measured for each type of selected BMSW fraction. The purpose of crystallinity determination is to understand how the cellulose structure changes after pre-treatment in order to explain the glucose yield obtained during enzymatic hydrolysis. The measurement was carried out for these five selected BMSW fractions. In order to identify if the chemical or heat change the cellulose structure, the biomass with different treatment methods (dilute acid, steam treatment, acid-impregnated steam treatment) are analysed. Table 7.7 shows the crystallinity content for each biomass.

Table 7.7 Cellulose crystallinity after pre-treatment process

Biomass/ pre-treatment type	Before pre-treatment	Dilute acid treatment (1% H_2SO_4)	Steam treatment (121 °C)	H_2SO_4-impregnated steam treatment (1% H_2SO_4, 121 °C)
CP	16.8	18.7	29.4	29.0
PP	21.2	22.3	29.3	30.7
GR	37.3	38.5	46.6	47.0
NP	70.8	72.6	79.1	81.7
SP	67.4	69.3	78.9	79.5

Note: CP-carrot peelings, PP-potato peelings, GR-grass, NP-newspaper, SP-scrap paper.

Table 7.7 shows that heat treatment (steam treatment) increases the cellulose crystallinity. Chemicals, like acids, do not significantly increase the cellulose crystallinity. Although some small changes can be observed before pre-treatment and after dilute acid treatment, they are more likely to be due to measurement errors. The crystallinity of carrot peelings has increased from 16.8% to 29.4% after steam treatment. The increases also have been observed for potato peelings (from 21.2% to 29.3%), grass (from 37.7% to 46.6%), newspaper (from 70.8% to 79.1%) and scrap paper (from 67.4% to 78.9%). Similar increases for all the biomass are found with acid-impregnated steam treatment that has the same temperature (121 °C) as steam treatment.

Bhuiyan (1999) has observed the increase of wood crystallites was doubled with the heat treatment in a drying oven at 220 °C. Other researchers (Fuller *et al.*, 1940; Creely and Conrad, 1962; Conrad, 1962) have reported on the change of crystallinity of cellulose after heat treatment. As higher temperature results in

increase of crystallites, lower temperature is better for the pre-treatment. The increase in crystallinity may be explained as crystallization in quasi crystalline of amorphous regions due to rearrangement or reorientation of cellulose molecules inside these regions; the more crystallization in wood cellulose may be due to the crystallization in hemicelluloses and wood cellulose contain more crystalline regions than pure cellulose.

As the sample has to be oven-dried at 105 °C before the measurement, which might have caused the small increase of cellulose crystallites of dilute H_2SO_4 treated biomass. Another reason for cellulose crystallites increase may be due to the reduction of amorphous cellulose as part of it has been hydrolysed by the acid.

7.4 Conclusions

In conclusion, among all the selected pre-hydrolysis methods, sulphuric acid-impregnated steam treatment is the most appropriate method for the selected BMSW fractions. Residence time has a greater influence on the lignin/hemicellulose removal than acid concentration and temperature. With the conditions of temperature 121 °C, acid concentration 1% and residence time 60 min, the suggested method, sulphuric acid-impregnated steam treatment, can remove more than 80% of lignin and hemicellulose for all the studied biomass.

The cellulose crystallites structure increases after dilute-acid pre-treatment and steam treatment. Increase of CrI has been observed with any methods that involve heat treatment (such as steam treatment and acid-impregnated steam treatment). The reasons for the small increase after acid treatment may contribute to the oven drying process (105 °C) before the compositional analysis and the reduction of amorphous cellulose that has been hydrolysed during the process. The results of this work have shown that the heat does increase the crystallites of the materials studied that agrees with most research findings in the literature.

Chapter 8

Enzymatic hydrolysis and process optimisation

8.1 Introduction

Enzymatic hydrolysis is a process by which enzymes (biological catalysts) are used to break down cellulose into sugar (i.e. glucose). Enzymatic hydrolysis of BMSW is of crucial importance during the bioconversion of BMSW-to-bio-ethanol. According to Wayman (1999), current enzymatic hydrolysis process contributes to 35% of the total ethanol cost (2.1k€/KW). Hence, this research seeks to investigate how the process can be optimised in order to improve the product yield and reduce the cost.

The study reported in this chapter begins with the selection of cellulase for hydrolysis by comparing the two most commonly used enzymes (*Trichoderma virid* and *Trichoderma seerei*). The selected cellulase is then used for all the enzymatic hydrolysis involved in this work. The purpose of the study is (i) to better understand the role of the conditions of the process as a factor of performance, (ii) to identify the limiting factors in order to (iii) optimise the process. Various factors, considered as important in the literature, are initially selected. Evaluating the process performance under different conditions is used to identify the most significant factors. In order to study the interaction between factors, a two-level fractional experimental design is introduced. Detailed methodology including ANOVA analysis and P-value is presented in Section 5.6. After the effects of each factor are discussed, the optimal conditions for each type of selected waste fraction or

combination are given. Experimental results from the best conditions are compared with the predicted value generated from the software package model.

All the biomass used is pre-treated with acid-impregnated steam treatment under the optimal conditions given in Section 7.3, 1% sulphuric acid, 60 min residential time and 121 °C, within the range investigated.

8.2 Enzyme selection

Efficient hydrolysis of cellulose requires the synergistic activities of three types of enzymes. Endo-b–1, 4-glucanases hydrolyse accessible regions on cellulose chains to provide new sites for attack by exo-acting cellobiohydrolases, which removes successive cellobiose units from newly created chains ends. Finally, β-glucosidase hydrolyses cellobiose, and smaller amounts of higher cellooligomers, to glucose. Two cellulose digesting enzymes used in this study (*T. reesei* and *T. viride)* contain endogenous β-glucosidase activity; however, the activities of this enzyme are generally insufficient to prevent the accumulation of cellobiose, resulting in product inhibition of endoglucanases and cellobiohydrolases (Tengborg *et al.*, 2001). Consequently, β-glucosidase was supplemented during the enzymatic hydrolysis of BMSW fractions.

Figure 8.1 shows the glucose yield after 96 h hydrolysis for each type of biomass with two different cellulases. The results of this study indicated that using *T. viride* resulted in higher glucose yields as compared to using *T reesei*, which corroborates previous studies indicating an activity (units glucose produced per mg enzyme) of *T. viride* and *T. reesei* of 3 – 10 and 1.0, respectively.

From Figure 8.1, it can be seen that with the same cellulase- *T. virid*, potato peelings give the highest glucose yield (96%), followed by combination of carrot and potato peelings (88%), carrot peelings (80%), grass (64%), combination of all the selected BMSW fractions (55%), scrap paper (48%), combination of newspaper and scrap paper (48%), and finally newspaper (18%). Obviously, kitchen waste

gives the highest glucose yield. The lowest product yield is found in paper and card waste which contains highest cellulose contents among the selected BMSW fractions. The same trend is found with the cellulase of *T. reesei.*

All the samples were pre-treated before hydrolysis using the methods of acid-impregnated steam treatment which has been detailed in Section 5.3. As shown in Section 7.3.4, after pre-treatment, potato peelings have highest cellulose content (65.26%), followed by combination of newspaper and scrap paper (60.71%), newspaper (60.07%), scrap paper (59.49%), combination of carrot peelings and potato peelings (53.65%), combination of the selected BMSW fractions (53.39%), carrot peelings (50.46%), and grass (43.12%). From the cellulose content point of view, potato peelings will give the highest glucose yield which agrees with the experimental findings. Paper and card waste (newspaper and scrap paper) should give relatively high glucose yield according to the cellulose contents. However, this was not the case of experimental results. In contrast, paper and card waste give the lowest yield compared to others.

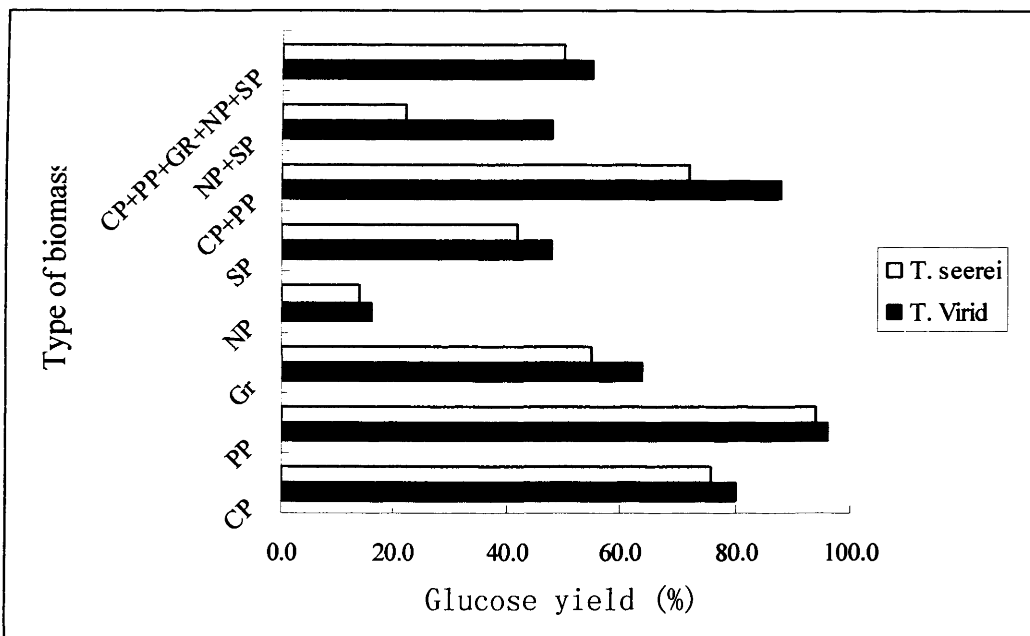


Fig.8.1 Effects of cellulase (T. viride, T. reesei, 100 FPU/ g) during enzymatic hydrolysis of selected BMSW fractions in glucose yield (50 °C, 96 h). CP- carrot peelings, PP- potato peelings, Gr- grass, NP- newspaper, SP- scrap paper

As the glucose yield is not only affected by cellulose content, but also the cellulose structure (.i.e. crystallinity), it is important to examine the influence from cellulose

crystallinity. As observed in Section 7.4.4, after pre-treatment, scrap paper has the highest CrI (79.5%), followed by newspaper (81.7%), grass (47%), potato peelings (30.7%), and carrot peelings (29.0%). Therefore, the relative low glucose yield from paper and card waste can be explained because of the highest crystallinity structures which is difficult to be broken down by the enzyme. The biodegradation of crystalline cellulose generally involves the action of both endo- and exo-acting cellulases. Classical endoglucanases nick the cellulose internally, thus disrupting its crystallinity and generating new free ends in the polymer. Cellobiohydrolases (exoglucanases) act processively from these free ends, remaining attached to the cellulose and releasing soluble cellobiose molecules, which are subsequently hydrolysed to assimilable glucose by β-glucosidases (Beguin and Aubert, 1994). As paper and card waste has higher portions of crystallinity cellulose, they require more endoglucanases to provide free ends for exoglucanases to attack. As the same amount of celluloses are provided during the experimental work, it is very likely that there is not enough endoglucannases for paper and card waste to be hydrolysed resulting in low glucose yield.

Mansfield *et al.* (1999) mentioned that during enzymatic hydrolysis, the structural differences in cellulose and the intricate association with other biomolecules in lignocellulosic substrates are very important factors in controlling their susceptibility to degradation, particularly hydrogen-bonded and ordered crystalline cellulose. The research findings of this work agree with his findings. Furthermore, due to the inherent insolubility and physical complexity of cellulose moieties, several different enzymes are needed for complete solubilisation. It is apparent that the efficiency of the cellulase enzymes to hydrolyse cellulose is inextricably linked to the structural characteristics of the substrate, such as crystallinity, as reported by Walker and Wilson (1991) and Mansfield *et al.* (1999). A possible approach in the future is to increase the productivity of cellulase production by mutation of cellulolytic fungi and optimizing culture conditions.

8.3 Process optimisation

8.3.1 Experimental results

Experiment setups were designed using fractional approach. Six factors were chosen according to their effects on glucose yield from experimental results. These include: (1) substrate concentration, (2) enzyme loading, (3) beta-glucosidase loading, (4) pH, (5) temperature and (6) particle size. The design method employing the six particular factors 'randomly' generates 32 sets of experimental runs represented on Table 8.1. Then experiments were carried out in the laboratory according to the 32 selected runs. The experimental results are analysed and compared with the results generated from ANOVA. ANOVA is a technique that uses p-value in order to examine the significance of the effect of the main factors or the interactions between them. P-value is the probability that shows the significance of a factor which means the rejection of the null hypothesis which is that all the factors have the same effect in the experiments. If the p-value is smaller than 0.05 then the effect of the factor itself or the interaction between factors should be considered as significant. Certainly if the p-value is smaller than the limit of 0.0001 then the factor or the interaction of the factors is considered very significant. On the other hand when the p-value is higher than 0.1 then the factor or the interaction between factors should not be considered significant (Montgomery and Runger, 2004). Detailed description can be found in Section 5.5 3.

Results from experimental work, according to the randomized setup under different conditions (see Table 8.1), are presented in this section. Figures 8.2-8.9 present a diagram of the variation of the produced glucose from the cellulose amount of the pre-treated biomass for the respective run of the experiments. The product - glucose is presented as theoretic yield (%). It can be seen from Figure 8.2 to Figure 8.9 that the number of runs for various ranges of glucose% produced is as follow: From carrot peelings: 2 runs in the range 0-20%, 12 runs in the range 20-40% , 4 runs in the range 40-60%, 9 runs in the range 60-80% and 5 runs in the ranges 80-100% with the lowest product - glucose 15.4% and the highest 94.38%; From potato peelings: 1 run in the range 0-20% , 13 runs in the range 20%-40%, 6 runs in the

range 40%-60%, 5 runs in the range 60%-80% and 7 runs in the range 80%-100% with the lowest product - glucose 18.96% and the highest 90.78%; From grass: 0 run in the range 0-20%, 15 runs in the range 20%-40%, 3 runs in the range 40%-60%, 7 runs in the range 60%-80% and 7 runs in the range 80%-100% with the lowest product - glucose 21.87% and the highest 92.86%; From newspaper: 2 runs in the range 0-20%, 14 runs in the range 20%-40%, 3 runs in the range 40%-60%, 8 runs in the range 60%-80% and 5 runs in the range 80%-100% with the lowest product - glucose 16.61% and the highest 91.40%; From scrap paper: 0 runs in the range 0-20%, 13 runs in the range 20%-40%, 5 runs in the range 40%-60%, 7 runs in the range 60%-80% and 7 runs in the range 80%-100% with the lowest product - glucose 20.81% and the highest 92.20%; From a combination of carrot peelings and potato peelings: 3 runs in the range 0-20%, 9 runs in the range 20%-40%, 9 runs in the range 40%-60%, 7 runs in the range 60%-80%, and 4 runs in the range 80%-100% with the lowest product - glucose 13.96% and the highest 90.36%; From a combination of newspaper and scrap paper: 2 runs in the range 0-20%, 10 runs in the range 20%-40%, 8 runs in the range 40%-60%, 8 runs in the range 60%-80%, and 4 runs in the range 80%-100% with the lowest product - glucose 18.07% and the highest 90.78%; From mixed substrates: 1 run in the range 0-20%, 11 runs in the range 20%-40%, 6 runs in the range 40%-60%, 4 runs in the range 60%-80% and 10 runs in the range 80%-100% with the lowest product - glucose 16.58% and the highest 97.58%.

Table 8.1 Randomised experimental setup generated according to fractional experimental design

Randomised Runs	Particle (mm) A	Substrate con.(g/L) B	Cellulase (FPU/g) C	Beta-glucosidase (PNPGU/g) D	pH E	Temperature °C F
11	0.2	5	100	100	3.7	37
8	1.2	15	100	100	5	50
30	0.2	5	100	100	5	50
15	1.2	5	100	10	5	50
31	1.2	5	100	100	3.7	50
5	1.2	15	10	10	5	50
9	0.2	5	10	10	3.7	37
10	0.2	5	100	10	5	37
20	1.2	5	10	100	3.7	37
21	0.2	5	10	100	3.7	50
25	0.2	15	100	10	3.7	37
22	0.2	15	10	100	3.7	37
6	1.2	5	10	100	5	50
27	1.2	15	100	100	3.7	37
13	0.2	15	10	10	3.7	50
3	1.2	15	100	10	5	37
12	0.2	15	10	10	5	50
24	0.2	15	100	10	5	50
18	1.2	15	10	100	3.7	50
1	1.2	15	10	100	5	37
26	1.2	5	10	10	5	37
29	0.2	15	100	100	5	37
14	1.2	15	100	10	3.7	50
7	0.2	5	10	10	5	50
16	1.2	5	100	10	3.7	37
23	0.2	5	100	10	3.7	50
32	0.2	5	10	100	5	37
28	1.2	5	100	100	5	37
17	1.2	15	10	10	3.7	37
2	1.2	5	10	10	3.7	50
4	0.2	15	10	10	5	37
19	0.2	15	100	100	3.7	50

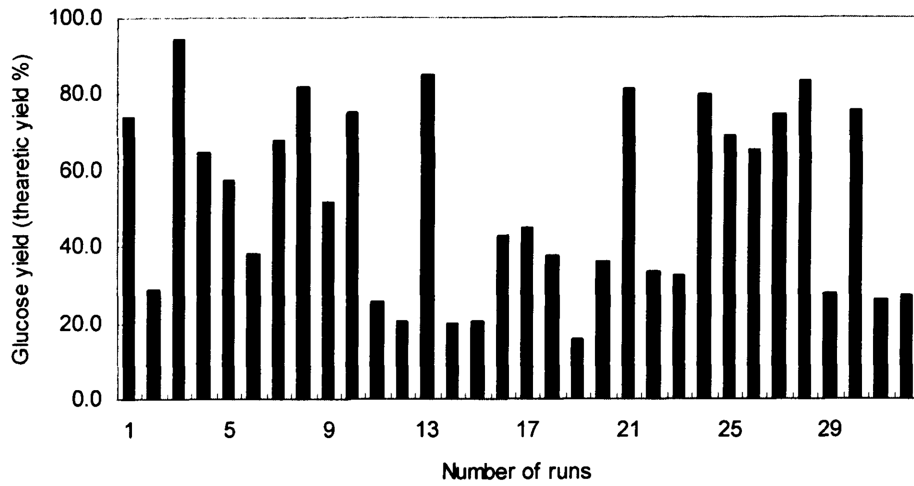


Figure 8.2: Produced glucose % from CP for each run of the experiments (feedstock: CP)

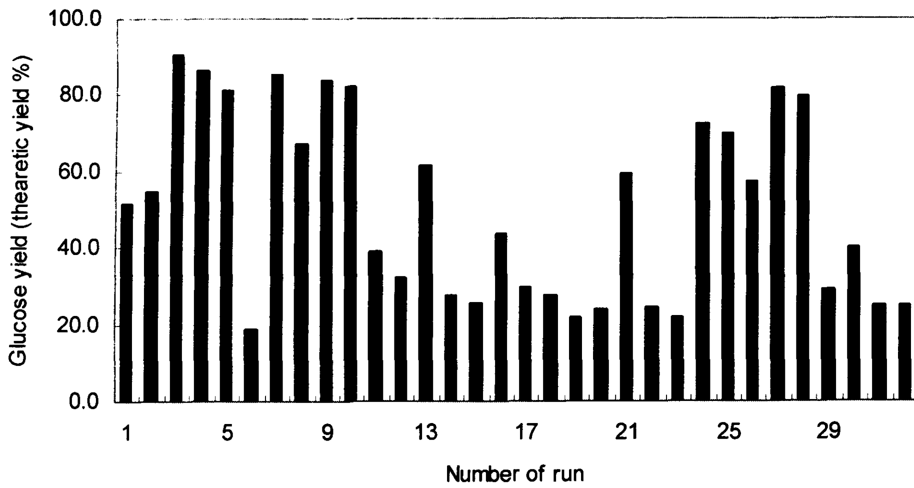


Figure 8.3: Produced glucose% from each run of the experiments (feedstock: PP)

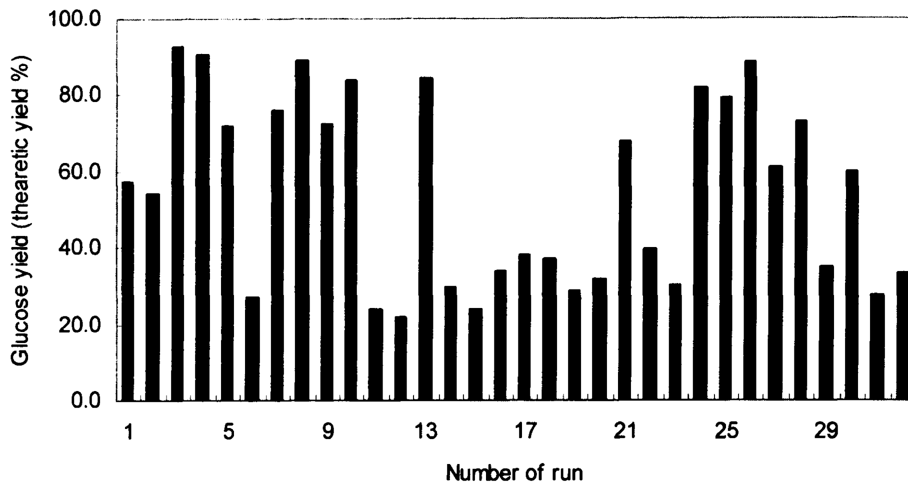


Figure 8.4: Produced glucose% from each run of the experiments (feedstock: Gr)

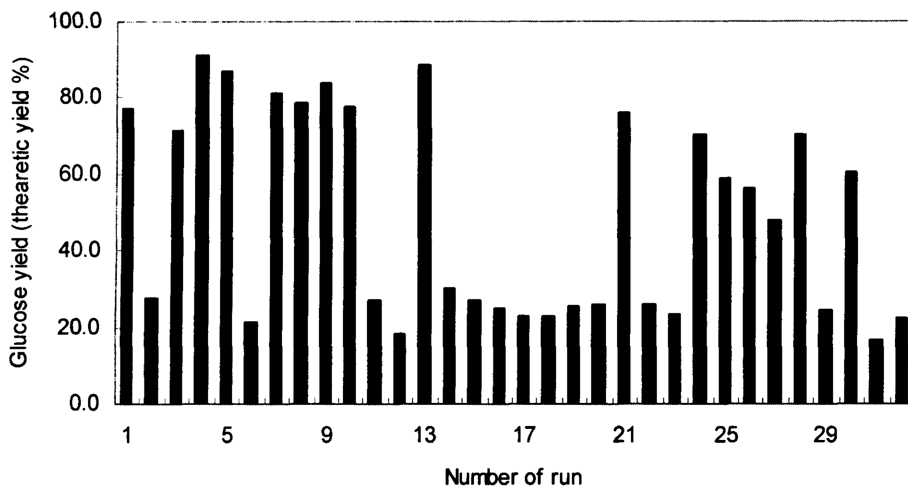


Figure 8.5: Produced glucose% from each run of the experiments (feedstock: NP)

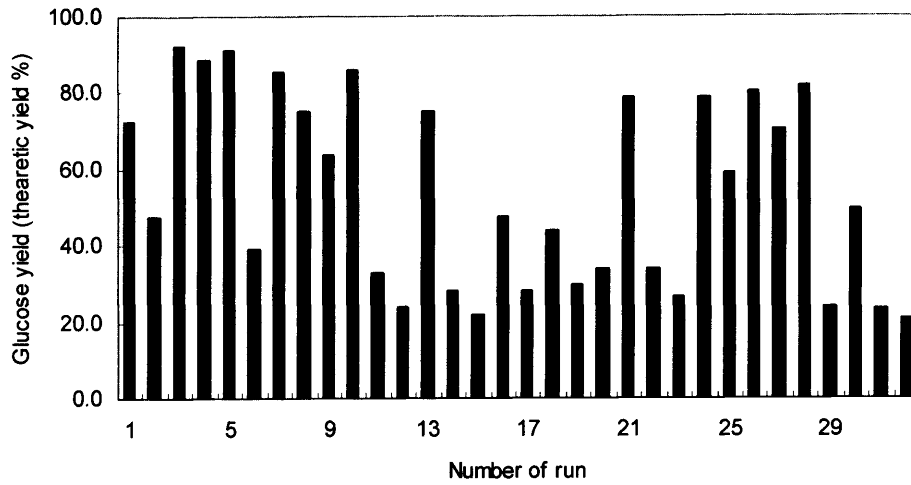


Figure 8.6: Produced glucose% from each run of the experiments (Feedstock: SP)

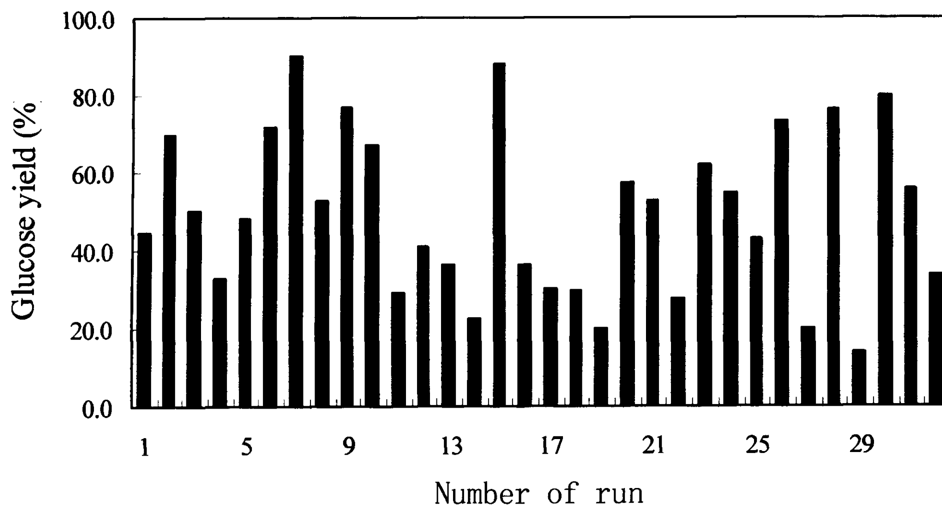


Figure 8.7: Produced glucose% from each run of the experiments (feedstock: CP+PP)

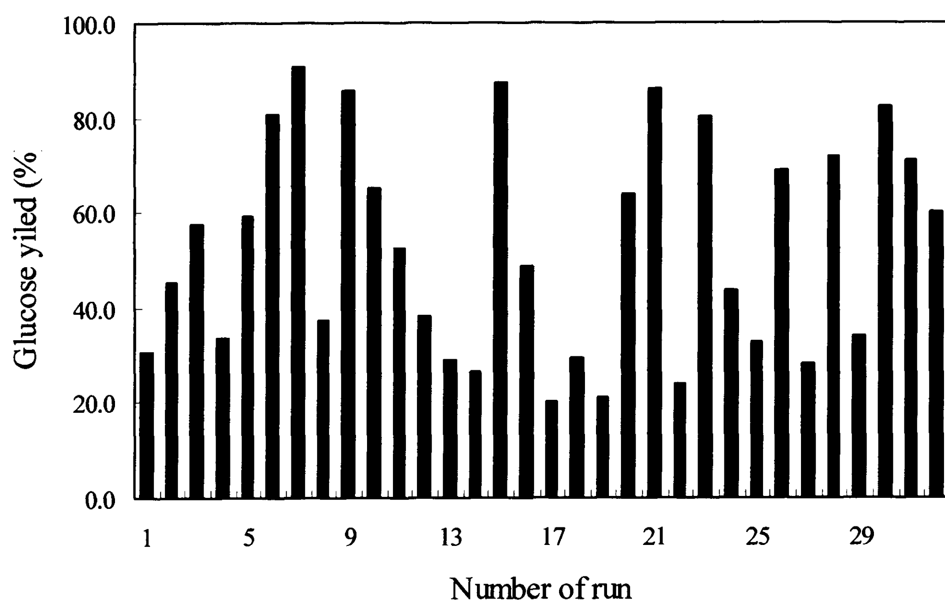


Figure 8.8: Produced glucose% from each run of the experiments (feedstock: NP+SP)

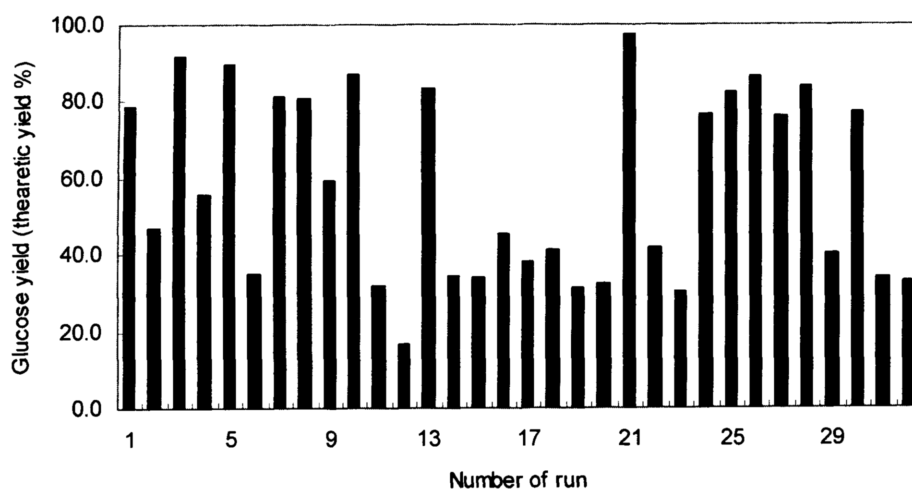


Figure 8.9 Produced glucose% from SP for each run of the experiments (feedstock: Mixed)

8.3.2 Analysis of the controlling parameters

In this section, the experimental results obtained were analysed in order to identify the controlling parameters. The results of analysis are also compared and confirmed with ANOVA model. The assumption of ANOVA model can be found in Chapter 5. Residuals are estimates of experimental error obtained by subtracting the observed responses from the predicted responses. The predicted response is calculated from the chosen model, after all the unknown model parameters have been estimated from the experimental data. Examining residuals is a key part of all statistical modelling. It can indicate whether the assumptions are reasonable and the choice of model is appropriate.

The normal probability plot of studentised residuals is a graphical tool for assessing whether a dataset has approximately a normal distribution (Chambers, 1983). The studentised residual has the form of standard deviation of the error. Studentised residuals have constant variance when the model is appropriate. The data are plotted against a theoretical normal distribution in such a way that the points should form an approximate straight line. Departures from this straight line traduce departures from normality.

The results of the analysis are described in the figures and tables that followed, and presented in accordance to the type of biomass. For each type of biomass, a graph with the normal probability is presented to examine if the chosen model is appropriate for the observed data, followed by a table of significant factors controlling the process with their p-value. Finally a cube graph shows the change of response (glucose yield) in response to the most significant factors.

Figures 8.10 - 8.17 are diagnostic graphs showing the relationship between Normal Probability and Studentised Residuals by using the analysis of variance (ANOVA) method. Most of the data lay on a straight line which indicates that the model statistics and diagnostic are appropriate, ensuring that further analysis can be based on the data obtained.

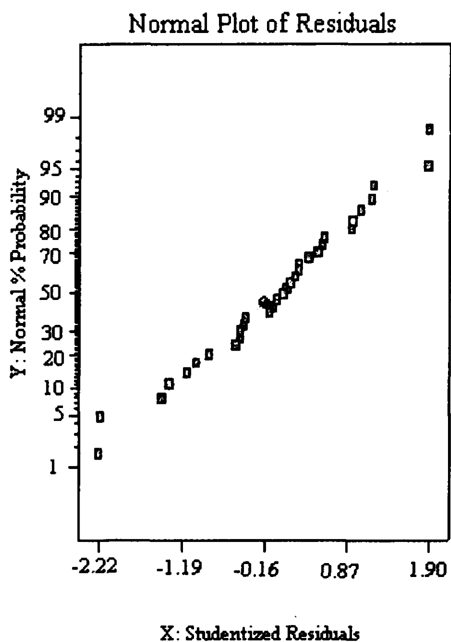


Figure 8.10 Normality of studentised plot (substrate: carrot peelings)

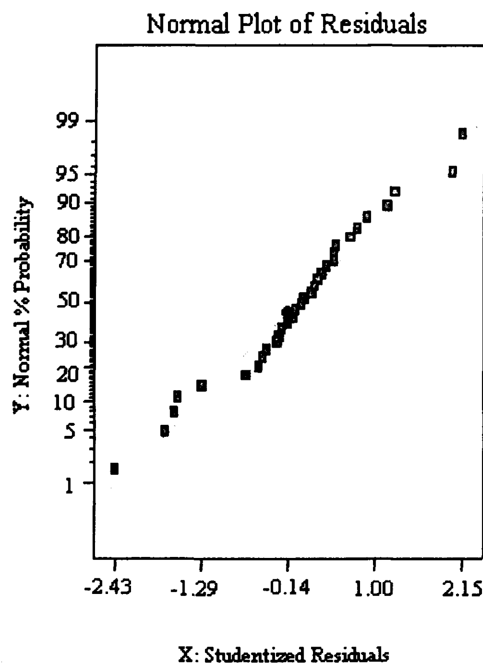


Figure 8.11 Normality of studentised plot (substrate: potato peelings)

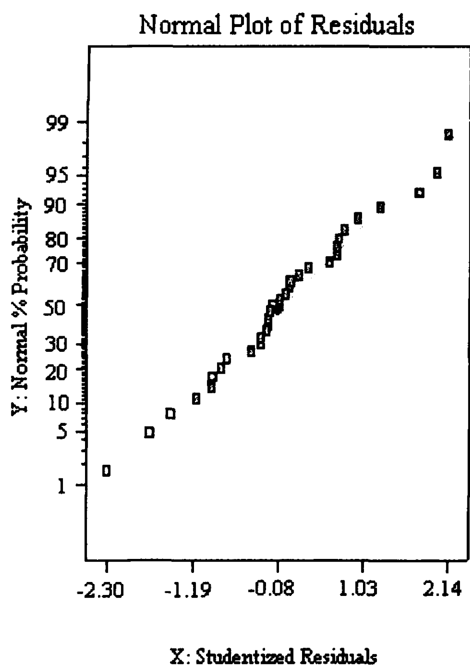


Figure 8.12 Normality of studentised plot (substrate: grass)

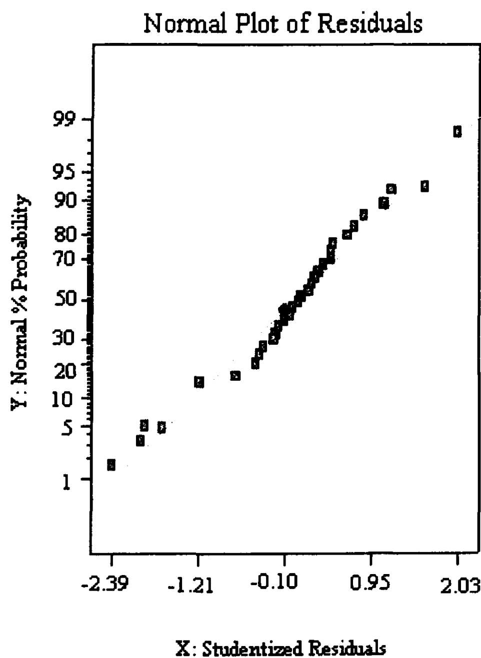


Figure 8.13 Normality of studentised plot (substrate: newspaper)

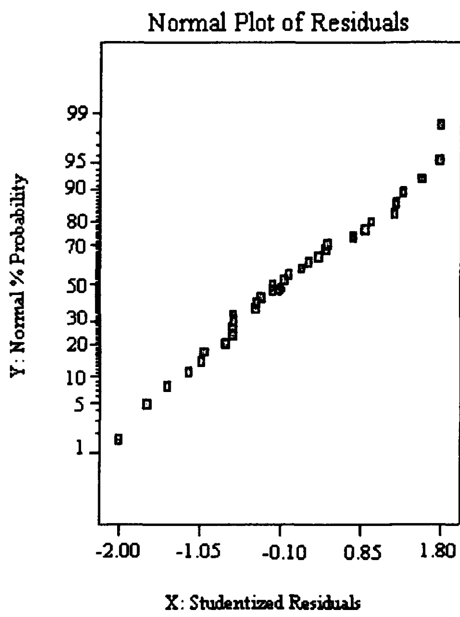


Figure 8.14 Normality of studentised plot (substrate: scrap paper)

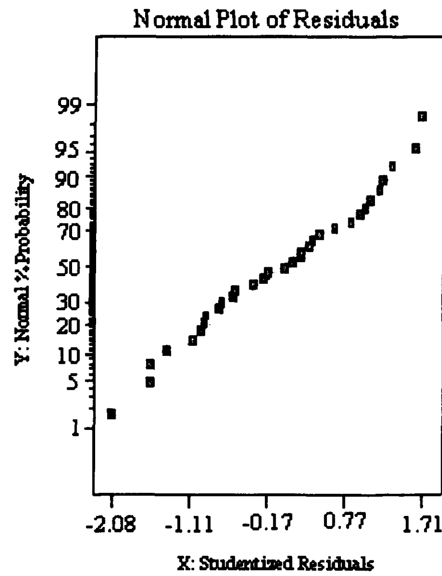


Figure 8.15 Normality of studentised plot (substrate: combination of carrot peelings and potato peelings)

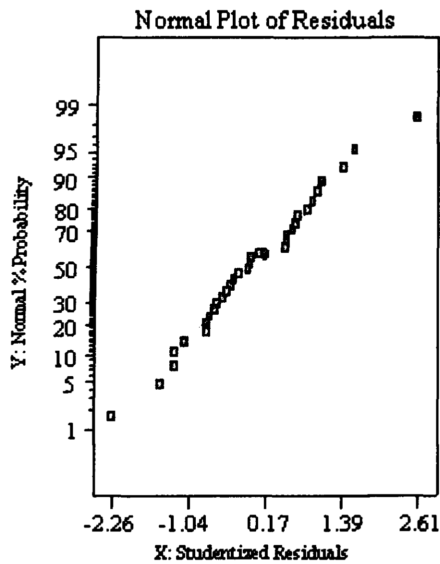


Figure 8.16 Normality of studentised plot (substrate: combination of newspaper and scrap paper)

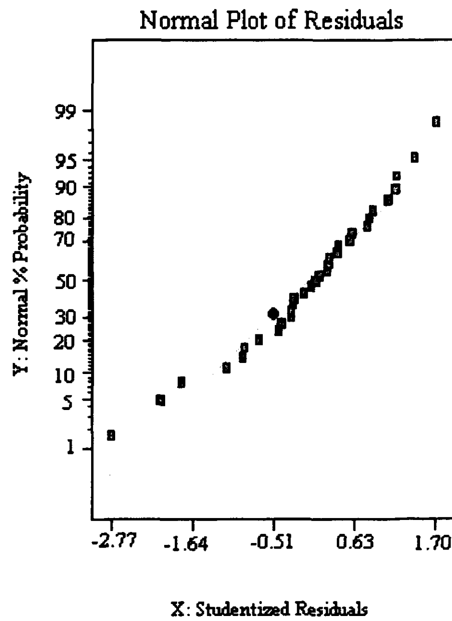


Figure 8.17 Normality of studentised plot (substrate: mixed substrates)

Table 8.2 shows the p-value of the affecting factors during the enzymatic hydrolysis for each type of biomass. The first column of the table shows the factors which by themselves or their interactions have an effect on the model, while the second column shows the significance of the factor or the interaction of the factors. It can be deduced that the process is very sensitive to B and E (p-value < 0.0001). In other words, the enzymatic hydrolysis process with carrot peeling used as biomass feedstock is mainly controlled by substrate concentration and pH value. Table 8.2 also shows the factors which by themselves or their interactions have an effect on the model. It can be deduced that B (substrate concentration) is very significant for the process (p-value < 0.0001). Although particle size and cellulase concentration is not significant (more than 0.05), the interaction between these two factors has significant contribution to the process. As other biomass, the concentration of substrate has a very significant contribution to the reaction process (see Table 8.2). Temperature and pH are considered as significant factors when using grass as feedstock. As for newspaper, the concentration of substrate has very significant contribution to the reaction process. Particle size, cellulase concentration and temperature are not considered as significant factors when using grass as feedstock, but their interaction make significant contribution as shown in the Table 8.2 is less than 0.05. It can also be seen that the concentration of substrate has very significant contribution to the reaction process. Particle size, cellulase concentration and temperature are not considered as significant factors when using grass as feedstock, but their interaction make significant contribution as shown the p-value is less than 0.05.

The factors with the most significant for the process identified are the same when different substrate samples are used. It shows the concentration of substrate has very significant contribution to the reaction process. Particle size, cellulase concentration and temperature are not considered as significant factors when using grass as feedstock, but their interaction make significant contribution; as shown in the Table 8.2, the p-value is close to 0.07.

From the p-value analysis, it can be concluded that substrate concentration is extremely important for each selected biomass type, followed by pH. It is found that it is not necessary to have high cellulase concentration providing that the cellulase

is enough for the presented cellulose level. The analysis continues by looking at the cube graph which represents the range of glucose yield produced according to different combination of factors values. Detailed effects of each factor are discussed in the following sub-sections.

Table 8.2 P-value of the significant factors

Factors /biomass	A (particle size)	B (substrate concentration)	C (cellulase concentration)	D (beta-glucosidase concentration)	E (pH)	F (temperature)	AC	ACF	AD	BE
Carrot peelings (CP)	–	<0.0001	–	–	<0.0001	–	–	–	–	–
Potato peelings (PP)	0.8387	<0.0001	0.2359	–	–	–	0.0082	–	–	–
Grass (GR)	–	<0.0001	–	–	0.0101	0.0131	–	–	–	–
Newspaper (NP)	0.1423	<0.0001	0.5475	–	–	0.5370	–	0.0168	–	–
Scrap paper (SP)	–	<0.0001	–	–	0.0032	–	–	–	–	–
CP+PP	–	<0.0001	–	–	0.0136	–	–	–	–	–
NP+SP	0.7933	<0.0001	–	0.2304	0.0002	–	–	–	0.0089	–
CP+PP+GR+NP+SP	–	<0.0001	–	–	0.0703	–	–	–	–	0.0722

From Figure 8.18, it can be observed that as the substrate concentration increases from 5 to 15 g/L the glucose yield (%) decreases from 4.40 to 3.59 in transferred nature log format. Figure 8.18 shows that the increase of the pH value gives an increase to glucose yield % (about 14% from 3.11 to 3.55 in transferred nature log format). The model also shows that factors of C (cellulase concentration), D (Beta-glucosidase concentration), and F (Temperature) have limited effect on the reaction. To improve the glucose yield produced, it is important to keep the substrate concentration as low as possible but also pH as high as possible within the provided range.

Figure 8.19 shows with certain conditions of factor D, E, and F, the changes of glucose yield according to difference values of factor A, B and C. From Figure 8.19 it can be observed that as the substrate concentration increases from 5 to 15 g/L then the glucose % decreases from 4.31 to 3.40% in transferred nature log format. The increase of the particle size value results in a small decrease of glucose yield (from 4.31 to 4.07% in transferred nature log format). In contrast, the increase of cellulase concentration gives an increase on glucose yield (from 4.07 to 4.22% in transferred nature log format). The interaction of these factors indicates that the higher glucose yield is obtained when lower substrate concentration, smaller particle size and higher cellulase concentration within the study range.

From Figure 8.20 it can be observed that as the substrate concentration increases from 5 to 15 g/L then the glucose % decreases (from 3.87 to 3.15% in transferred nature log format) and the difference of glucose % in transferred nature log format is 0.72%. Higher pH value gives higher glucose yield (4.19% in transferred nature log format when pH is 5.0% and 3.87% when pH is 3.7%). Similarly higher temperature results in higher glucose yields (3.47% when temperature is 50°C and 3.15% when temperature is 37°C). However, the effects of pH value and temperature are unlikely as strong as substrate concentration.

From Figure 8.21 it can be observed that as the substrate concentration increases from 5 to 15 g/L then the glucose % decreases and the difference of glucose % in transferred nature log format is 1.1 (from 4.32 to 3.22%). Higher pH value gives slightly higher glucose yield. Similarly higher temperature results in higher glucose yield. However, the effect of particle size is unlikely as strong as substrate

concentration.

From Figure 8.22 it can be observed that as the substrate concentration increases from 5 to 15 g/L then the glucose % decreases from 4.23 to 3.16% in transferred nature log format. Higher pH value gives slightly higher glucose yield. Similarly higher temperature results in higher glucose yield. However, the effect of particle size is unlikely as strong as substrate concentration.

Figure 8.23 indicates that the decrease of substrate concentration from 15g/L to 5g/L results in increase of glucose yield for the biomass of combination of carrot peelings and potato peelings. Higher pH value gives slightly higher glucose yield.

Similar result is found for combination of newspaper and scrap paper as shown in Figure 8.24. Higher glucose yield is obtained with the lower substrate concentration and higher pH value.

From Figure 8.25, the glucose % decreases when the substrate concentration increases from 5 to 15 g/L and the difference of glucose % in transferred nature log format is 0.95% (from 4.37 to 3.42%). Higher pH value gives slightly higher glucose yield. Similarly higher temperature results in higher glucose yield. However, the effect of particle size is unlikely as strong as substrate concentration.

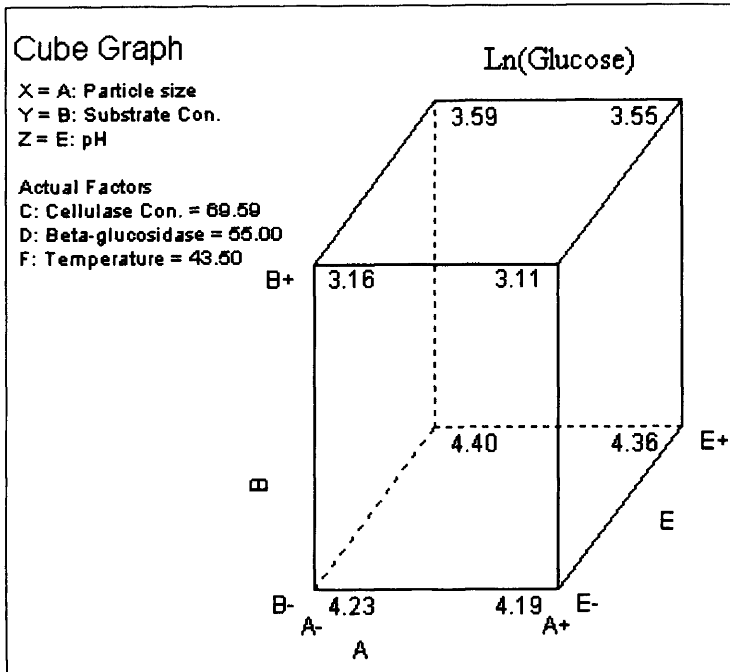


Figure 8.18 Effects on produced glucose yield (in nature log format) in response to factor A, B & E (Feedstock: carrot peelings)

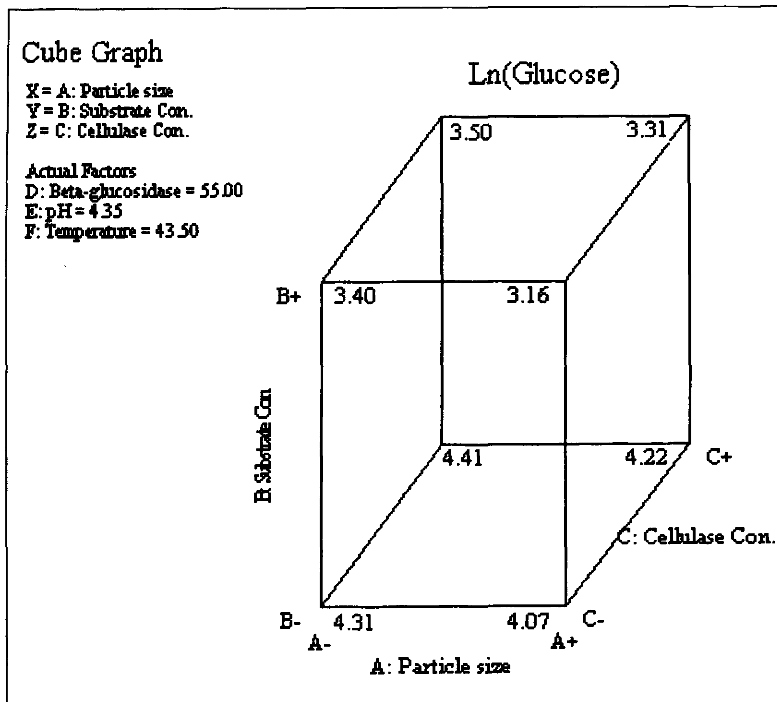


Figure 8.19 Effects on produced glucose yield (in nature log format) in response to factor A, B & C (Feedstock: potato peelings)

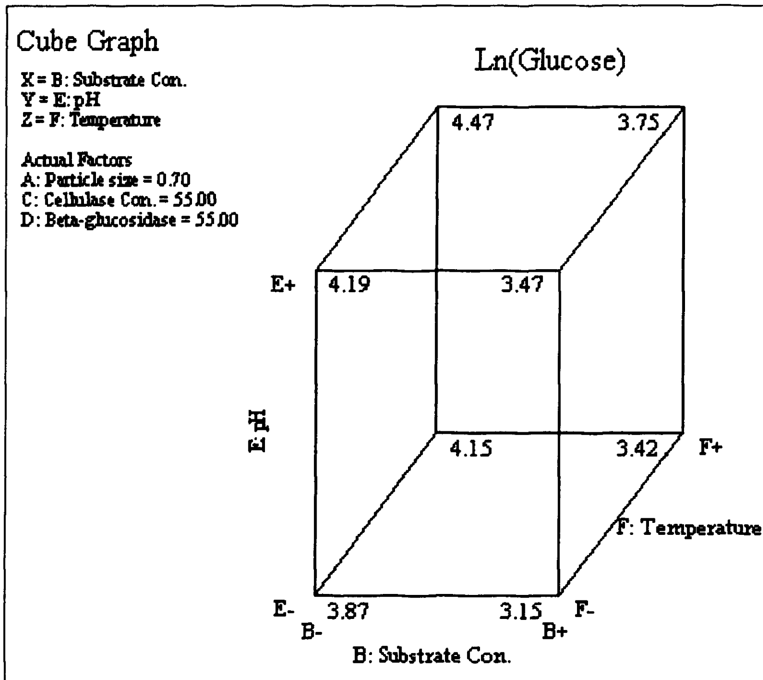


Figure 8.20 Effects on produced glucose yield (in nature log format) in response to factor B, E & F (Feedstock: grass)

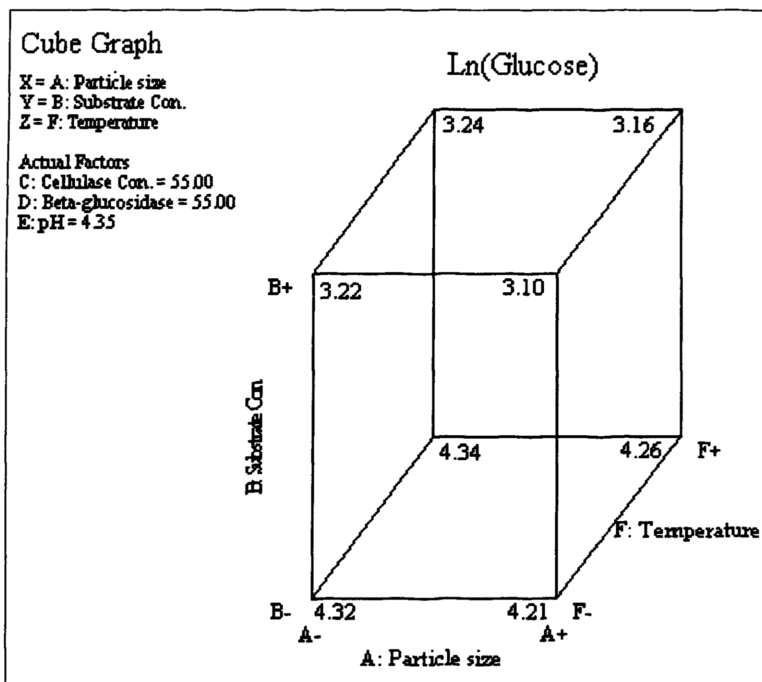


Figure 8.21 Effects on produced glucose yield (in nature log format) in response to factor A, B & E (Feedstock: newspaper)

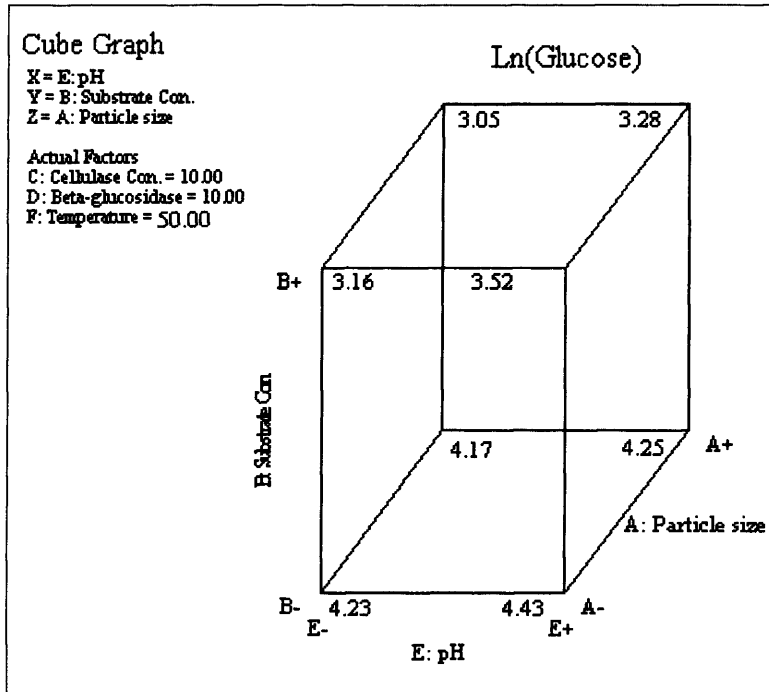


Figure 8.22 Effects on produced glucose yield (in nature log format) in response to factor A, B & E (Feedstock: scrap paper)

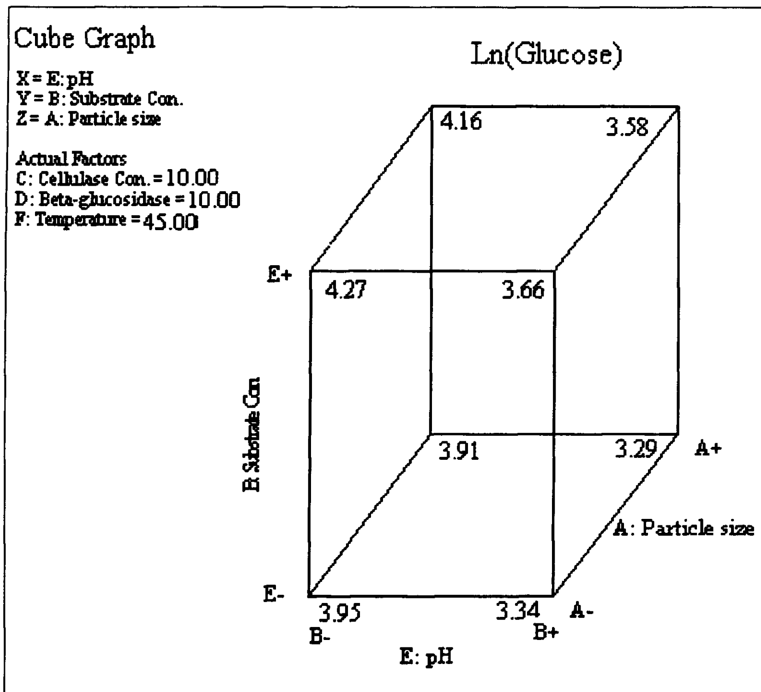


Figure 8.23 Effects on produced glucose yield (in nature log format) in response to factor A, B & E (Feedstock combination of: carrot peelings and potato peelings)

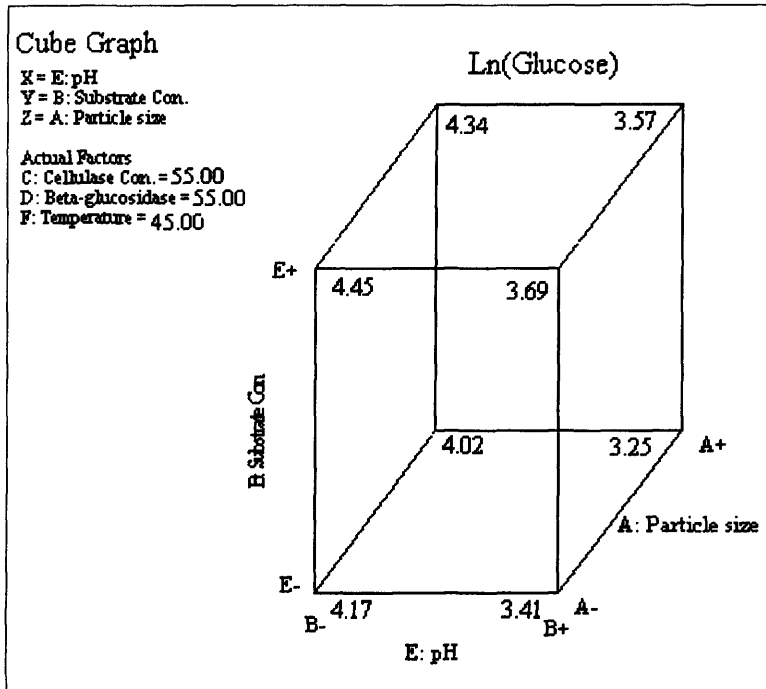


Figure 8.24 Effects on produced glucose yield (in nature log format) in response to factor A, B & E (Feedstock: combination of newspaper and scrap paper)

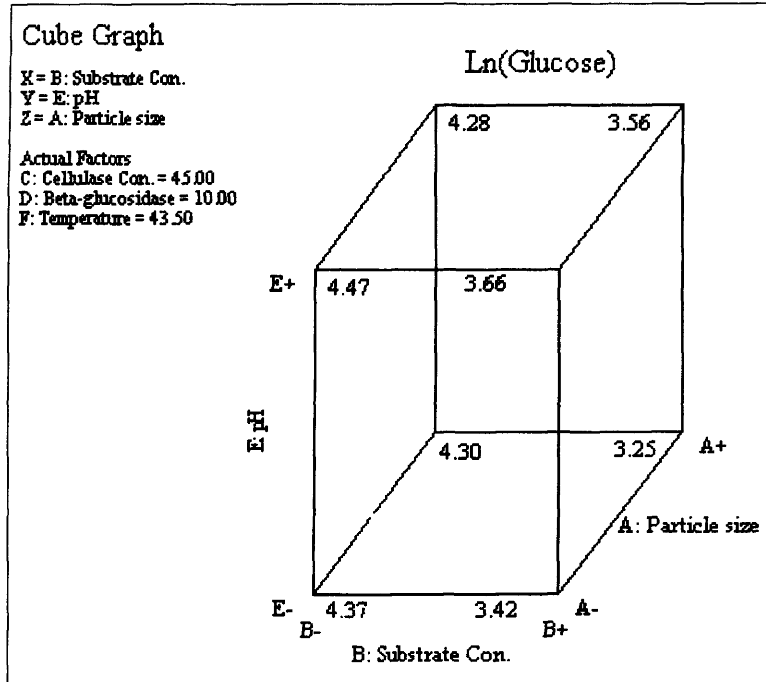


Figure 8.25 Effects on produced glucose yield (in nature log format) in response to factor A, B & E (Feedstock: mixed waste)

8.3.3 Effects of substrate concentration

From the above analysis, substrate concentration appears to be one of the main factors that affect the yield and initial rate of enzymatic hydrolysis of cellulose. Figure 8.26 shows that a maximum of 50% increase of glucose yield can be observed when the substrate concentration decreases from 15g/L to 5g/L. Same trends also happen to other biomass with maximum 43.6% increase for carrot peelings, 35.0% increase for potato peelings, 24.6% for grass, 50.2% for newspaper, 45.1% for scrap paper, 23.1% for combination of carrot peelings and potato peelings, 41.2% for a combination of newspaper and scrap paper, and 48.5% for mixed waste. Low substrate levels result in an increase of the yield and reaction rate of the hydrolysis which agrees with the findings from Cheung and Anderson (1997). High substrate concentration can cause substrate inhibition, which substantially lowers the rate of the hydrolysis. As Huang and Penner (1991) and Penner and Liaw (1994) suggested, the extent of substrate inhibition depends on the ratio of total substrate to total enzyme. Huang and Penner (1991) found that the substrate inhibition occurred when the ratio of the microcrystalline substrate Avicel pH 101 to the cellulase from *Trichoderma reesei* (grams of cellulose/FPU of enzyme) was greater than 5. The investigation of this work is not subject the inhibition effect due to the fact the maximum cellulose to enzyme ratio is 1.5. Penner and Liaw (1994) reported that the optimum substrate to enzyme ratio was 1.25 g of the microcrystalline substrate Avicel pH 105 per FPU of the cellulase from *T. reesei.*

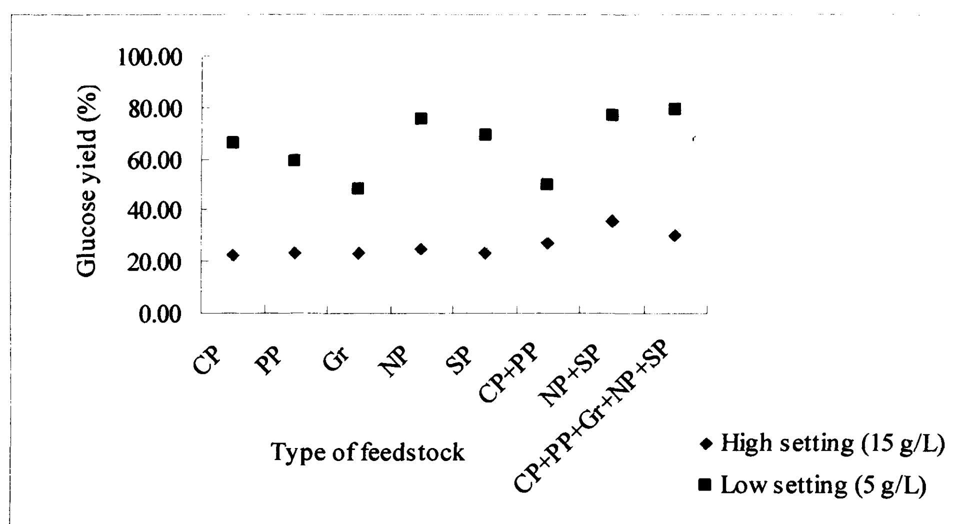


Figure 8.26 Difference of glucose yield corresponded to different substrate concentration. (High setting – substrate concentration 15 g/L, low setting – substrate concentration (5 g/L)

8.3.4 Effects of cellulase and beta-glucosidase concentration

By increasing the dosage of cellulases in the process from 10 FPU to 100 FPU, a maximum of 15.5% increase of glucose yield is observed when using grass as biomass (Figure 8.27). With such a limited contribution the increase of cellulase would significantly increase the cost of the process. Experimental results indicate that 10 FPU/g of cellulase dosage is enough to hydrolyse 5-10 g/L substrate, which provides a hydrolysis profile with high levels of glucose yield in a reasonable time (24 h) at a reasonable enzyme cost. Gregg and Saddle (1996) found the similar glucose yield (80%) with the same amount of cellulase (10 FPU) but after 48-72 h hydrolysis. Use of a cellulase mixture from different microorganisms or a mixture of cellulases and other enzymes in the hydrolysis of cellulosic materials has been extensively studied (Beldman *et al.*, 1988; Excoffier *et al.*, 1992; Xin *et al.*, 1993). The addition of β-glucosidases into the *T. reesei* cellulases system achieved better saccharification than the system without β-glucosidases (Excoffier *et al.*, 1991; Xin *et al.*, 1993). β-glucosidases hydrolyse the cellobiose which is an inhibitor of cellulase activity. A mixture of hemicellulases or pectinases with cellulases exhibited a significant increase in the extent of cellulose conversion (Ghose and Bisaria, 1979; Beldman *et al.*, 1984). A cellulose conversion yield of more than 90% was achieved in the enzymatic saccharification for all the selected biomass

with 10 FPU cellulase g substrate and 10 units' β-Glucosidases in this work. Higher enzyme loading such as 100 FPU/ g substrate does not greatly increase the hydrolysis yield. This can be explained that high concentration of enzyme has inhibition to the glucose product, which has been observed by Ooshima *et al.* (1986).

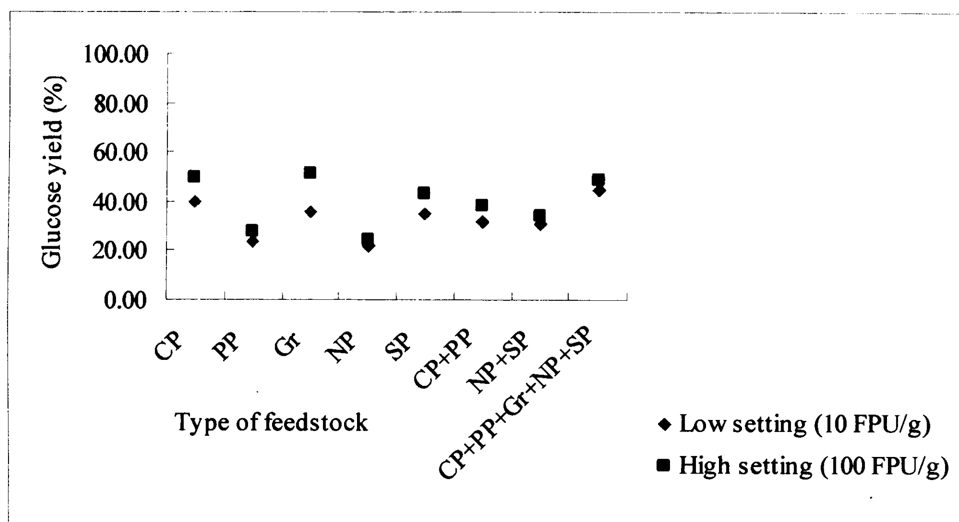


Figure 8.27 Effects of cellulase concentration on glucose yield (High setting – cellulose concentration 100 FPU/g, low setting – cellulase concentration 10 FPU/g)

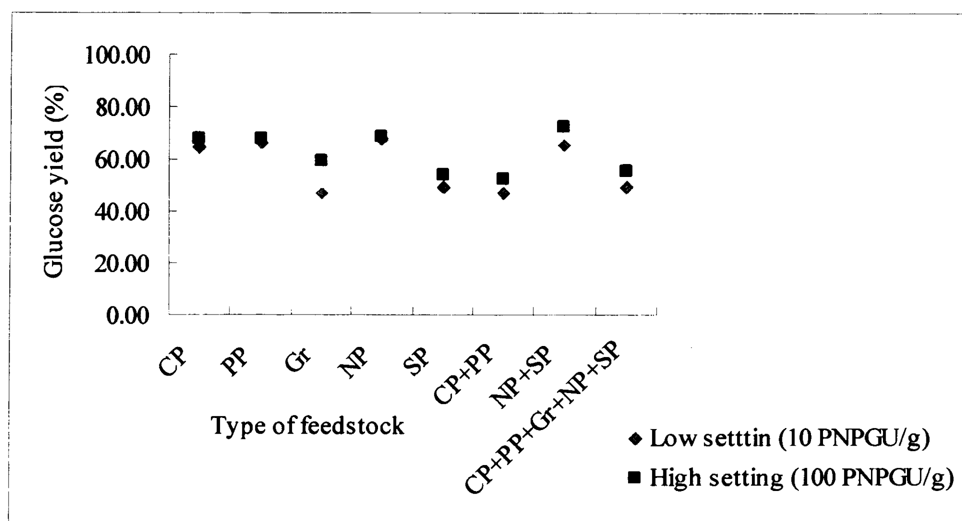


Figure 8.28 Effects of β-glucosidase concentration on glucose yield (High setting –β-glucosidase concentration 100 PNPGU /g, low setting –β-glucosidase concentration 10 PNPGU/g)

8.3.5 Effects of particle size

Particle size is indicative of the macro-accessibility of the substrate and may be representative of substrate surface readily available for enzymatic hydrolysis. Particle size has also been considered to be a major deterrent to enzymatic hydrolysis by limiting accessibility to 1, 3-1, 4-glucosidic bonds through restrictions on the surface area of the substrate. Therefore, as particle size decreased, a concomitant increase in conversion to either glucose or ethanol was expected.

From Figure 8.29, it is obvious that particle size does not have a significant effect on the hydrolysis reaction when using grass as biomass, and have very limited effect on other biomass. When the particle size decreases from 1.2 mm to 0.2 mm, it only results in maximum 1.2% increase of glucose yield from carrot peelings, 6.4% from potato peelings, 7.8% from newspaper, 2.5% from scrap paper, 3.0% from a combination of carrot peelings and potato peelings, 4.5% from a combination of newspaper and scrap paper, and 4.8% from mixed waste. The results of this work, suggested that particle size has very limited effects on glucose yield within the study range, though slighter higher product yields were observed by decreasing the particle size from 1.2 mm to 0.2 mm.

Rivers and Emerts (1987) stated that particle size may not be as important to the yield of enzymatic hydrolysis as once thought, which agrees with the findings from this work. With little improvement of the product yield; the reduction of particle size requires more energy input for mechanical performance such as milling. Therefore, it might be concluded that particle size is not as important in determining product yield to enzymatic hydrolysis within the range studied.

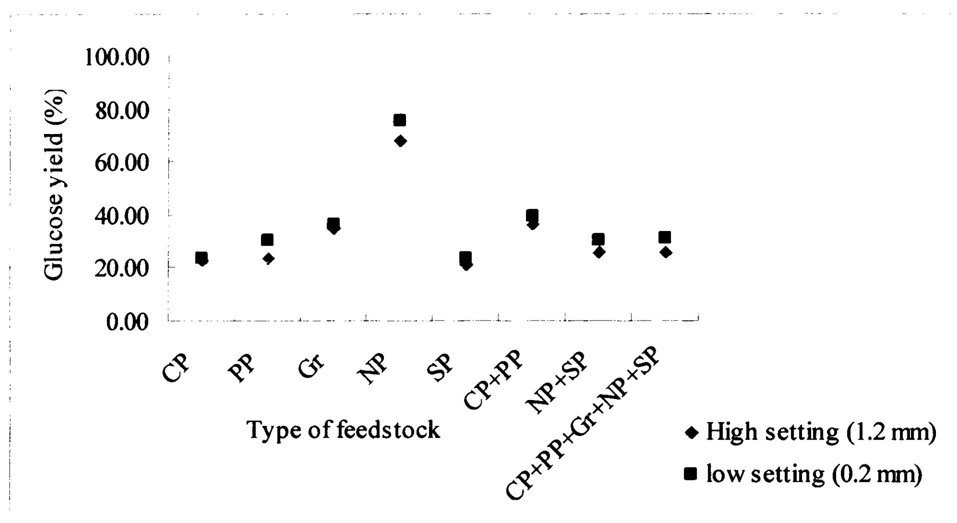


Figure 8.29 Effects of particle size on glucose yield (High setting – particle size 1.2 mm, low setting –particle size 0.2mm)

8.3.6 Effects of pH and temperature

Studies (Saha and Cotta, 2007; Wilkins *et al.*, 2005) have suggested that the optimal pH value is between 4.5 and 5.0. From the experimental results (Figure 8.30), it is obvious that pH can affect significantly the reaction rate. By increasing the pH value from 3.7 to 5.0, effects are observed except when using potato peelings or newspaper as biomass. A maximum of 12.4% increase in glucose yield has been seen for carrot peelings, 8.8% for grass, 10.2% for scrap paper, 9.0% for combination of carrot peelings and potato peelings, 9.8% for combination of newspaper and scrap paper, and 9.4% for mixed waste. Lower pH decreases the glucose yield. In contrast, higher pH value gives higher hydrolysis rate within the study range.

As for the hydrolysis temperature, the study range is within 37-50 °C, which is a suitable temperature for enzymes (Ball *et al.*, 1985; Rogalski *et al.*, 1993). Xu *et al.* (2006) suggests that the initial hydrolysis rate increases with enhancing temperature, and that maximum hydrolysis rate was observed at 50 °C. Higher up, hydrolysis rate decreased when temperatures exceeded 50 °C. This result could be attributed to the thermal inactivation of endoglucanase I and cellobiohydrolase I (Dominguez *et*

al., 1992) and (Jimenez, *et al.*, 1995). This work suggests that when the temperature is within the range 37-50 °C, the increase of temperature improve the hydrolysis rate when grass or newspaper is used as biomass, but does not significantly improve the product yield from other biomass (see Figure 8.31).

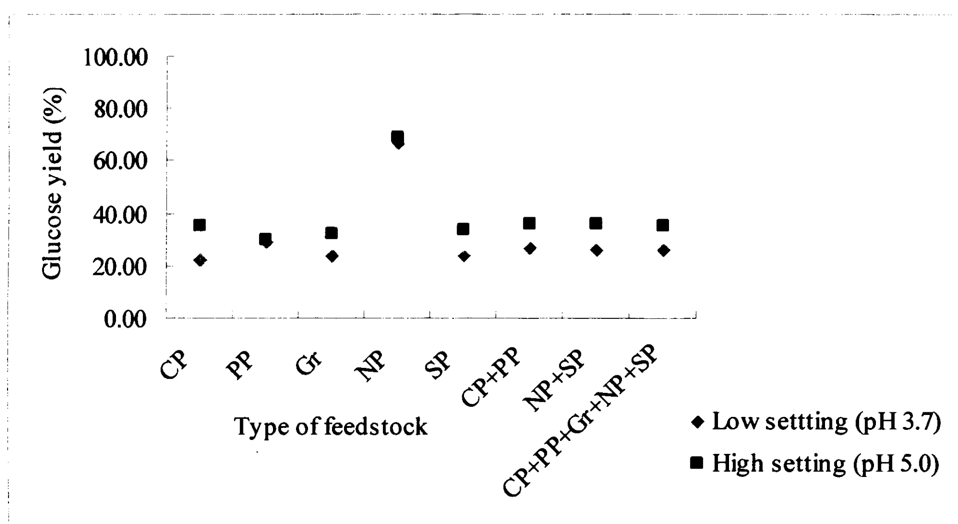


Figure 8.30 Effects of pH on glucose yield (High setting – pH 5.0, low setting – pH 3.7)

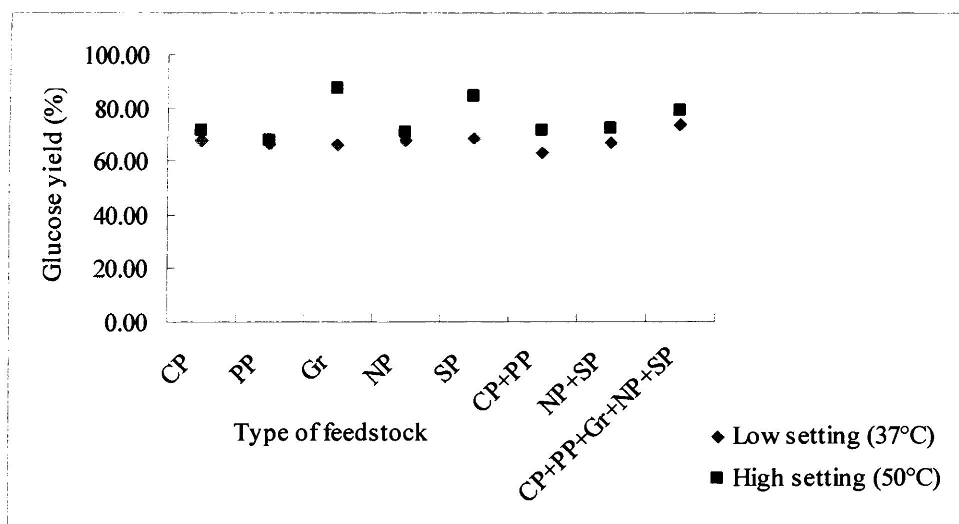


Figure 8.31 Effects of temperature on glucose yield (High setting – temperature 50 °C, low setting – temperature 37 °C)

8.4 Optimisation solution

Based on the analysis of experimental data and factor's contribution to the reaction in Section 8.3, the optimal conditions of each type of biomass are determined and presented in this section, as shown in Table 8.3 and Figures 8.32 to 8.39.

According to the analysis of factors in the previous section, it is obvious that substrate concentration is the most critical limiting factor for hydrolysis process, followed by temperature, particle size or pH value depending the type of feedstock. Figure 8.32-8.39 shows the optimisation solutions with the variation of substrate concentration and other limiting factors. For example, in Figure 8.32, the x-axis corresponds to substrate concentration, while the y-axis corresponds to glucose yield (in nature log format); two lines represent two different parameter conditions (the red one under condition of pH value is equal to 5.00 and the black line is the variation situation corresponding to the condition when pH value is equal to 3.70). For this figure, it can be seen that when using carrot peelings as biomass, the optimum predicted glucose yield is produced when substrate concentration is 5.00 g/L and the reaction pH is 5.00.

The optimum level for each factor and the best glucose yield predicted are given in Table 8.3 (below). Experimental work with the optimum conditions was carried out. The actual and predicted yield can be compared from Figure 8.40. The glucose yields were obtained after 24 h of hydrolysis.

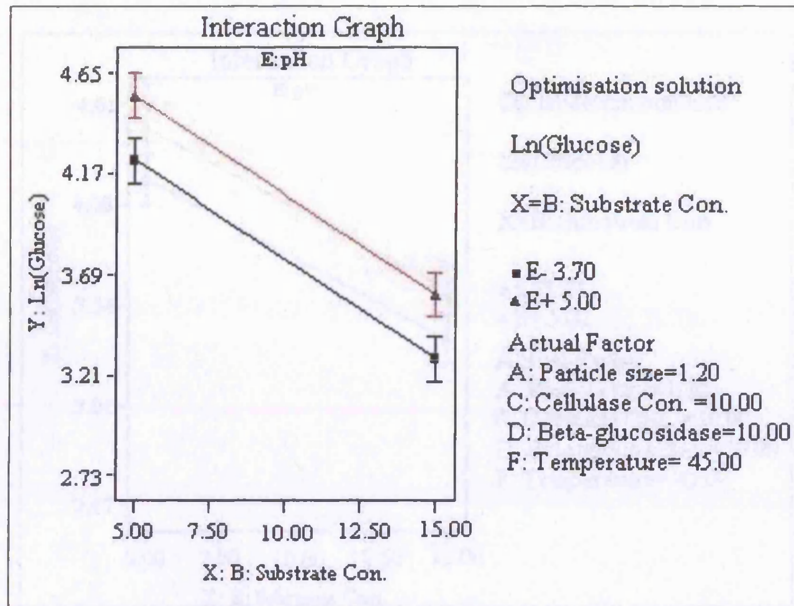


Figure 8.32 Graph of predicted optimum point (feedstock: CP)

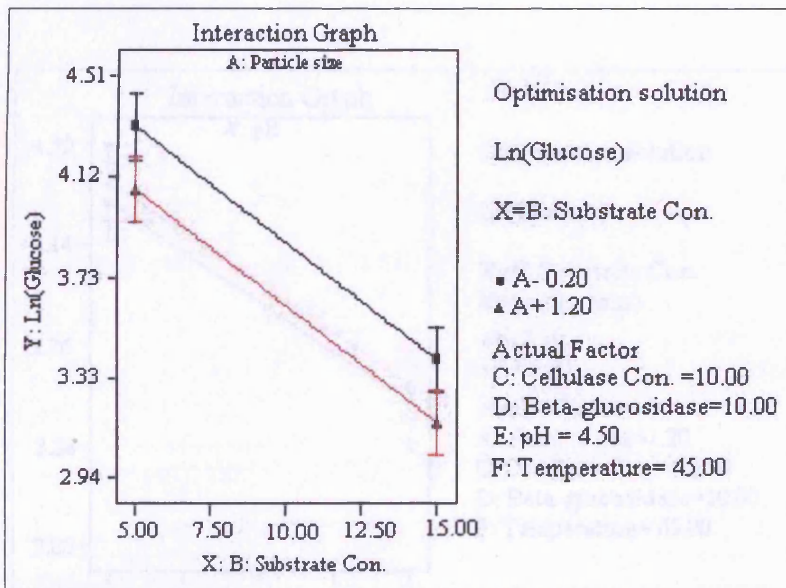


Figure 8.33 Graph of predicted optimum point (feedstock: PP)

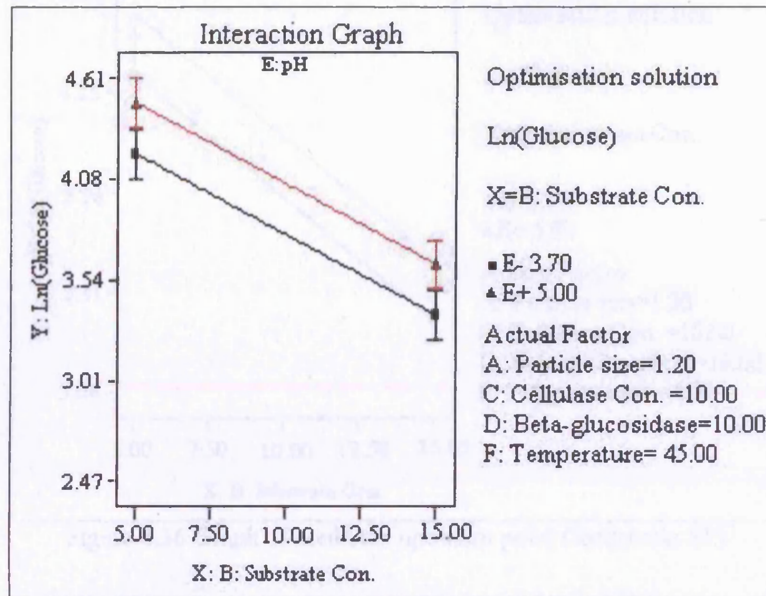


Figure 8.34 Graph of predicted optimum point (feedstock: Gr)

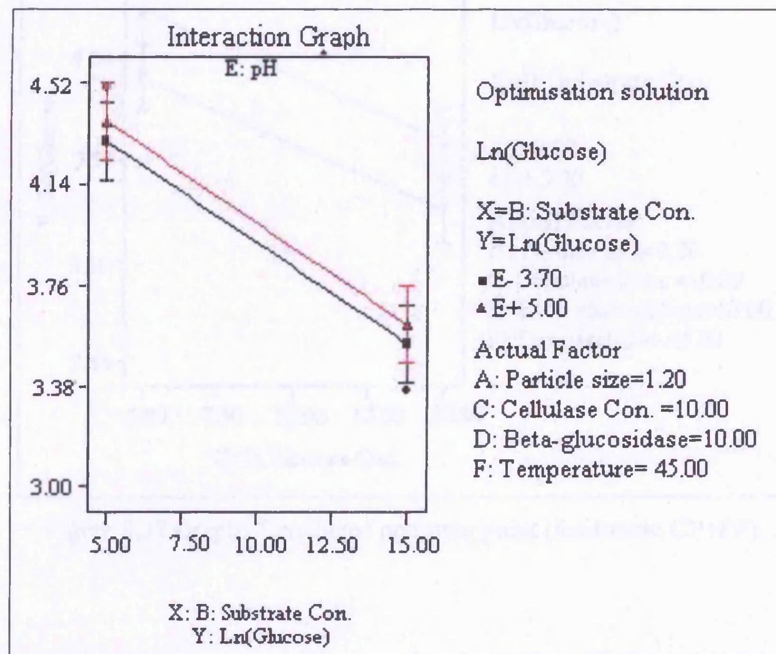


Figure 8.35 Graph of predicted optimum point (feedstock: NP)

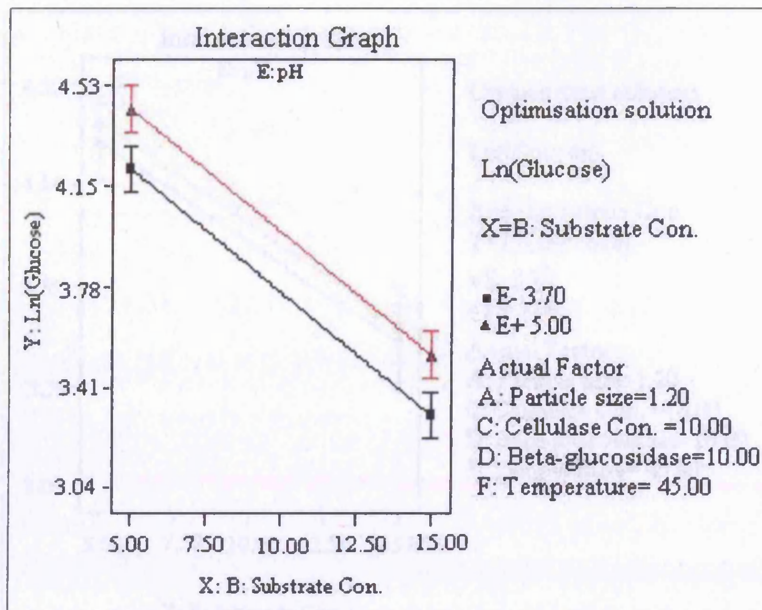


Figure 8.36 Graph of predicted optimum point (feedstock: SP)

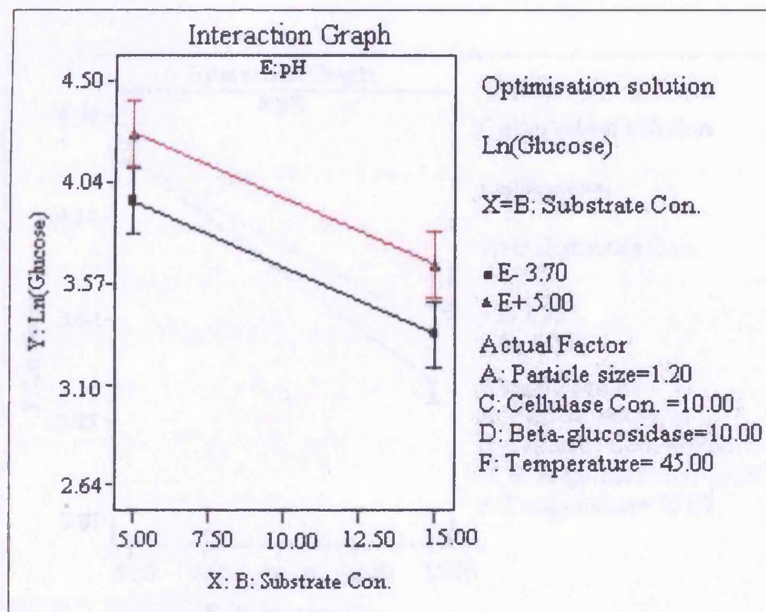


Figure 8.37 Graph of predicted optimum point (feedstock: CP+PP)

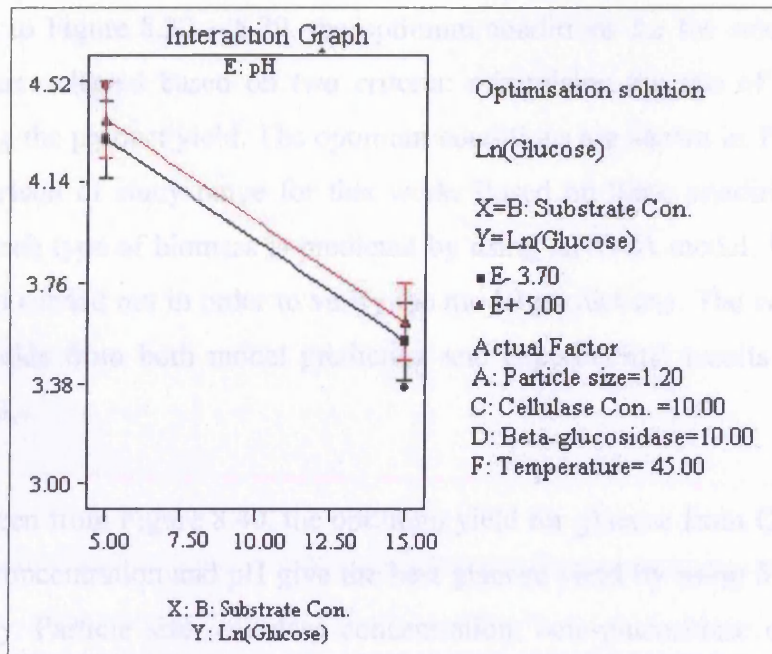


Figure 8.38 Graph of predicted optimum point (feedstock: NP+SP)

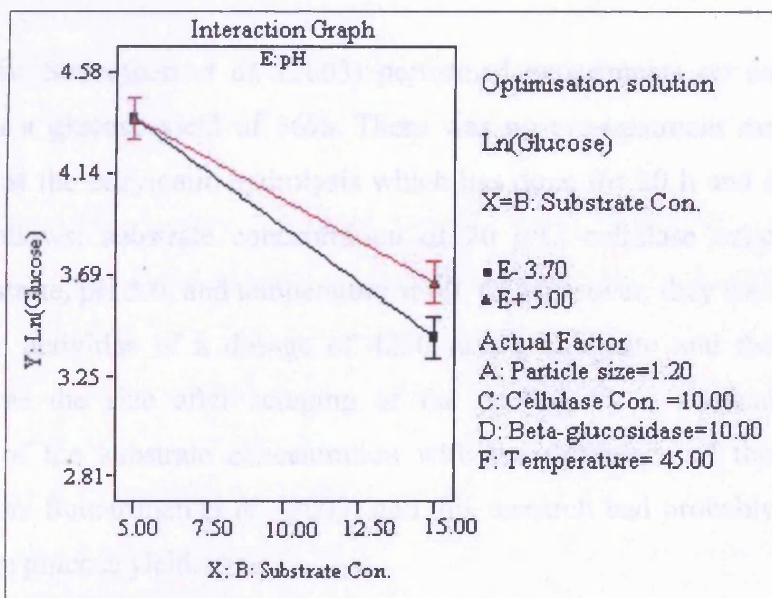


Figure 8.39 Graph of predicted optimum point (feedstock: Mixed BMSW fractions)

According to Figure 8.32 – 8.39, the optimum conditions for the selected BMSW fractions are selected based on two criteria: minimising the use of enzyme and maximising the product yield. The optimum conditions are shown in Table 8.5 with the comparison of study range for this work. Based on these conditions, glucose yield for each type of biomass is predicted by using ANOVA model. Experimental wok is also carried out in order to verify the model predictions. The comparison of glucose yields from both model prediction and experimental results is shown in Figure 8.33.

It can be seen from Figure 8.40, the optimum yield for glucose from CP is 80.26%. Substrate concentration and pH give the best glucose yield by using 5 g/L and 5.0, respectively. Particle size, cellulase concentration, beta-glucosidase concentration and temperature give the best glucose yield by using the whole range of values. Certainly in order to reduce the cost of the process, the low level of cellulase and beta-glucosidase concentration and temperature can be used. Additionally the high level of particle size can be used in order to reduce energy use during milling.

Additionally, Suutarinen *et al.* (2003) performed experiments on carrot peeling; they obtain a glucose yield of 36%. There was no pre-treatment method for the biomass, just the enzymatic hydrolysis which has done for 20 h and its conditions were as follows: substrate concentration of 20 g/L, cellulase concentration 30 FPU/g substrate, pH 5.0, and temperature at 40 °C. Moreover, they used enzyme for pectinolytic activities of a dosage of 4200 nkat/g substrate and the size of the biomass was the size after scraping of the peeling by a surgical knife. The difference of the substrate concentration with the difference of the size of the biomass from Suutarimen *et al.* (2003) and this research had probably the greatest effect on the glucose yield.

As shown in Figure 8.40, the optimum yield for glucose from PP is 79.44%. Particle size, substrate and cellulase concentration give the best glucose yield by using 1.2 mm, 5 g/L and 10 FPU/g substrate, respectively. Beta-glucosidase concentration, pH and temperature give the best glucose yield by higher values. Certainly in order to reduce the cost of the process, the low level of beta-glucosidase concentration and temperature can be used.

Suutarinen *et al.* (2003) used potato peeling in order to produce glucose by enzymatic hydrolysis; the conditions were as follows: substrate concentration of 20 g/L, cellulase concentration 30 FPU/g substrate, pH 5.0, and temperature at 40 °C. Moreover they used enzyme for pectinolytic activities of a dosage of 4200 nkat/g substrate and the size of the biomass was the size after scraping of the peeling by a surgical knife. The enzymatic hydrolysis was performed for 20 h and the glucose yield was 22%. It can be seen that the difference from the glucose of this research is large and this happens probably because of substrate and cellulase concentration and the size of the biomass.

As can be seen from Figure 8.40, the optimum yield for glucose from GR is 80.41%. Substrate concentration and pH give the best glucose yield by using 5 g/L and 5.0, respectively. Particle size, cellulase concentration, beta-glucosidase concentration and temperature give the best glucose yield by using the whole range of values. Certainly in order to reduce the cost of the process, it can be used the low level of cellulase and beta-glucosidase concentration and temperature. Additionally the large particle size can be used in order to reduce energy use during milling. Experiments have been performed by using grass biomass which particle size and substrate concentration was 0.2 mm and 10 g/L, respectively.

Sun and Cheng (2005) used a biomass that is similar to the grass used for this research. They used Bermuda grass in particle size of 3.13 mm. Bermuda grass treated with 1.2% sulphuric acid and placed in autoclave at 121 °C for 90 min while in this research grass treated with 1% and for 60 min. During the enzymatic hydrolysis, they used substrate of 50 g/L, 25 FPU/g substrate, 75 PNPGU/g substrate, pH 4.8 and 50 °C. They achieved a glucose yield of 83% but the hydrolysis was performed for 48 h and at 100rpm while in this research, hydrolysis was performed for 24 h and at 68 rpm. The conditions are similar as regards the pre-treatment method and the hydrolysis except the particle size, the stirring velocity and residence time of the enzymatic hydrolysis. Probably the residence time was the factor that affected the glucose production from Bermuda grass.

It has been observed that the optimum yield for glucose from NP is 82.10% (see Figure 8.40). Particle size, substrate, cellulase concentration and temperature give

the best glucose yield by using 1.2 mm, 5g/L, 10 FPU/g substrate and 50 °C, respectively. *β*-glucosidase concentration and pH give the best glucose yield by using the whole range of values. Certainly in order to reduce the cost of the process, the low level of beta-glucosidase concentration can be used.

Additionally Sung and Jin (2004) have shown that the ammonia-hydrogen peroxide pre-treatment of newspaper biomass and the enzymatic hydrolysis for 24 h gives a glucose yield about 73%. when using pieces of 0.5 x 0.5 cm, substrate concentration of 10 g/L, cellulase concentration 30 FPU/g substrate, pH 4.8 and temperature at 50 °C. Probably the use of large pieces of biomass helped the hydrolysis as Figure 8.22 shows that an increase of particle size gives better glucose yield. This difference in the size of biomass might cover the reduction that could become from the substrate concentration of 10 g/L and cellulase concentration of 30 FPU/g substrate which, as it seems from Figure 8.29, reduces the glucose yield. Certainly the different method of pre-treatment should have affected the glucose yield.

The optimum glucose yield from SP is 84.62%, as shown in Figure 8.40. Particle size, substrate, cellulase concentration and pH give the best glucose yield by using 0.2 mm, 5 g/L, 100 FPU/g substrate and 5.0, respectively (Table 8.3). *β*-glucosidase concentration and temperature give the best glucose yield by using the whole range of values. Certainly in order to reduce the cost of the process, the low level of beta-glucosidase concentration and the low level of temperature can be used.

Wyk (1999) did experiments on office paper and the glucose yield was 17.2%. Wyk used for the pre-treatment method materials of 1cm x 1cm and the biomass was prepared in 0.05 M Tris–HCl buffer, pH 4.5 (2.5%; w/v) and was pre-treated by homogenization (Ultra-Turrax homogenizer). During enzymatic hydrolysis, Wyk used substrate and cellulase concentration 10 g/L and 40 FPU/ g substrate, respectively. The pH and temperature was 4.5 and 45 °C, respectively. The large difference in glucose yield from Wyk (1999) research and this research has potentially come from the larger particle size, substrate and cellulase concentration. Additionally the difference at the pH value probably affected the glucose yield.

Figure 8.40 indicates that 78.15% of glucose yield can be obtained after 24 h of

hydrolysis under the optimal conditions (0.5 g/L substrate concentration, 1.2mm particle size, 10 FPU cellulase, 10 PNPGU beta-glucosidase, pH 5.0 and temperature of 50 °C) from the combination of carrot peelings and potato peelings. Under the same conditions (as shown in Table 8.3), 79.86% of glucose yield are generated from combination of newspaper and scrap paper.

From Figure 8.40, it can be seen that the optimum yield for glucose from mixed BMSW fractions is 84.71%. Substrate concentration, pH and temperature give the best glucose yield by using 5.00 g/L, 5.00 and 50 °C, respectively. Particle size, cellulase concentration and beta-glucosidase give the best glucose yield by using any point within the whole range of values (Table 8.3). In order to reduce the cost of the process (30% coming from cellulase), requires using low level of cellulase concentration and high level of particle size in order to reduce the amount of energy used for milling.

Table 8.3 Optimum point prediction with best factor setting

Factor	Optimum level	Study range
A-particle size (mm)	1.20	0.20 - 1.20
B-substrate concentration (g/L)	5.00	5.00 - 15.00
C-cellulase conc. (FPU/g substrate)	10.00	10.00 - 100.00
D-beta-glucosidase conc. (PNPGU/g substrate.)	10.00	10.00 - 100.00
E-pH	5.00	3.70 - 5.00
F-temperature (°C)	50.00	37.00 - 50.00

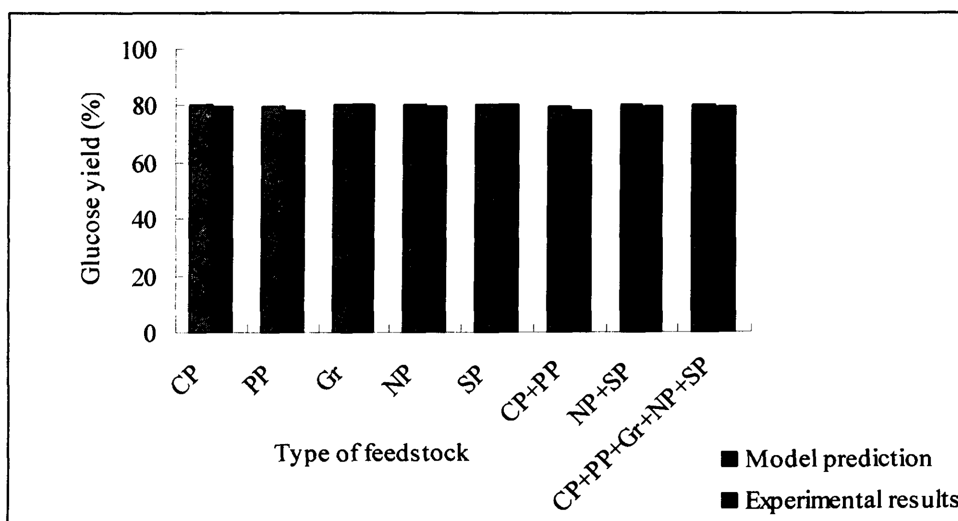


Figure 8.40 Comparison of glucose yield from model prediction and experimental results at optimum conditions

8.5 Composition analysis of hydrolysed residues

After the enzymatic hydrolysis, hydrolysed residue generally contains ash, acid insoluble lignin (AIL) and protein (Tengborg *et al.*, 2001). The composition of the residues was analysed based on the optimal hydrolysis conditions. Ash content has been measured and is presented in Table 8.4.

Table 8.4 Hydrolysed solid residue ash content

Hydrolysed solid residue	Ash content (%)
CP	42.48
PP	31.72
Gr	23.64
NP	34.84
SP	31.58
Mixed BMSW fractions	34.41

The ash content is an average of hydrolysed solid residues. As a result there were some hydrolysed solid residues whose cellulose was not totally converted to glucose.

The weight which is lost at every step of the over whole process from the raw material pre-treatment till the glucose production for every biomass was calculated. This measurement helps to assess the reduction of the waste because the hydrolysed residues will be potential transferred to the landfill. Additionally the UK has the obligation to reduce its BMSW to the landfill according to an EU Directive, the reduction of landfill waste should be 25% by 2010, 50% by 2013 and 65% by 2020 (EUROPA, 2005). The results of mass balance are presented in Table 8.12. The numbers for the column of pre-treatment come from a single type of experiments (1% H_2SO4 and steam treatment at 121 ^oC for 1h). Contrarily the column of hydrolysis is an average of the weight of the hydrolysed solid residues and not the optimal combination of factors which has a better conversion of cellulose to glucose.

Table 8.5: Solid residues from each step, for 1kg of raw material

Biomass	Raw material (kg)	Pre-treatment (kg)	Hydrolysis (kg)
CP	1	0.54	0.29
PP	1	0.41	0.21
Gr	1	0.47	0.22
NP	1	0.78	0.38
Sp	1	0.75	0.43
Mixed	1	0.59	0.33

After the acid and steam pre-treatment the samples were dried in the oven at 105 °C and then weighed in order to measure the loss weight. Then the portion of the loss was calculated. The same process was repeated for the hydrolysed solid residues. Table 8.5 shows that there is a reduction of waste of 71, 79, 78, 62, 57 and 67% for CP, PP, GR, NP, SP and Mixed, respectively. These results show that PP and GR have lost more weight than the others. This can be explained because raw materials of PP and GR have the largest amount of hemicellulose and acid soluble lignin which are removed during the pre-treatment method. Certainly from Tables 7.2, 7.6, 7.7 and 7.8 it can be inferred that some materials have lost larger amount than hemicellulose and ASL but hemicellulose and ASL have not been fully removed. Obviously some amounts of cellulose have been removed by the acid and steam pre-treatment but these are relatively small (Soderstrom *et al.*, 2003). Hydrolysis solid residues contains amounts of AIL, ash, protein, cellulose that did not convert to glucose. Certainly if the hydrolysed solid residues from the optimum enzymatic hydrolysis were measured, their weight would be smaller.

8.6 Conclusions

From this chapter, it can be concluded that the enzyme *T.virid* leads to higher performance of hydrolysis than *T. seerei*. It can also be seen that substrate concentration and pH are key parameters for most of the substrates (such as CP, GR, SP and mixed). For potato peeling and newspaper, apart from substrate concentration, the controlling parameters include the interaction of particle size and cellulase concentration and interaction of particle size, cellulase concentration and temperature respectively. For the final goal of maximizing the glucose yield from mixed waste, the key is to control the substrate concentration, pH value, and

temperature within the study range.

After optimisation, most of the substrates used for this study produced more than 80% glucose yield after 24 h hydrolysis. While higher glucose yield was produced, less cost is considered by choosing low level of value (such as cellulase concentration and beta-glucosidase concentration) and less energy use (such as particle size).

Chapter 9

Effects of substrate features on cellulase-cellulose adsorption

9.1 Introduction

Although the mechanisms of enzymatic hydrolysis are still not completely understood, it is widely recognised that cellulase-cellulose adsorption is a critical step in controlling the hydrolysis rate. Quantitative models have been proposed based on different kinetics modes, mainly Michaelis-Menten kinetics and Langmuir isotherm adsorption. Very few models were developed with consideration of substrate features since most of them were initially developed for first generation biomass based on pure cellulose. As lignocellulosic substrates with complex structural features (e.g. waste) are considered to be promising for second-generation biofuels, it is important to provide some fundamental understanding on the effects of substrate features in the process.

HCH-1 model described in Section 4.5 is selected and fitted to the experimental data obtained from the laboratory. This HCH-1 model is based on Langmuir isotherm adsorption corresponding to substrate concentration. The initial model was developed for pure cellulos. It was not sure if this model can be used for MSW-feedstock due to MSW's more complex structures. For this reason, new parameters were developed for the selected BMSW. This simulation would provide some information whether the cellulose-cellulase adsorption for MSW-biomass abides the same mechanism as the first generation biomass. With the roles of substrate features studied, further fundamental understanding is provided by

developing a new regression model simulating the effects of substrate features on cellulase-cellulose adsorption process.

9.2 Kinetics studies

The hydrolysis conversion rates are observed over a period of 96 h. Figure 9.1 shows the hydrolysis rate according to the experimental data for each type of biomass. It is obvious that within the reaction time the conversion rate increases for every different type of biomass. However, the increase in the conversion rate slows down with the increase of time. With the same substrate concentration (5g/L), mixed BMSW was found to have a glucose yield of 3.15 g/L followed by combination of newspaper and scrap paper 2.95 g/L, scrap paper 2.8 g/L, combination of carrot peelings and potato peelings 2.72 g/L, newspaper 2.65 g/L, grass 2.49 g/L, and potato peelings 2.38 g/L, and carrot peelings 2.10 g/L.

Hydrolysis of cellulose in a batch reactor or mode is generally characterised by an initial logarithmic phase, associated with the rapid release of soluble sugar, followed by a declining rate of sugar production as the reaction proceeds. Several studies (Mandels *et al.*, 1981; Nutor and Converse, 1991; Wang and Converse, 1992; Yang *et al.*, 2006) have shown that the specific hydrolysis rate declines rapidly with increased conversion of the substrate. There are a number of proposed explanations for the diminishing rate of hydrolysis. One possibility is as follows: during the hydrolysis, the substrate becomes enriched in the more recalcitrant cellulose as the less recalcitrant amorphous cellulose is hydrolysed (Phillippidis, 1994). The other possible contributing factors to the declining hydrolysis rate including enzyme adsorptive loss to lignin; deactivation of the enzyme through thermal, mechanical, and chemical actions; and enzyme endproduct inhibition by the hydrolysis products (Gregg and Saddler, 1996). However, the complete mechanism of cellulose hydrolysis has not been fully determined, due primarily to the complexity of both the substrate and the enzymatic system required to hydrolyse crystalline cellulose. Consequently, the characteristics of a typical batch hydrolysis reaction, such as hydrolysis yield or initial rate, probably reflect the influences of a number of factors

including the susceptibility of the cellulases to mechanisms such as denaturation or inhibition, the intrinsic structural features of the substrate, and the changes that occur to the substrate as the reaction progresses (Ramos *et al.*, 1993). Only some of these factors are readily available for manipulation within the design or operation of the enzymatic hydrolysis step.

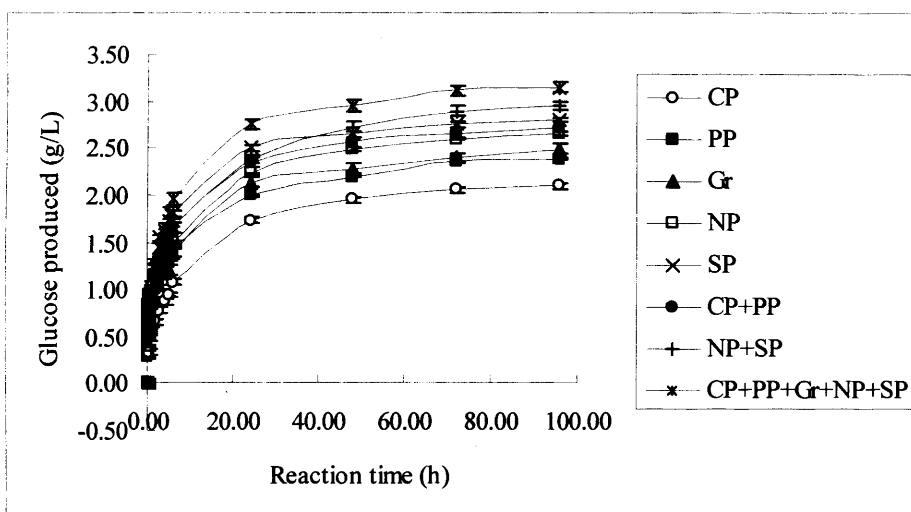


Figure 9.1 Glucose produced over a period of 96 h reaction time. CP-carrot peelings, PP-potato peelings, GR-grass, NP-newspaper, SP-Scrap paper, CP+PP-combination of carrot peelings and potato peelings (50% each), NP+SP-combination of newspaper and scrap paper (50% each), CP+PP+GR+NP+SP-combination of all five BMSW fractions (20% each).

9.3 Cellulase-cellulose adsorption

9.3.1 Adsorption trends

Enzymes are proteins that catalyse (i.e. accelerate) and control the rates of chemical reactions, in this case the hydrolysis (Garrett and Grisham, 1999). Protein can be measured with UV-spectrophotometer. In this work, during enzymatic hydrolysis, samples were taken to measure the protein which indicates the free enzyme in the reaction at the time when the samples are taken. The experimental work was carried out based on the optimal conditions found in this work as stated in Chapter 8. The amount of protein is 120 mg/L.

The adsorbed protein for each type of substrate is shown in Figures 9.2 - 9.9, respectively. For a better visualisation, the adsorbed protein plots in the first 30 min were enlarged for each figure. From these figures (9.2 - 9.9), it is obvious that the adsorbed protein reaches to a maximum level after a few minutes of the reaction and then decrease to a relatively steady state within 30 min. This may be due to the cellulase being adsorbed into the cellulose before being desorbed. The observation is consistent to Lynd *et al.* (2002) who reported that adsorption of enzyme reaches steady-state conditions within half an hour from the start of the reaction. As shown by the data presented in Figures 9.2 - 9.9, the fraction of the enzyme that is adsorbed falls off as the hydrolysis proceeds. However, adsorbed enzyme reaches at a different level with different type of substrate. The adsorbed protein reaches a maximum level of 72.3 mg/L when using carrot peeling as substrate and then fall to 16.5 mg/L after 30 min. Likewise, the maximum level of 96.9 mg/L is found for potato peelings with 59.8 mg/L within 30 min; 51.1 mg/L for grass with 11.4 mg/L after half an hour; 100.4 mg/L for newspaper with 79.4 mg/L after 30 min; 103.6 mg/L for scrap paper with 102.1 with 30 min, 93.5 mg/L for combination of carrot peelings and potato peelings with 39.3 within 30 min, 119.2 mg/L for combination of newspaper and scrap paper with 114.0 mg/L after 30 min, and 103.6 mg/L for mixed waste fractions with 69.9 mg/L after 30 min of reaction.

The maximum level of adsorbed enzyme shows adsorption capacity which is less than total protein provided (120 mg/L). Although adsorbed enzyme is desorbed after the maximum capacity is reached, the level of adsorbed enzyme does not reach zero within the study period (96 h). This observation agrees with the findings from Ooshima *et al.* (1983) that the cellulase adsorptive capacity decreases during enzymatic hydrolysis. As the experimental work for all the selected pre-treated biomasses are in the same conditions (temperature, pH, particle size, substrate concentration, total protein amount), it is speculated that the cause for this observation may come from the substrate features, such as the presence of lignin and cellulose crystallinity.

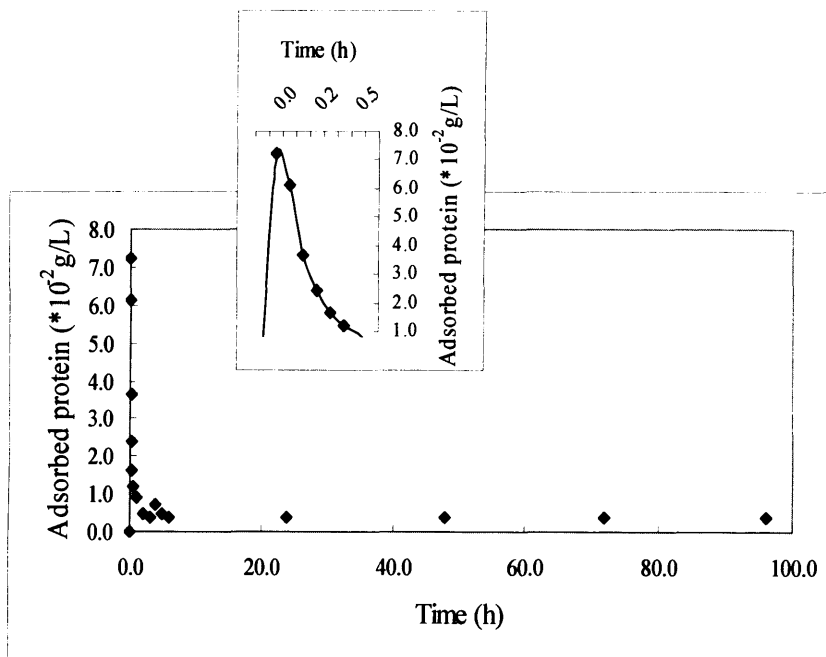


Figure 9.2 Protein adsorption during enzymatic hydrolysis (substrate: carrot peelings)
Note: the enlarged part of the graph shows the adsorbed protein in the first 30 min

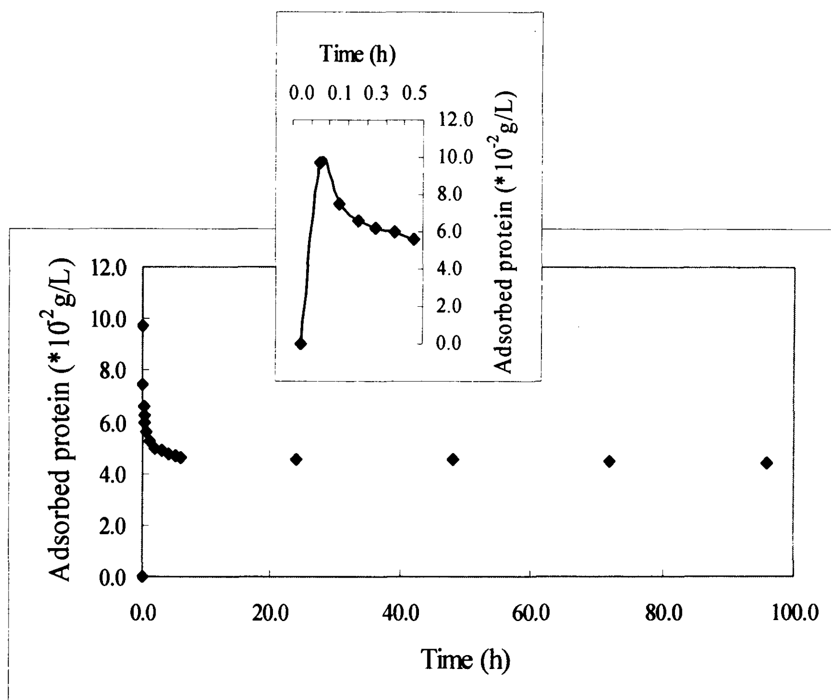


Figure 9.3 Protein adsorption during enzymatic hydrolysis (substrate: potato peelings)
Note: the enlarged part of the graph shows the adsorbed protein in the first 30 min

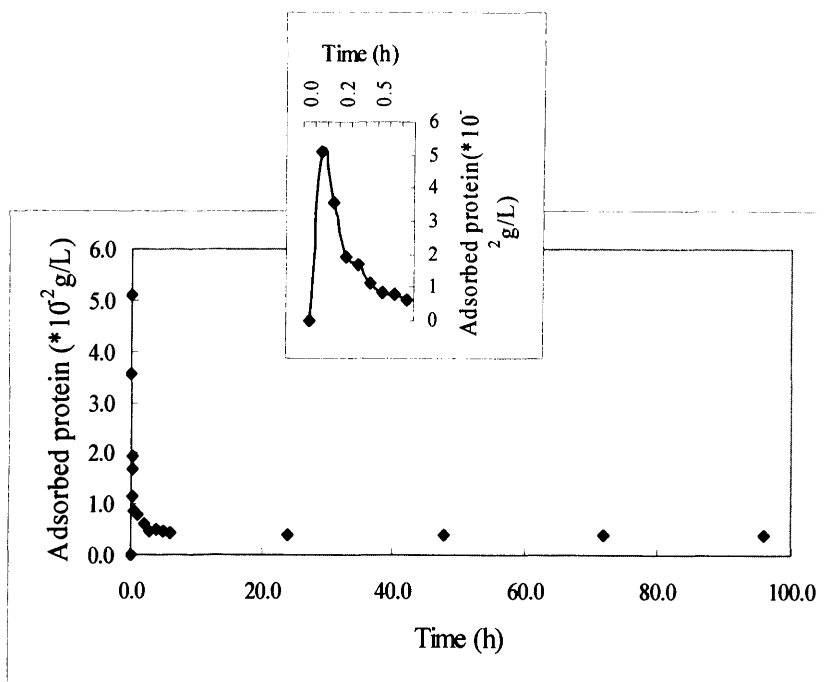


Figure 9.4 Protein adsorption during enzymatic hydrolysis (substrate: grass)
Note: the enlarged part of the graph shows the adsorbed protein in the first 30 min

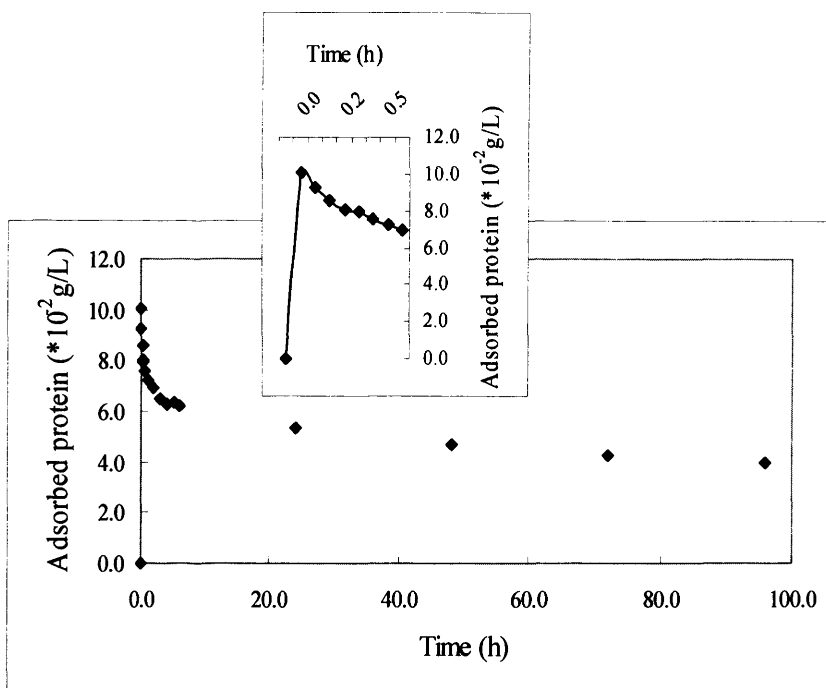


Figure 9.5 Protein adsorption during enzymatic hydrolysis (substrate: newspaper)
Note: the enlarged part of the graph shows the adsorbed protein in the first 30 min

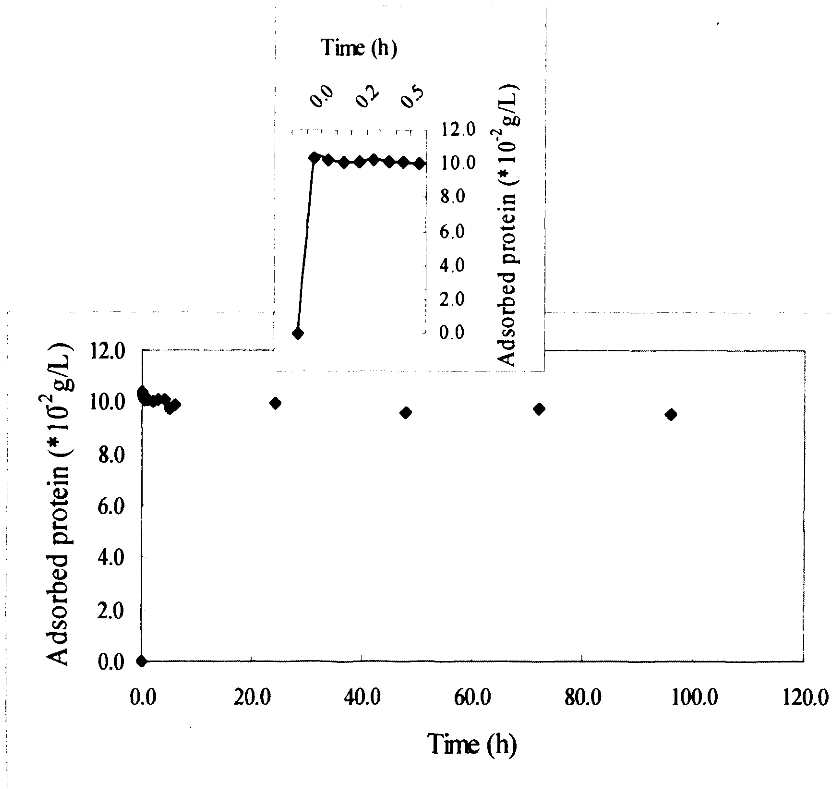


Figure 9.6 Protein adsorption during enzymatic hydrolysis (substrate: scrap paper)
Note: the enlarged part of the graph shows the adsorbed protein in the first 30 min

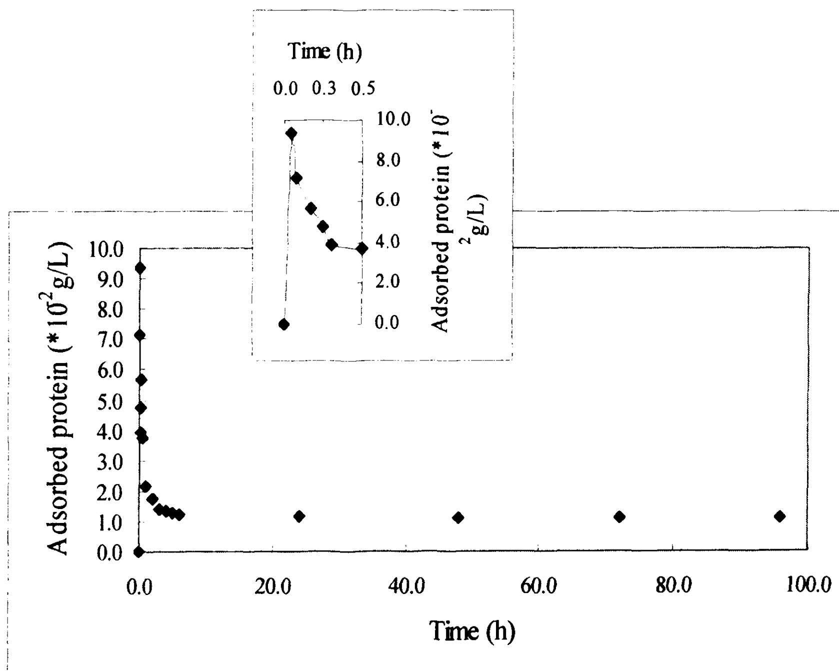


Figure 9.7 Protein adsorption during enzymatic hydrolysis (substrate: CP+PP)
Note: the enlarged part of the graph shows the adsorbed protein in the first 30 min

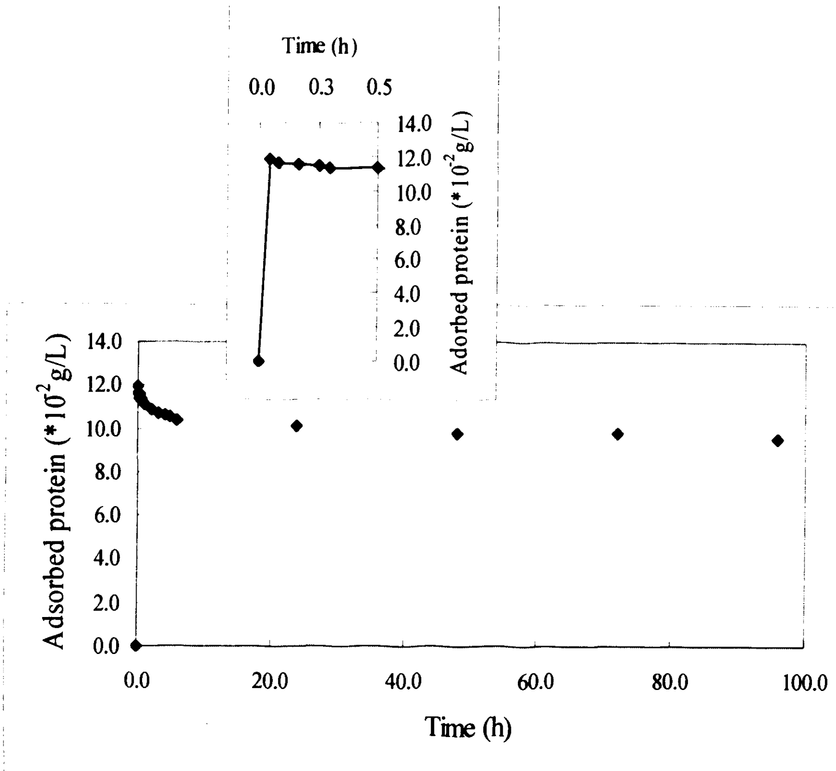


Figure 9.8 Protein adsorption during enzymatic hydrolysis (substrate: NP+SP)
Note: the enlarged part of the graph shows the adsorbed protein in the first 30 min

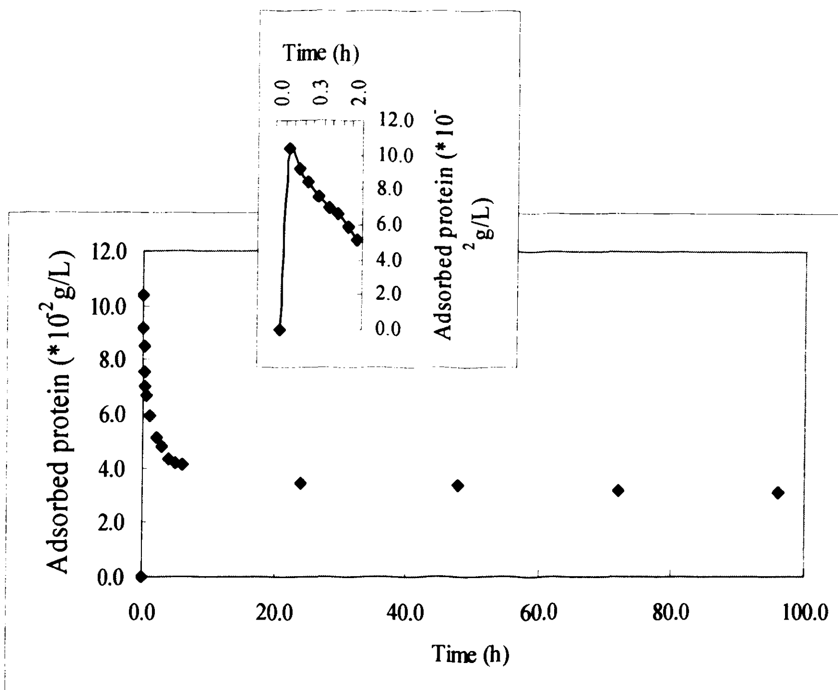


Figure 9.9 Protein adsorption during enzymatic hydrolysis (substrate: mixed waste)
Note: the enlarged part of the graph shows the adsorbed protein in the first 30 min

9.3.2 Relationship between glucose yield and cellulose-cellulase adsorption

As presented in Section 9.2, the protein adsorption increases suddenly in the very beginning of the reaction (the first few minutes) and then decrease over the time. The reserve phenomena are observed from hydrolysis conversion rate. In Figures 9.10 – 9.17 are plotted the relationship between the the protein adsorption and glucose yield over a period of reaction time (96 h) for every type of biomass. The logarithmic line between these two sets of data shows there is a correlation between glucose yield and adsorbed enzyme. This finding agrees with the results obtained by Nidetzky and Steiner (1993) that used the first generation biomass; there is a difference between both results; they stated a linear relationship instead of logarithmic one. This may be explained by the fact that in this work biomass from selected BMSW fractions received more effects from substrate features. Hence, in order to understand the glucose yield, it is important to look into the cellulose-cellulase adsorption.

By comparing the protein adsorption presented, the hydrolysis rate increases to three times more than the initial rate within 2 h during which protein adsorption fails to a minimum level. It has previously been noted that the concentration of adsorbed enzyme is a central feature in several kinetic models (Fan and Lee, 1983; Holtzapple *et al.*, 1984). Despite this, one can notice that the prediction of the adsorbed enzyme concentration as well as the hydrolysis product as a function of time have not previously been used in evaluating the model and in determining the parameters. Ohmine *et al.* (1983) concluded that the fall-off in adsorbed protein concentration could not be fully explained by the changes in substrate crystallinity and product inhibition. They concluded that there must be some other "rate-retarding factor." Matsuno *et al.* (2004) has suggested that such a factor might be the reversible inactivation of the adsorbed enzyme due to diffusion into the cellulose fibrils. It is hypothesized that the arrangement of the enzyme components needed for their synergistic effect is sterically hindered in small pores. The correlation between the adsorbed enzyme and glucose concentration produced (Figures 9.10 - 9.17) strongly support such a slow deactivation of the adsorbed

enzyme. Fan and Lee (1983) and Holtzapple *et al.* (1984a) also assume deactivation of the adsorbed enzyme. However, they assume that the deactivation is due to the formation of a complex with the product, whereas in this treatment the deactivation rate is proportional to the concentration of adsorbed enzyme or, in an alternate mechanism, the concentration of enzyme in solution but not the product concentration. Furthermore, Fan and Lee (2002) assume that the substrate becomes less reactive as the hydrolysis proceeds. In their study, it was assumed that the concentration of adsorption sites is proportional to the square of the remaining substrate concentration. While these two approaches differ, they both have the same effect of reducing the rate of reaction as the hydrolysis proceeds. In agreement with Holtzapple *et al.*, (1984), the author of this work also recognises that the adsorption of enzyme decreases the concentration of available adsorption sites.

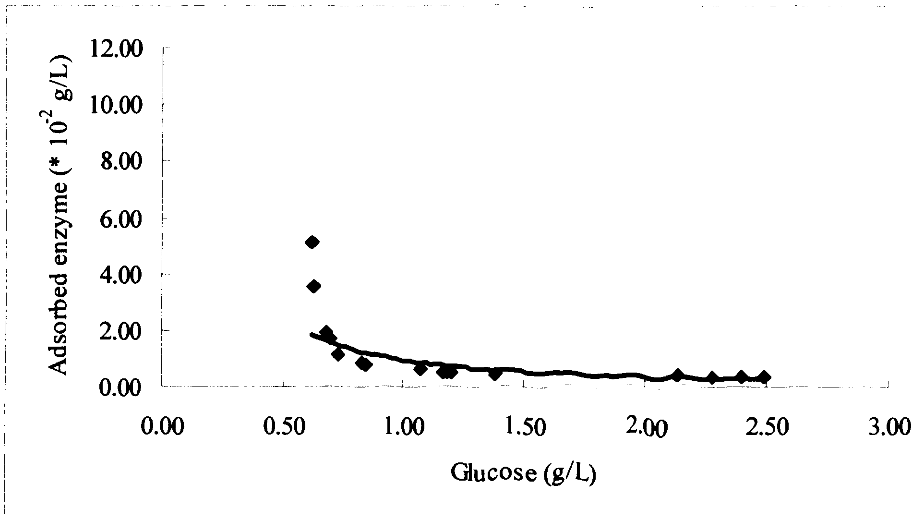


Figure 9.10 Correlation between glucose and adsorbed enzyme over reaction time (substrate – carrot peelings)

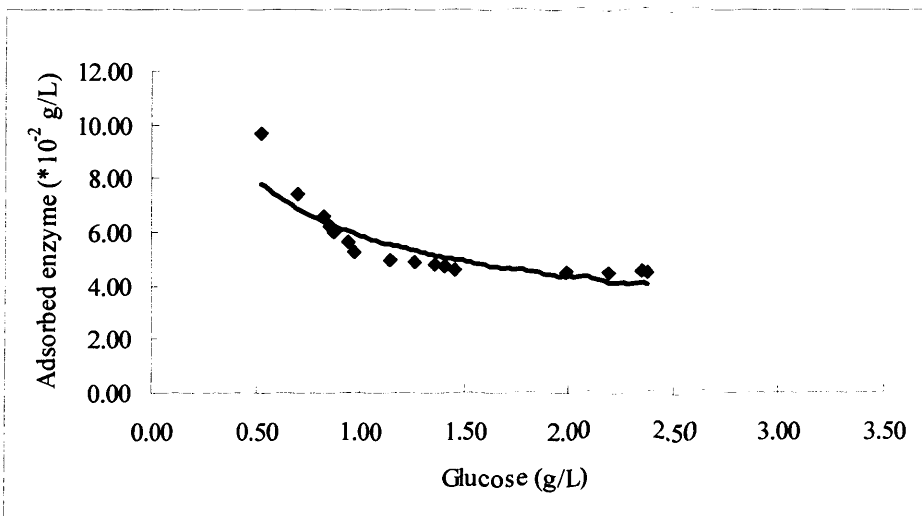


Figure 9.11 Correlation between glucose and adsorbed enzyme over reaction time (substrate – potato peelings)

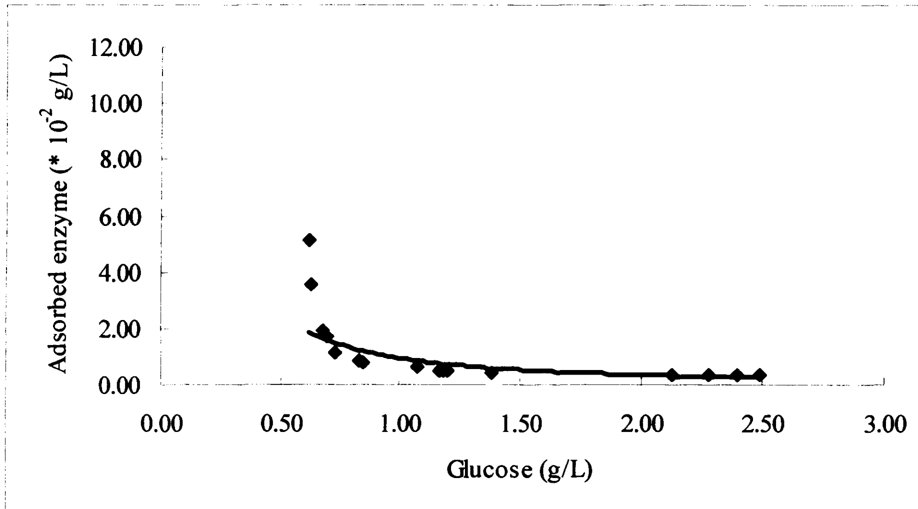


Figure 9.12 Correlation between glucose and adsorbed enzyme over reaction time (substrate – grass)

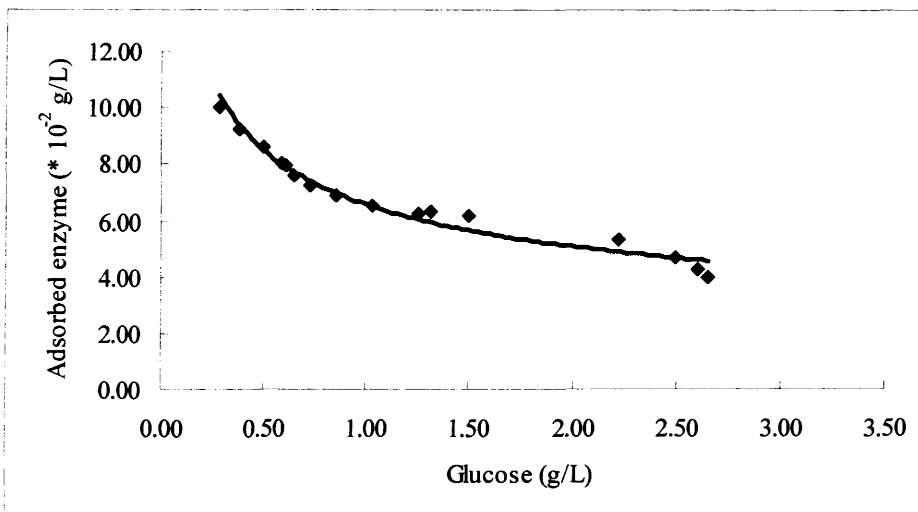


Figure 9.13 Correlation between glucose and adsorbed enzyme over reaction time (substrate – newspaper)

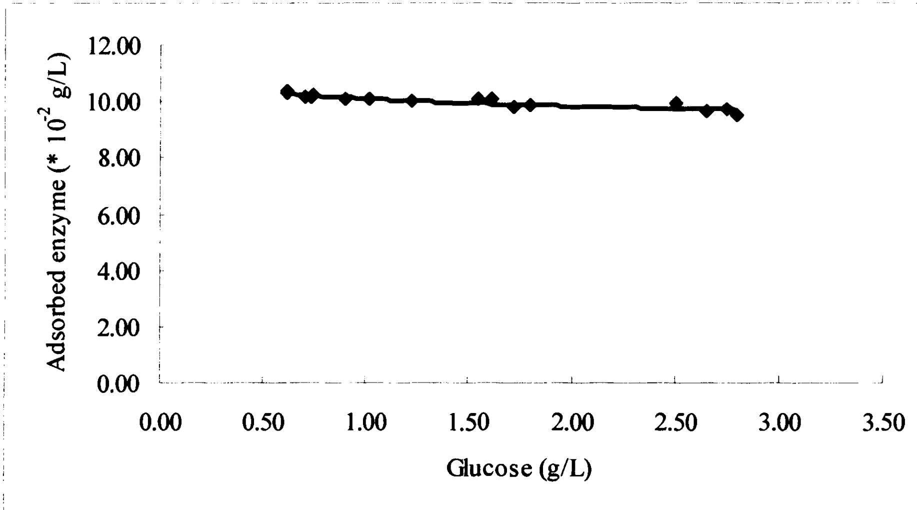


Figure 9.14 Correlation between glucose and adsorbed enzyme over reaction time (substrate – scrap paper)

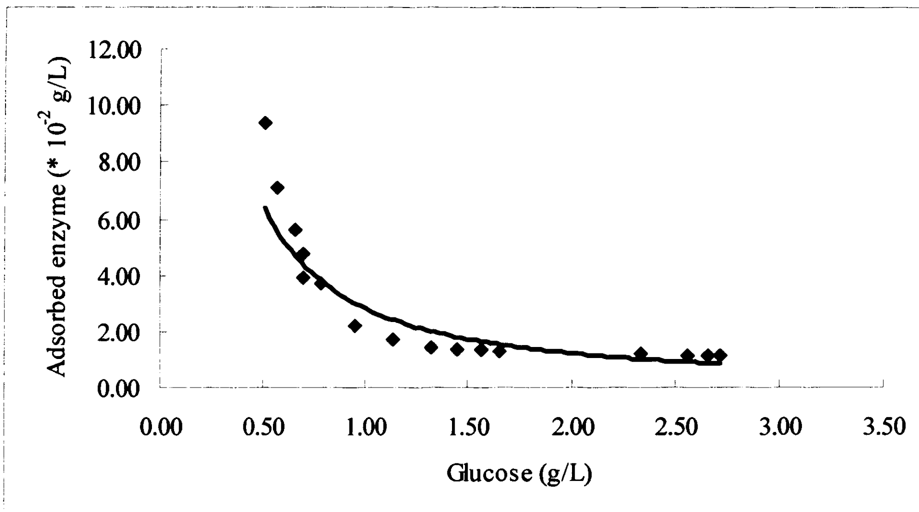


Figure 9.15 Correlation between glucose and adsorbed enzyme over reaction time (substrate – combination of carrot peelings and potato peelings)

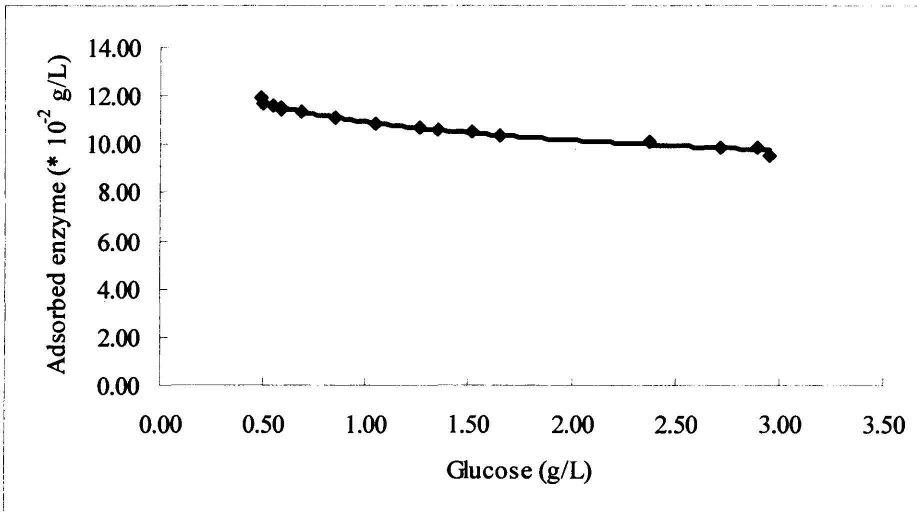


Figure 9.16 Correlation between glucose and adsorbed enzyme over reaction time (substrate – combination of newspaper and scrap paper)

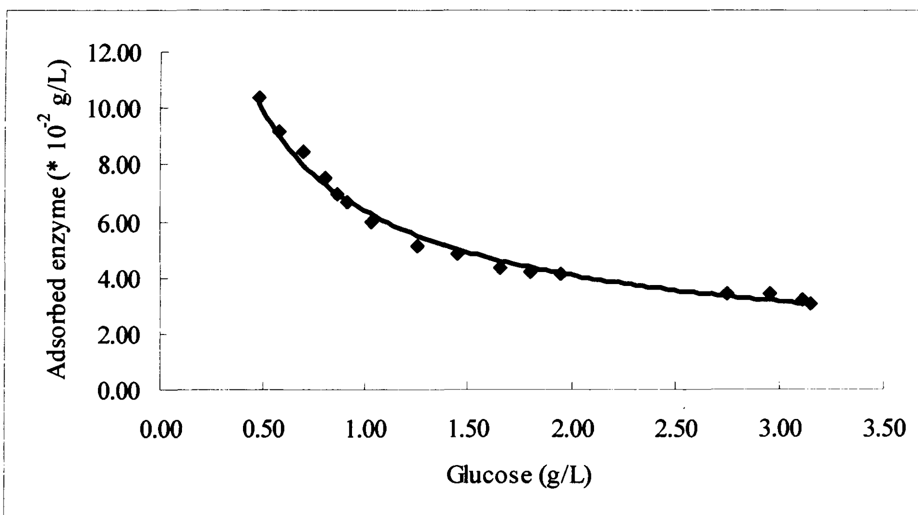


Figure 9.17 Correlation between glucose and adsorbed enzyme over reaction time (substrate – mixed BMSW fractions)

9.4 Simulation with HCH-1 model

From the discussion in Section 9.3, the cellulase-cellulose adsorption trends from the second generation biomass are very similar with the first generation biomass. The comparison of measured data from experimental results in this work with the results from HCH-1 model shows whether the cellulase-cellulose adsorption for MSW-feedstock follows the same or similar mechanisms. As mentioned in Section 4.5, HCH-1 model developed by Holtzapple *et al.* (1984a) based on Langmuir adsorption is selected to simulate the experimental data from this work. Tables 9.1 and 9.2 present the HCH-1 model and simplified HCH-1 model. The detailed information such as mechanisms, assumption and equations about this HCH-1 model can be found in Chapter 4.

The HCH-1 model was developed for pure cellulose Solka Floc BW200. Solka Floc BW200 is fibrous powdered cellulose, with bulk density of 1.5 g/cm^3 and 30% of crystallinity index. This work adopts the model on the selected BMSW fractions which has been characterised in Chapter 6 to simulate the experimental results with the optimal conditions as suggested in Chapter 8. Triplicate experiments were carried out for each type of sample. This work starts from using the parameter given by Holtzapple (1984) as shown in table 9.3.

Table 9.1 presents HCH-1 model and simplified HCH-1 model

Model	Equation
Simplified HCH-1 model	$\frac{d[G_s]}{dt} = \frac{k\,[G_x][E]\left(\frac{1}{1+\beta\,[G_s]}\right)}{\alpha + [G_x] + \varepsilon\,[E]}$
HCH-1 model	$\frac{d[G_s]}{dt} = \frac{k\,[G_x][E]\left(\frac{1}{1+\beta\,[G_s]}\right)}{\alpha + \phi\,[G_x] + \varepsilon\,[E]}$ where $\phi = \frac{[G_x] - \alpha - \varepsilon\,[E] + \sqrt{([G_x] - \alpha - \varepsilon\,[E])^2 + 4\,\alpha\,[G_x]}}{2\,[G_x]}$

Table 9.2 presents HCH-1 model and simplified HCH-1 model in their integrated forms

Model	Equation
Simplified HCH-1 model	$0 = \kappa[E]t + ef\ln([G_x]/[G_x^0]) + (f - e\beta)([G_x] - [G_x^0]) - \beta/2([G_x]^2 - [G_x^0]^2)$ $where\ e = \alpha + \varepsilon[E] \quad f = 1 + \beta[G_x^{'}]$
HCH-1 model	$0 = 2\kappa[E]t + ef\ln([G_x]/[G_x^0]) + (f - e\beta)([G_x] - [G_x^0]) - \beta/2([G_x]^2 - [G_x^0]^2)$ $+ \{f - \beta/4(2[G_x^0] + b)\}X + \{\beta/4(2[G_x^0] + b) - f\}X^0$ $+ [fb/2 - \beta/8(4a - b^2)]\ln\frac{2X + 2[G_x] + b}{2X^0 + 2[G_x^0] + b} + ef\ln\frac{[G_x](2eX^0 + b[G_x^0] + 2a)}{[G_x^0](2eX + b[G_x] + 2a)}$ $where \quad X = \sqrt{a + b[G_x] + [G_x]^2} \quad a = (\alpha + \varepsilon[E])^2 = e^2$ $X^0 = \sqrt{a + b[G_x^0] + [G_x^0]^2} \quad b = (\alpha - \varepsilon[E])$

Both HCH-1 model and the simplified HCH-1 model were used with the constants in table 9.3. The agreements between the model and the experimental data for each biomass are presented in Figures 9.18 to 9. 25. It can be seen that the glucose concentration produced from the experimental work from the selected waste biomass follows the same trend given in both models, although the data do not fall in the same line as model. This may be due to the fact that both models were developed based on using pure cellulose which has different cellulose content and different free cellulose sites compared to the selected BMSW fractions. However, the HCH-1 model shows a closer correlation to the experimental data compared with the simplified HCH-1 model. It is suggested that this is due to the fact that simplified HCH-1 model assumes that the free cellulose sites are equal to the total amount of cellulose; this is not the case for all the selected BMSW fractions, since part of the cellulose has a crystallinity structure. However, the hydrolysis of crystalline cellulose is not as easy as amorphous cellulose. Hence, development of the new parameters is needed to describe the processes involved in biomass that exhibits special amount of free cellulose sites.

Table 9.3 Constants value for HCH-1 value from Holtzapple et al. (1984a)

Constants	$K(h^{-1})$	$\alpha(g/L)$	τ	$\beta(L/g)$	Average absolute error (%)
HCH-1 model	26.3	8.97	36.8	0.45	9.98
Simplified HCH-1 model	31.1	22.5	9.22	0.47	12.7

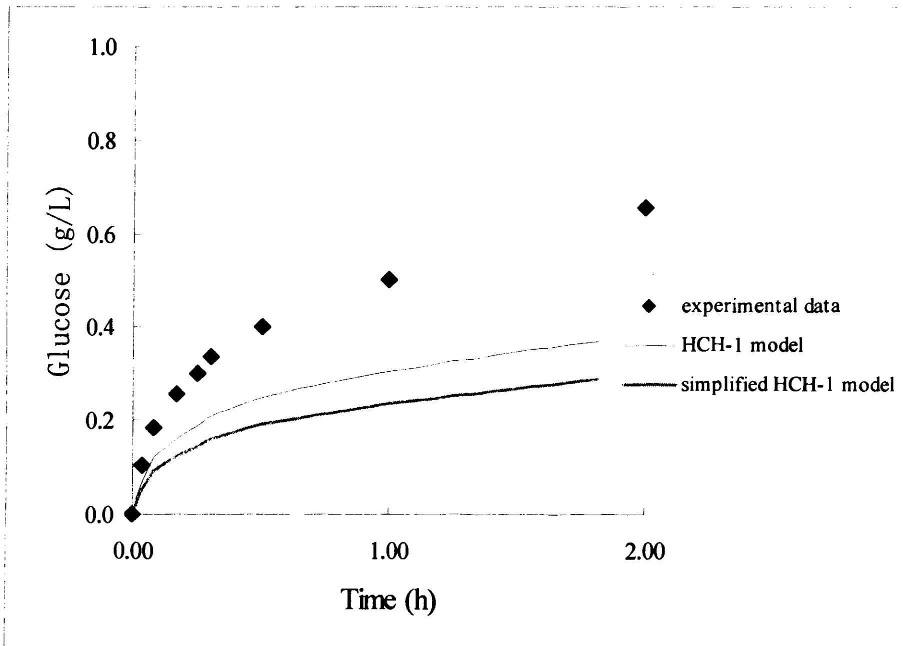


Figure9.18 Agreement of glucose yield with HCH-1 model and simplified HCH-1 model with the constants determined by Holtzapple et al. (1984a); substrate: carrot peelings.

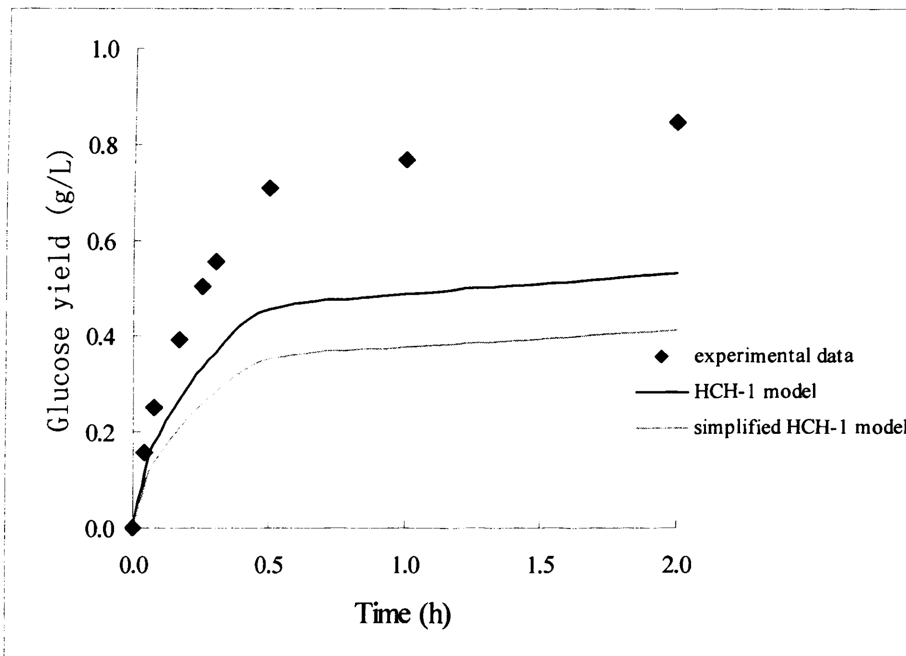


Figure 9.19 Agreement of glucose yield with HCH-1 model and simplified HCH-1 model with the constants determined by Holtzapple *et al.* (1984a); substrate: potato peelings

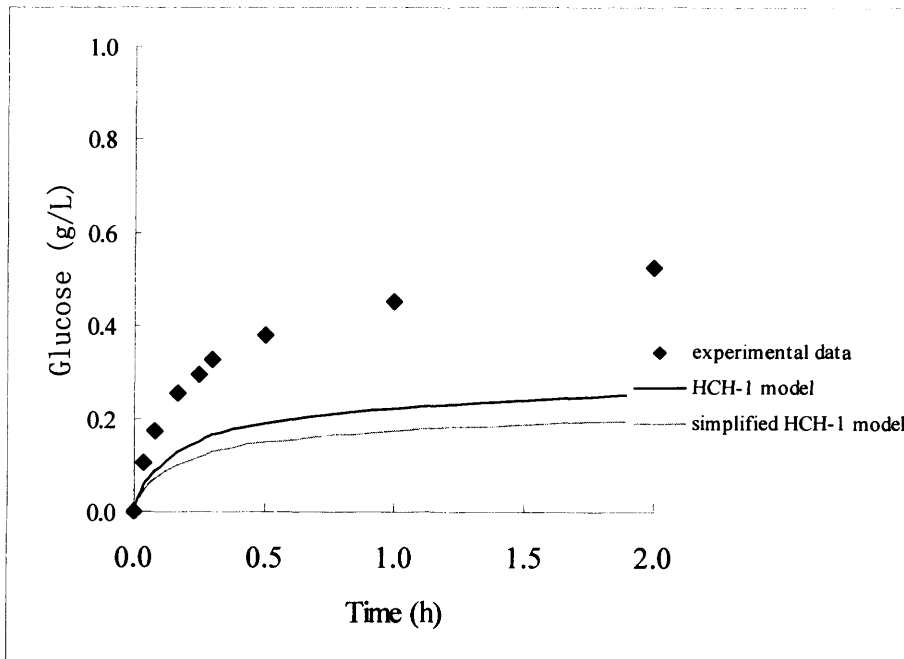


Figure 9.20 Agreement of glucose yield with HCH-1 model and simplified HCH-1 model with the constants determined by Holtzapple *et al.* (1984a), substrate: grass

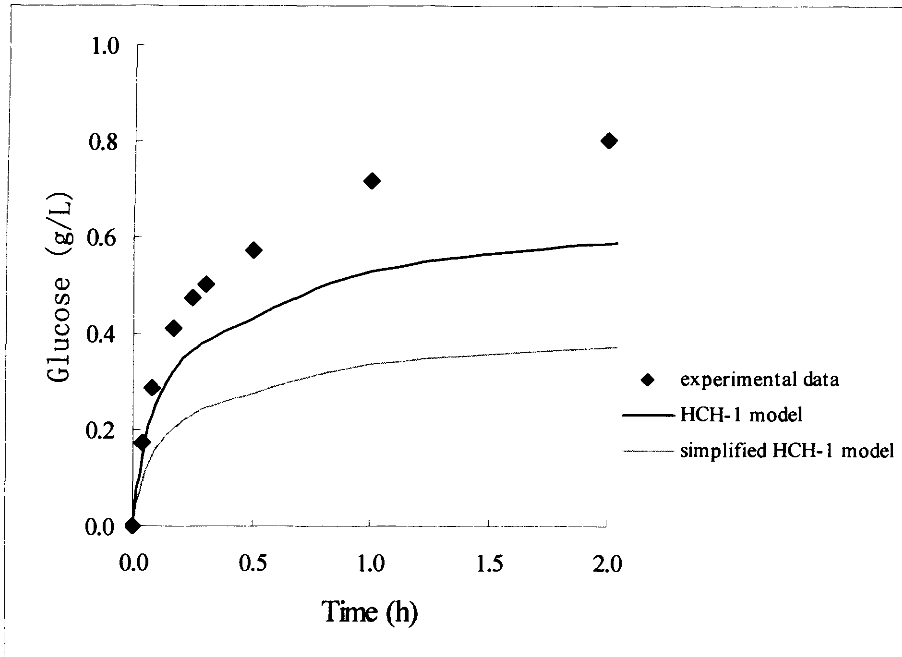


Figure 9.21 Agreement of glucose yield with HCH-1 model and simplified HCH-1 model with the constants determined by Holtzapple *et al.* (1984a), substrate: newspaper

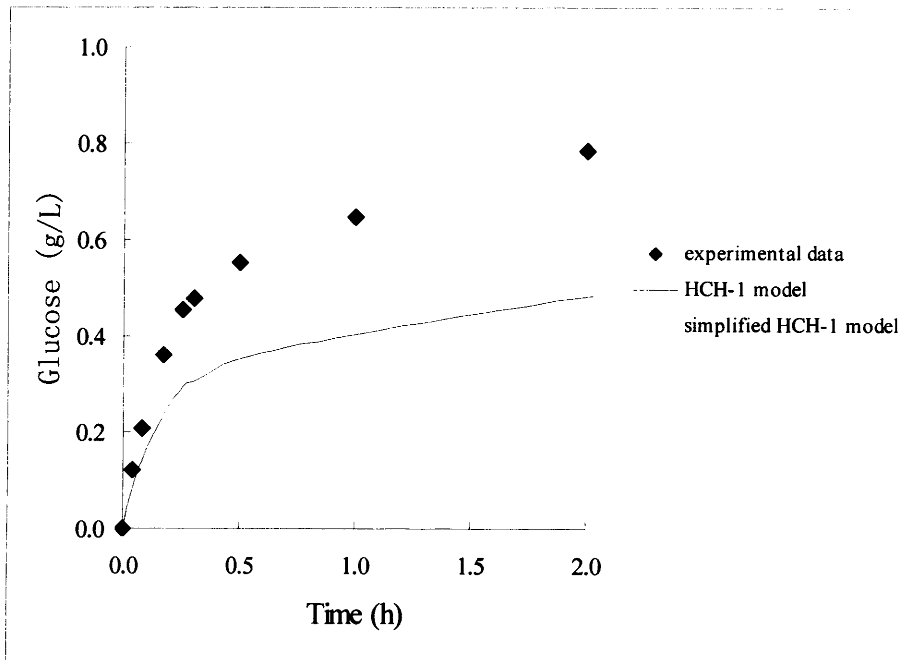


Figure 9.22Agreement of glucose yield with HCH-1 model and simplified HCH-1 model with the constants determined by Holtzapple *et al.* (1984a), substrate: scrap paper

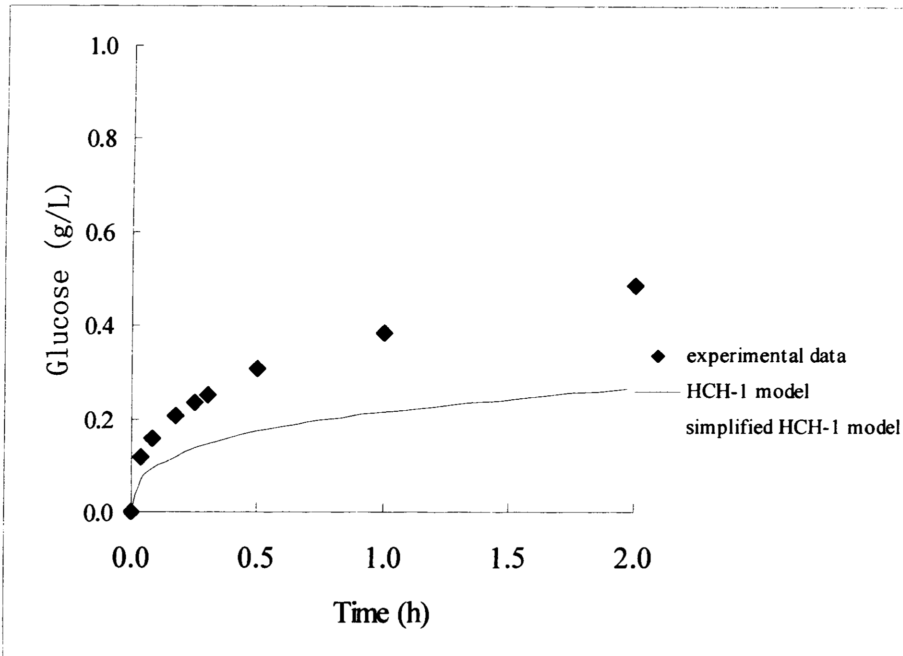


Figure 9.23 Agreement of glucose yield with HCH-1 model and simplified HCH-1 model with the constants determined by Holtzapple *et al.* (1984a), substrate: combination of carrot peelings and potato peelings

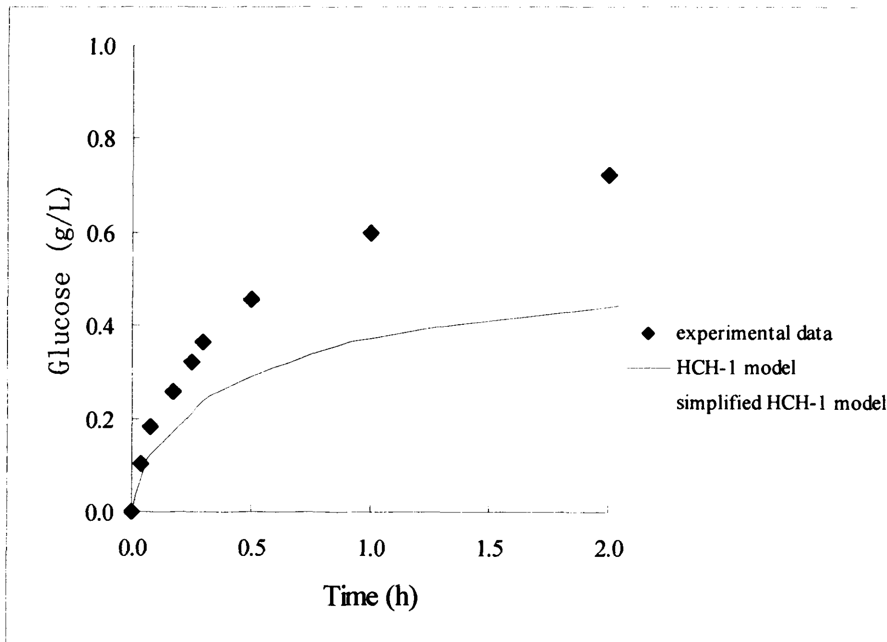


Figure 9.24 Agreement of glucose yield with HCH-1 model and simplified HCH-1 model with the constants determined by Holtzapple *et al.* (1984a), substrate: combination of newspaper and scrap paper

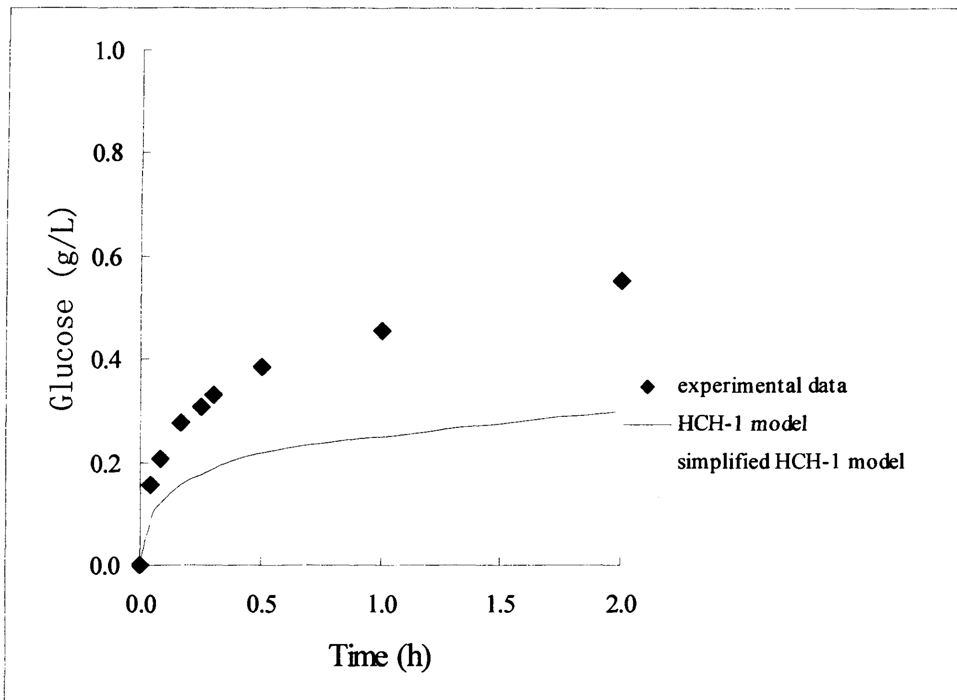


Figure 9.25 Agreement of glucose yield with HCH-1 model and simplified HCH-1 model with the constants determined by Holtzapple *et al.* (1984a), substrate: mixed waste

Holtzapple *et al.*, (1984a) states that in general, the parameters determined can be converted into appropriate constants by multiplying by an appropriate scaling factor. The developed model has been used for simulating the hydrolysis process for corn stover (Holtzapple *et al.*, 1996, Holtzapple *et al.*, 2002). The difficulty lies in finding this particular factor. This work adopts this method, and new constants are found by multiplying a certain scaling factor as shown in Table 9.4. The reason for the new parameter is due to the fact that the selected BMSW has more complicated substrate features than pure cellulose. The discussion on the relationship of substrate features with the new parameter is presented in Section 9.4.1.

Table 9.4 Parameter fitting

Constants	$K(h^{-1})$	$\alpha(g/L)$	τ	$\beta(L/g)$
Parameter 1.6	42.08	14.35	58.88	0.72
Parameter 1.4	36.82	12.56	51.52	0.63
Parameter 1.3	34.19	11.66	47.84	0.59
Parameter 1.2	31.56	10.76	44.16	0.54
Parameter 1.1	28.93	9.87	40.48	0.50
Parameter 0.9	23.67	8.07	33.12	0.41
Parameter 0.5	13.15	4.49	18.4	0.23
Parameter 0.1	2.63	0.90	3.68	0.05

Table 9.4 shows the value of constants after multiplying a certain scaling factor. The first column of the table shows the scaling factors. For instance, parameter 1.6 means that the value of each constant is obtained after multiplying a scaling factor by 1.6. The maximum ratio of free cellulose sites to total cellulose can only be 1, which is indicative of free cellulose sites. This ratio depends not only on the parameter constants but also the cellulose concentration for each biomass but also on the enzyme concentration as it can be seen from HCH-1. The agreement of experimental data and the HCH-1 model with different constants are produced in Figures 9.20 - 9.27. The agreements are produced in the format of glucose concentration related to time. For each type of biomass, figures are produced for the reaction duration of 2 h. The reasons for producing figures in such short reaction time (2 h) because the cellulose-cellulase adsorption happens within 30 min which has been discussed in Section 9.3. But in general, long period produced more product yield; 24 h of hydrolysis is required at least in order to produce product rates greater than 80%.

Results from Figures 9.26 - 9.33 indicate that with the new constants found in Table 9.4, the glucose concentration from experimental data agrees with the model when the constants of parameter found by Holtzapple multiplying a factor of 1.6 for the first 2 h. Exceptions were found with the biomass from grass where the experimental data did not agree with the model after a reaction time of 15 min. The likely explanation is that grass has lower cellulose content after the pre-treatment than other biomass. However, after 2 h of reaction, the glucose concentration obtained from experiments is generally higher than that predicted by the model regardless the parameters. For this observation, two possible reasons can be found: First, because the experimental work was carried out under the optimal conditions, the use of the enzyme is minimal but enough for the reaction to take place. As many researchers (Szczodrak and Targonski, 1989; Saxena *et al.*, 1992; Philippidis *et al.*, 1993; Zheng *et al.*, 1998) suggest, enzyme inhibition is found during hydrolysis after a few hours of reaction. In this work, the minimum use of cellulase avoids the inhibition at the maximum level, which is not the case when the HCH-1 model was developed. Second, HCH-1 model was developed based on pure cellulose which has simpler cellulose structure than the selected BMSW fractions. As found in the current literature, adsorption models were developed base on pure cellulose; thus there is a need for the current model to be adapted to the secondary biomass such as BMSW. Moreover, HCH-1 model used the viables such as glucose concentration, enzyme concentration and substrate concentration. It does not take into account the concentration of adsorbed enzymes in the model equation. Hence, the following section (9.5) will explore the relationship between the enzyme adsorption and substrate features with the purpose of providing the understanding of the role of substrate features during the cellulose-cellulose adsorption.

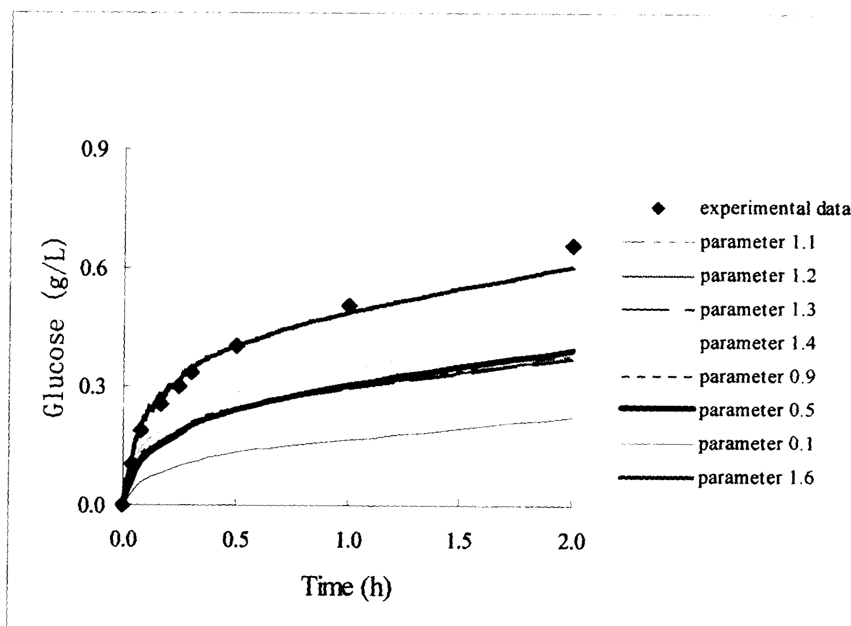


Figure 9.26 Agreement of glucose yield from experimental data with HCH-1 model with different scaling factors for the constants of K, a, τ, β. Substrate: carrot peelings

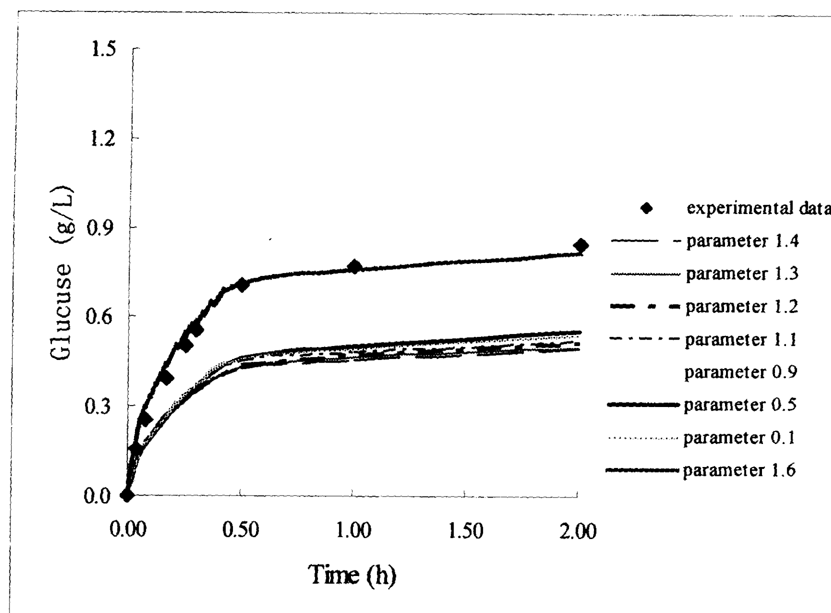


Figure 9.27 Agreement of glucose yield from experimental data with HCH-1 model with different scaling factors for the constants of K, a, τ, β. Substrate: potato peelings

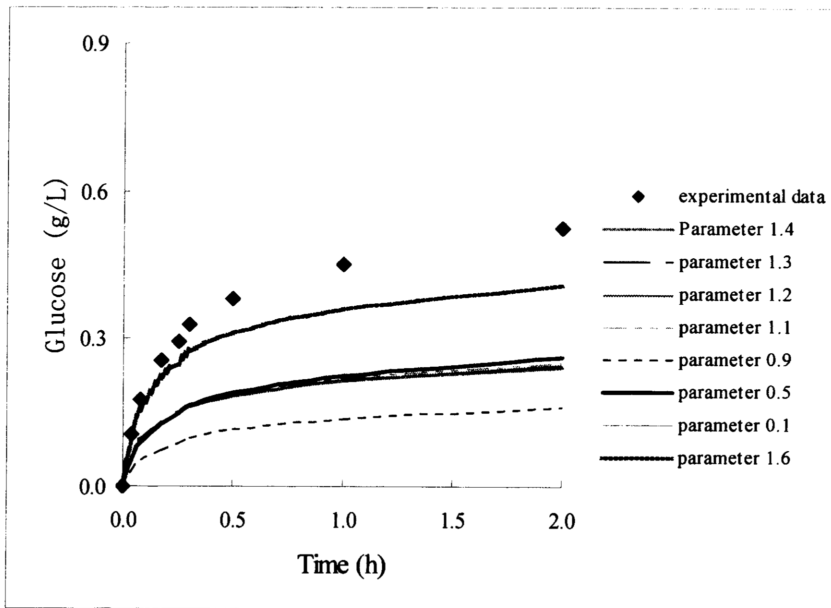


Figure 9.28 Agreement of glucose yield from experimental data with HCH-1 model with different scaling factors for the constants of K, a, τ, β. Substrate: grass

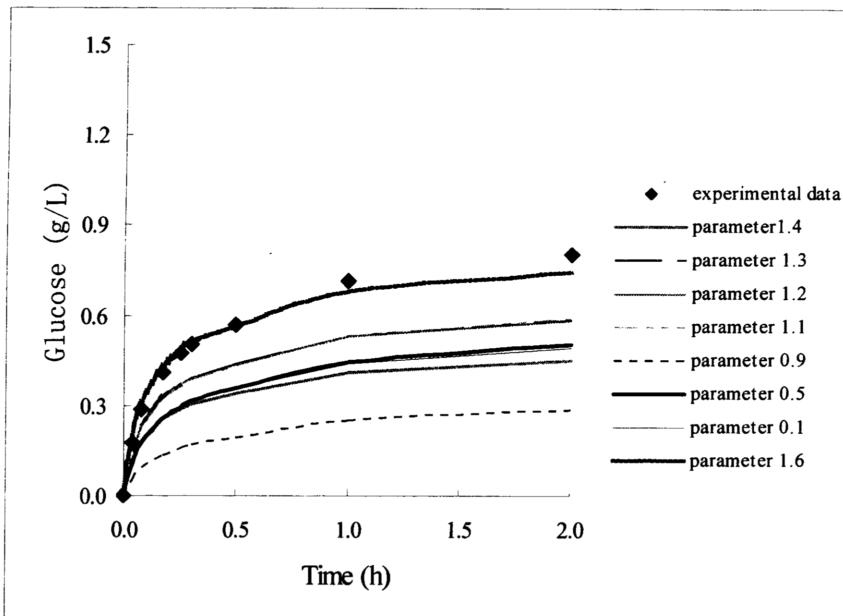


Figure 9.29 Agreement of glucose yield from experimental data with HCH-1 model with different scaling factors for the constants of K, a, τ, β. Substrate: newspaper

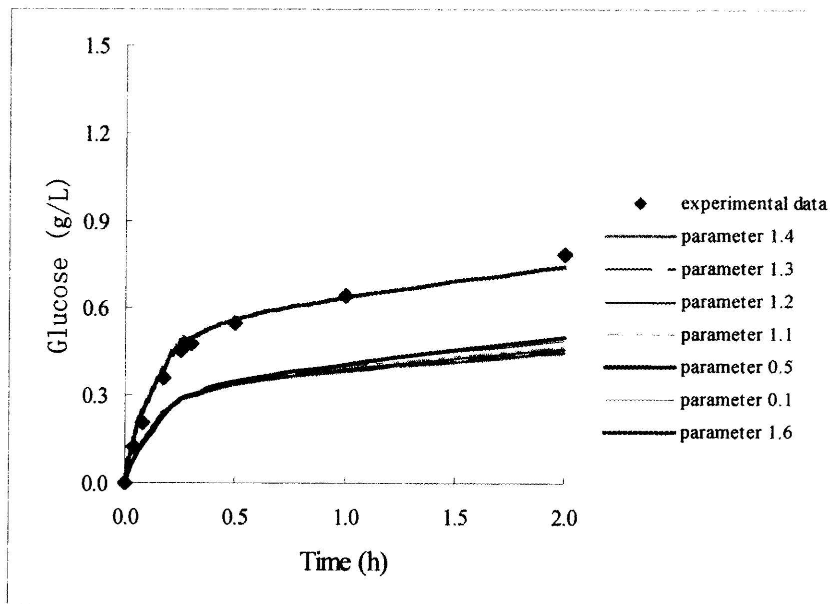


Figure 9.30 Agreement of glucose yield from experimental data with HCH-1 model with different scaling factors for the constants of K, a, τ, β. Substrate: scrap paper

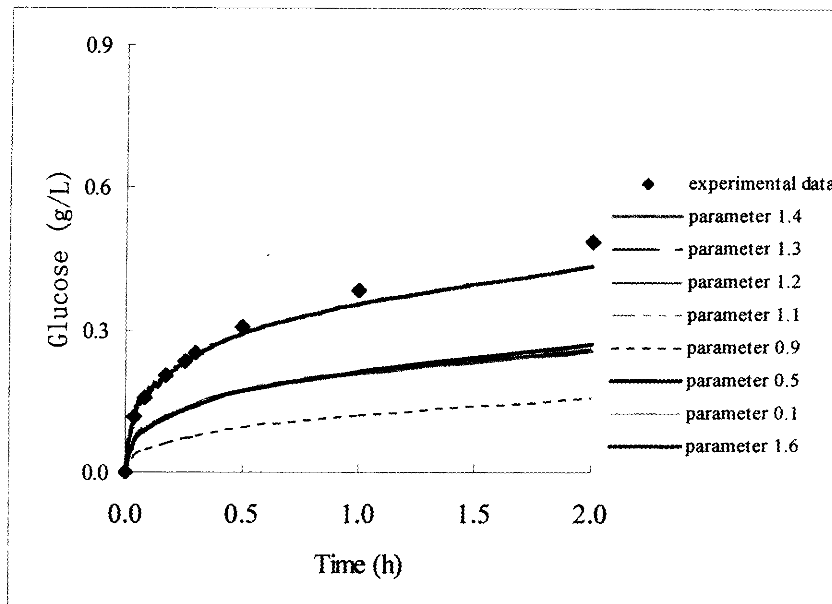


Figure 9.31 Agreement of glucose yield from experimental data with HCH-1 model with different scaling factors for the constants of K, a, τ, β. Substrate: combination of carrot peelings and potato peelings

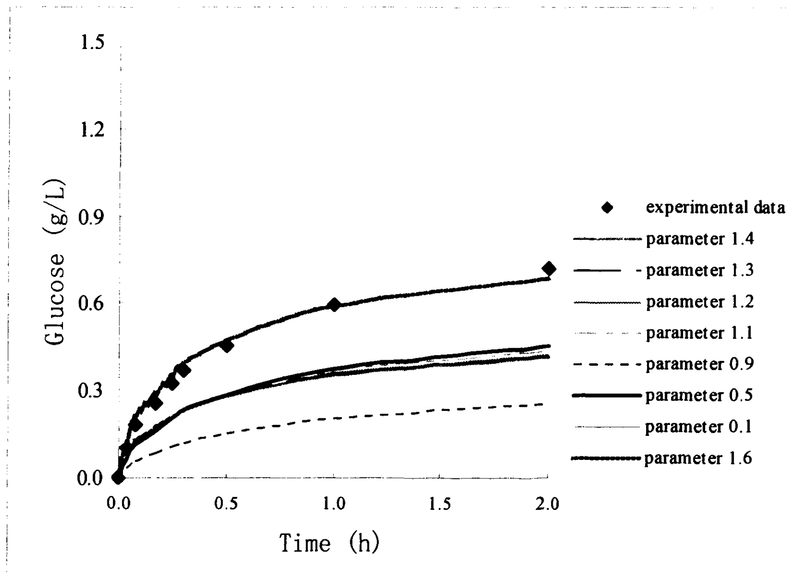


Figure 9.32 Agreement of glucose yield from experimental data with HCH-1 model with different scaling factors for the constants of K, a, τ, β. Substrate: combination of newspaper and scrap paper

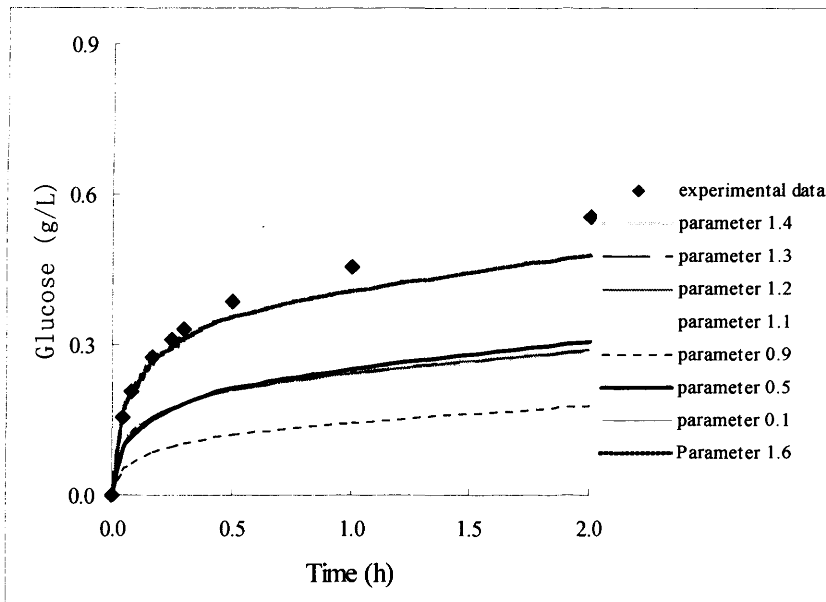


Figure 9.33 Agreement of glucose yield from experimental data with HCH-1 model with different scaling factors for the constants of K, a, τ, β. Substrate: Mixed BMSW

9.4.1 Relationship between free cellulose sites and cellulose crystallinity

As mentioned in this work, cellulose crystallinity is an important structural property which affects the pre-treatment process and the hydrolysis process. This part of cellulose is not easy to be converted to sugar compared to amorphous cellulose. In Section 9.4.2, Φ is defined as the ratio of free cellulose sites to the total cellulose. The importance of this factor has been discussed in the previous Section 9.4.3. Hence, a question is raised: does crystalline cellulose contribute to the free cellulose sites? This section studies the relationship between these two factors. Table 9.5 shows the amount of free cellulose sites, crystalline cellulose, and amorphous cellulose. The data of amorphous cellulose is obtained by using subtracting the total cellulose content to crystalline cellulose.

The amount of free cellulose sites is almost the same for every type of biomass as can be seen from Table 9.5. This is due to the fact that the ratio is affected by enzyme concentration, substrate concentration and the constants of the parameters presented in Table 9.4. As mentioned in Section 9.4.3, the same concentration of enzyme, substrate concentration and parameter constants are applied to all the biomass and the cellulose contents for each substrate are very similar, leading to the ratio of free cellulose sites to the total cellulose is almost the same.

The amount of free cellulose sites calculated according to HCH-1 model are higher than the amount of amorphous cellulose with carrot peelings and potato peelings, and the combination of both This indicates that crystalline cellulose does contribute to providing free cellulose sites, which is correlated with the attack of cellobiohydrolases (CBHs) on crystalline cellulose. The opposite situation was observed for newspaper, scrap paper and combination of both. For grass and combination of mixed substrate, the amount of free cellulose sites is almost as much as amorphous cellulose. A likely explanation is that lignin does contribute to the adsorption of enzyme during the reaction, resulting in fewer enzymes available to free cellulose sites as well as the bulk density. The difference of bulk density has been observed in Section 6.4. Carrot peelings and potato peelings are powdered

substrate with higher bulk density after milling to particle size of 1.2 mm. Newspaper and scrapper are floc type of fibres. The lower bulk density, that is higher volume, provides more chance for the contacts between enzyme and free cellulose, that is, more likely for CBHs to attack crystalline cellulose.

In studies with pure celluloses, amorphous regions were shown to degrade 5 - 10 times faster than highly crystalline celluloses by fungal enzymes (Gama *et al.*, 1994; Klyosov, 1990; Lynd *et al.*, 2002). This suggests that the high initial rates are due to preferential hydrolysis of the more easily degraded amorphous regions and the rate decreases as the enzymes encounter the more recalcitrant crystalline regions. In contrast, several researchers have observed no substantial change in crystallinity as enzymatic hydrolysis progresses beyond the initial stage (Lenz *et al.*, 1990; Ohmine *et al.*, 1983; Puls and Wood, 1991). The inconsistencies in the rate of hydrolysis and crystalline cellulose may be due to the crude/impure nature of the cellulase enzyme complex. The quantities of endoglucanases (EGs) relative to cellobiohydrolases (CBHs) can be inconsistent from batch to batch. Because CBHs have been shown to degrade crystalline cellulose whereas EGs are very ineffective, the differences in enzyme batches may lead to conflicting results when investigating the increase or decrease of crystallinity as the reaction progresses.

Table 9.5 Free cellulose site with cellulose crystallinity

Biomass	Ratio of free cellulose site to total cellulose (under HCH-1 model with parameter 1.6)	Ratio of Crystalline cellulose to total cellulose	Ratio of amorphous cellulose to total cellulose
CP	0.57	0.71	0.29
PP	0.58	0.69	0.31
GR	0.57	0.53	0.47
NP	0.58	0.18	0.82
SP	0.58	0.21	0.79
CP+PP	0.58	0.70	0.30
NP+SP	0.57	0.20	0.80
CP+PP+Gr+NP+SP	0.57	0.44	0.56

9.5 The role of Substrate features during adsorption

Modified HCH-1 model has a good data fitting for the experimental results obtained in this work. This means, for the second generation biomass, the cellulose-cellulase adsorption follows the similar adsorption mode (i.e. Langmiur adsorption). However, this model does not take into account of the adsorbed enzyme and substrate features. For example, the maximum enzyme adsorption capacity and steady-state adsorption (2 h) are very different for each type of biomass. As stated in Section 9.3, it is suspected that lignin and crystallinity contribute to controlling the maximum adsorption capacity and desorption ability resulting in different amount of enzyme adsorbed at each stage. This section investigates the effects of substrate features and aims at identifying the key features that affect the adsorption capacity.

As mentioned in Section 4.2, Lee and Fan (1983) suggested that the mechanism cellulase adsorption involves physical disruption of insoluble cellulose. Zhang *et al.* (1999) and Valjamae *et al.* (1999) observed that decrease of reaction was caused by the cellulose structure. The different level of cellulase adsorption for different types of biomass under the same condition from this work suggests the disruption of cellulose structure. Among the substrate features, it has been suggested that the presence of lignin, cellulose surface area, and crystallinity are the most influent factors (Sun and Cheng, 2002; Zhu, 2005). Hence, it is necessary to look at the relationship between the enzyme adsorptive capacity, steady-state adsorbed enzyme, lignin, cellulose and cellulose crystallinity.

From table 9.5, it is obvious that the substrates that have the highest enzyme adsorptive capacity (NP 100.4 mg/L, SP 1003.6 mg/L, NP+SP 119.2 mg/L, and mixed substrates 103.6 mg/L) happen to have the lowest amount of lignin content. In contrast, substrates with higher lignin contents (such as CP, PP, GR, CP+PP) are found to have lower enzyme adsorptive capacity. As Palonen (2004) reported that lignin has influence on hydrolysis rate, the findings of this work supports his statement. As lignin is present surrounding cellulose structure, it is very likely its presence preventing the enzyme being adsorbed by cellulose molecular. If this is the case, pre-treatment by removing lignin will play a more significant role than

expected, as the way to remove lignin content is by some pre-treatment methods (in Section 7.2).

Table 9.6 Relationship between the enzyme adsorptive capacity, steady-state adsorbed enzyme, lignin, cellulose and cellulose crystallinity for pre-treated biomass

Biomass	Enzyme adsorptive capacity (mg/L)	Steady-state adsorbed enzyme (mg/L)	Lignin content (%)	Cellulose content (%)	Cellulose CrI (%)
CP	72.3	16.5	20.7	50.5	29.0
PP	96.9	59.8	15.2	65.3	30.7
GR	51.1	11.4	19.3	43.1	47.0
NP	100.4	79.4	11.9	60.1	81.7
SP	103.6	102.1	6.9	59.5	79.5
CP+PP	93.5	39.3	19.1	53.7	29.5
NP+SP	119.2	114.0	10.2	60.7	80.3
CP+PP+GR+NP+SP	103.6	69.9	16.2	53.4	50.6

Although substrate with carrot peelings is found to have the highest lignin content, it does not result in the lowest enzyme adsorptive capacity, the cellulose content is found to be higher in carrot peelings (50.46%) than in grass (43.12 %). It is speculated that more cellulose content leads to more surface area which compensates the prevention from lignin. As Fan *et al.* (1980) concluded more cellulose content would tend to increase the accessibility of enzyme molecules to cellulose surface. This observation supports this statement, but the author also suspect the complex substrate features presented in the second generation biomass have influence on the kinetics of enzymatic hydrolysis.

However, it is interesting to find that the steady-state adsorbed enzyme for these substrates with higher enzyme adsorptive capacity is higher than the others. This means the substrate with higher adsorptive enzyme leads to fewer enzymes desorbed. By looking at table 9.5, it can be seen that these substrates with higher steady-state adsorbed enzyme happen to have higher crystalline content than others. It has been reported that the crystallite contents is one of the important factors that influences the cellulase-adsorption (Zhang *et al.*, 1999). But few have reported the influence on the cellulase desorption.

As a result, it is suggested that the presence of lignin limits the adsorption of enzyme, but once the enzymes get through the layer of lignin and adsorbed by

cellulose, crystalline structure determines the desorption of enzyme. Several researchers have found that delignification treatment of lignocellulosic biomass increases the yield of monosaccharides by enzymatic hydrolysis (Mooney *et al.*, 1998, Draude and Kurniawan, 2001). Lignin is known to coat the cellulose microfibrils in the plant cell wall (Saha, 2003), forming a physical barrier to access by hydrolytic enzymes, and removal of Lignin has been reported to increase the enzymatic hydrolysis of cellulose (Yang and Wyman, 2004; Ohgren *et al.*, 2007).

9.5.1 The presence of lignin

In this work, considering a residence time of 60 min, but using 1% acid concentration and lowest temperature, more than 80% of lignin and hemicellulose removal can be achieved for all the selected and combined BMSW fractions, which agrees with the findings by Wright (1998). Figure 9.34 shows the relationship between the lignin content and the maximum enzyme adsorbed capacity. As can be seen from Figure 9.34, substrate with lower lignin content results in higher maximum enzyme adsorbed capacity. For example, when the lignin content is in the range of 5% - 10%, the maximum adsorbed enzyme is about 10–12 * 10^{-2} g/L in contrast, when the lignin content increase (within the range of 20% - 25%), the amount of adsorbed enzyme decreases (within the range of 4-8 * 10^{-2} g/L).

As mentioned in the previous section, the cellulose-cellulase situation is further complicated for the second generation biomass because the action of cellulase enzyme systems is impacted by substrate properties in addition to concentration, such as crystallinity, accessible area, the presence of lignin, which depend on the particular substrate being investigated and change as the reaction proceeds. In this work, by examining the eight types of different selected BMSW fractions, it is obvious that lignin does have an influence on the adsorbed enzyme capacity. Hence, when seeking to understand enzymatic hydrolysis of cellulose that incorporates information about cellulose-cellulase adsorption, the presence of lignin and its amount need to be taken into account.

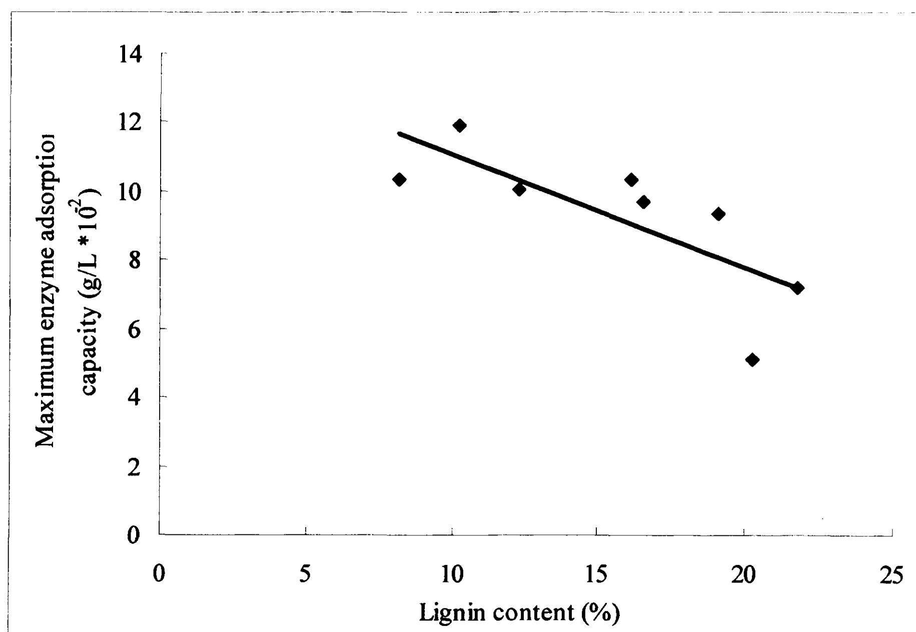


Figure 9.34 Relationship between lignin content and enzyme maximum adsorbed capacity

9.5.2 Cellulose content

Cellulose content is also an important factor during cellulose-cellulase adsorption, as it provides the active sites to access by cellulase resulting in further products. In general, for the first generation biomass, the more cellulose content one substrate has, the more glucose produced. Figure 9.35 plots the correlation between the amount of cellulose content and glucose yield. As it can be seen from figure 9.35, higher amount of cellulose content does not lead to higher glucose yield for the second generation biomass. This can be explained because the second generation biomass has more complex substrate properties including the presence of lignin. The lignin affects the adsorption of enzyme during the reaction process, which agrees with the finding in the previous Section 9.5.1.

Figure 9.35 shows that there exists no clear relationship between the cellulose content and glucose yield. The reason for this finding could be due to the fact that the cellulose consists of two main parts of structures: amorphous and crystallinity cellulose. From the discussion in Section 9.4.2, amorphous cellulose is very easily to be hydrolysed by enzyme, compared with crystallinity cellulose which is much

more difficult to be hydrolysed. Hence, it is necessary to look at the effects of cellulose structure on glucose yield and cellulase-cellulose interaction. The further discussion regarding the effects of crystallinity is presented in the following section.

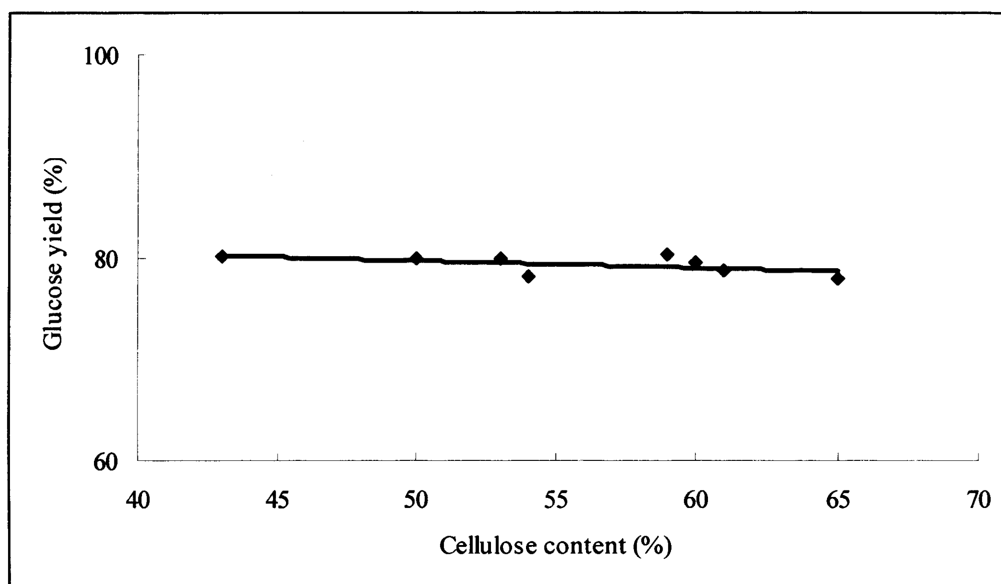


Figure 9.35 Correlation between cellulose content with glucose yield

9.5.3 Crystallinity

As mentioned in Chapter 2 and 6, the crystallinity (CrI) of cellulose is considered a major deterrent to enzymatic hydrolysis by limiting cellulase accessibility to cellulose (Grethlein, 1984; McMillan, 1994; Chang, and Holtzapple, 2000; Mosier *et al.*, 2005). It has been reported that a decrease in cellulose crystallinity especially influences the initial rate of cellulose hydrolysis by cellulase (Laureano-Perez *et al.*, 2005). Thus one of the purposes of pre-treatment is to disrupt the crystalline structure of cellulose to promote the hydrolysis of biomass.

From the discussion in Section 7.3.5, it is sure that heat treatment (steam treatment) does increase the cellulose crystallinity, which agrees with the finding from other researchers (Fuller *et al.*, 1940; Creely and Conrad, 1962; Conrad, 1962; Bhuiyan, 1999). Chemicals, like acids, do not significantly increase the cellulose crystallinity. As shown in Table 9.1, the crystallinity of carrot peelings has increased from 16.8%

to 29.4% after steam treatment. The increases also have been observed for potato peelings (from 21.2% to 29.3%), grass (from 37.7% to 46.6%), newspaper (from 70.8% to 79.1%) and scrap paper (from 67.4% to 78.9%). Similar increases for all the biomass are found with acid-impregnated steam treatment which has the same temperature (121 °C) as steam treatment. As higher temperature results in increase of crystallites, lower temperature is better for pre-treatment. The increase in crystallinity may be explained as crystallization in quasi crystalline of amorphous regions due to rearrangement or reorientation of cellulose molecules inside these regions; the more crystallization in wood cellulose may be due to the crystallization in hemicelluloses and wood cellulose contain more crystalline regions than pure cellulose.

Hence, based on the findings, the relationship between the degrees of cellulose crystallinity with the adsorbed enzyme capacities is represented in Figure 9.36. From this figure, it is obvious that the more cellulose crytallinity the substrates have, the more adsorbed enzyme remained in the cellulose at cellulose-cellulase adsorption steady state. For example, a substrate with about 80% crystalline cellulose has about 0.11 mg/L protein remained in cellulose structures when reaching at steady-state. This means more free enzyme is available at the reaction when the majority of cellulose is amorphous, which agrees with the findings of Negro (2003) that the more accessible (amorphous) portion of cellulose degrades more easily than the less accessible crystalline regions. There are two reasons responded to this change can be explained: 1_ enzymes adsorbed to crystallinity cellulose requires more time before it can be desorbed; 2_ the enzyme adsorbed by cellulose crystallinity is deacitived easily than those are adsorbed by amorphous cellulose. In this case, the decay of cellulose-cellulase adsorption can be explained by the removal of amorphous material resulting in accumulation of crystalline structures, or was attributed to the degradation and dissolution of easily accessible components of lignocellulose, namely, hemicellulose and lignin, thus resulting in a product with increased concentration of crystallinity.

Table 9.7 Cellulose crystallinity (%) after pre-treatment process

Biomass/ pre-treatment type	Before pre-treatment	Dilute acid treatment (1% H_2SO_4)	Steam treatment (121 °C)	H_2SO_4-impregnated steam treatment (1% H_2SO_4, 121 °C)
CP	16.8	18.7	29.4	29.0
PP	21.2	22.3	29.3	30.7
GR	37.3	38.5	46.6	47.0
NP	70.8	72.6	79.1	81.7
SP	67.4	69.3	78.9	79.5
CP+PP	18.6	19.7	29.1	29.5
NP+SP	68.5	69.9	81.2	80.3
CP+PP+Gr+NP+SP	42.1	43.6	52.0	50.6

Note: CP-carrot peelings, PP-potato peelings, GR-grass, NP-newspaper, SP-scrap paper.

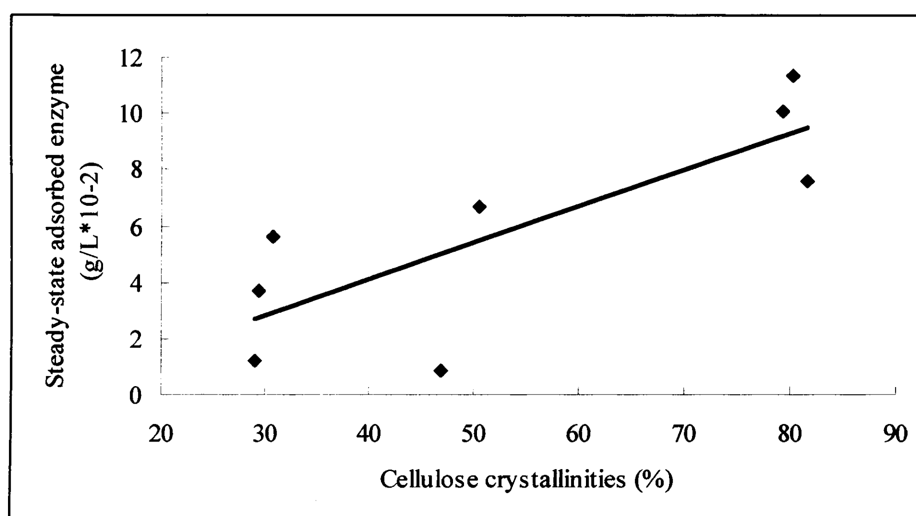


Figure 9.36 Relationship between cellulose crystallinity and steady-state adsorbed enzyme

9.6 Modelling the effects of substrate features on cellulose-cellulase adsorption

9.6.1 Key substrate features

Based on the understanding of pre-treatment and hydrolysis process gained through the previous chapters, it is clear that among all of the factors influencing enzymatic hydrolysis of lignocellulosic biomass, structural features is the most complicated one: they are interrelated and coupled with the extent of pre-treatment. It is broadly

accepted that crystallinity and lignin content play significant roles in influencing the biomass hydrolysis rate, which is also confirmed by the findings of this work as stated in Section 9.4. Biomass conversion rate is enhanced with an increase in accessible surface area and removal of lignin (Fan *et al.*, 1981; Grethlein, 1985; Thompson and Chen, 1992; Sinitsyn *et al.*, 1991); however, accessible surface area is not considered as a direct factor because it may correlate with other structural features (Sinitsyn *et al.*, 1991). Based on the previous sections, lignin content, cellulose content, and crystallinity are considered as key structural features that determine enzyme adsorption because these three features are independently controllable in pre-treatment processes and are easy to measure. Table 9.6 summarizes the three structural features and carbohydrate contents of the selected model lignocelluloses from waste.

Table 9.8 Three structural features

Biomass type	Lignin (g/L)	CrI (g/L)	Cellulose (g/L)	Maximum enzyme adsorbed (*1 0^{-2}g/L)	Adsorbed enzyme at 2 h (*10^{-2}g/L)
CP	1.0875	0.6235	2.15	7.23	0.49
PP	0.828	0.7675	2.5	9.69	4.99
Gr	1.0115	1.2455	2.65	5.11	0.61
NP	0.613	2.2005	2.70	10.04	6.92
SP	0.407	2.3373	2.95	10.36	10.03
CP+PP	0.955	0.885	3.00	9.35	1.73
NP+SP	0.511	2.4492	3.05	11.92	10.89
CP+PP+GR+NP+SP	0.8085	1.6445	3.25	10.36	5.12

As it was pointed out in Sections 9.4 and 9.5, three substrate structural features affect the glucose yield produced in hydrolysis process. Moreover, glucose yield has a logarithmic relationship with enzyme adsorption. Although models have been developed to simulate enzyme celluloase adsorption on pure cellulose (Holtzapple, 1984; 2002) and the correlation between glucose yield and some substrate features (Zhu, 2005). Very few studies have looked into the correlation between cellulase-cellulose adsorption and the effects from substrate features. From the findings of this work, it is suspected that lignin and crystallinity both affects the enzyme getting contact with cellulose through which glucose is produced. Hence, regression model is introduced in order to simulate the correlation between cellulose-cellulase adsorption and the substrate features.

9.6.2 Model development

Multiple linear regression models are often used as empirical models or approximating functions when more than one independent variable are involved. That is, the true functional relationship between the dependent variable and independent variables is unknown, but by utilising complex forms of independent variables, the multiple linear regression models adequately approximates the true unknown functions. In this study, regression models that include the quadratic terms of each independent variable and the interaction terms between the three structural features may take the following form:

$$\begin{aligned} E^a = {} & a_0 + a_1*L + a_2*CrI + a_3*C \\ & + a_{11}*L_2 + a_{22}*CrI^2 + a_{33}*C^2 \\ & + a_{12}*L*CrI + a_{13}*L*C + a_{23}*CrI*C + \varepsilon \end{aligned} \qquad (9.1)$$

Where E^a = adsorbed enzyme (g/L * 10^{-2})

L = Lignin content (g/L)

CrI= crystalline cellulose content (g/L)

C = Cellulose content (g/L)

$a_0 - a_{23}$ are correlation parameters, ε is random errors

Equation (9.1) is valid in the range

$0.4 \text{ g/L} < L < 1.2 \text{ g/L}$

$0.5 \text{ g/L} < CrI < 2.5 \text{ g/L}$

$2.0 \text{ g/L} < C < 3.5 \text{ g/L}$

$0.4 \text{ g/L} * 10^{-2} < E^a < 12 \text{ g/L} * 10^{-2}$

9.6.3 Parametric study

After variables are determined for each model, correlation parameters are obtained by using the Matlab (version 6.5) curve fitting tools. Table 9.2 summarises the correlation parameters. As can be seen from table 9.7, both maximum enzyme adsorption capacity and 2-h enzyme adsorption are influenced by lignin content, crystallinity, cellulose content, and their interaction.

Based on the observations of correlation parameters, lignin content (a_1 and a_{11}) showed significant negative influence on both maximum adsorbed enzyme and adsorbed enzyme at 2 h; cellulose crystallinity (a_2 and a_{22}) also had important influence on the maximum adsorbed enzyme and adsorbed enzyme at 2 h. Compared to lignin content and cellulose crystallinity, cellulose content (a_3 and a_{33}) had less effect on 2-h enzyme adsorption. From this work, it can be concluded that lignin content and crystallinity have significant effects on enzyme adsorption, which agrees well with Zhu's (2005) conclusion. It is apparent that the quadratic terms of lignin, crystallinity and cellulose content and interaction terms between three structural features also have important effects on cellulose-cellulase adsorption.

Table 9.9 Parameters from curve fitting for the proposed regression model

Parameters	Maximum E^a	2h E^a
a_0	51.71	18.24
a_1	-5.97	-0.23
a_2	-9.18	-2.90
a_3	-25.08	-4.94
a_{11}	-19.82	-4.41
a_{22}	3.45	1.71
a_{33}	2.94	1.91
a_{12}	-2.66	1.52
a_{13}	13.07	-3.72
a_{23}	0.26	-1.45

Note: E^a = adsorbed cellulase

9.6.4 Model simulation

Using Equation 9.1 with the parameters in Table 9.7, the maximum adsorbed cellulase and adsorbed cellulase at 2 h were calculated and compared with the measured data in Table 9.6, as shown in Figures 9.37 and 9.38, respectively. The R^2 values between measure data from experimental work and calculated data from proposed model were 0.97 and 0.91 for maximum adsorbed enzymes and the 2 h adsorbed enzyme respectively, indicating that Equation 9.1 describes the maximum enzyme adsorption capacity and 2 h cellulase adsorption satisfactorily.

The agreement between the measured and predicted values shows that lignin content, cellulose crystallinity and cellulose content are key factors that determine cellulose adsorption, and the cellulase adsorption has a logarithmic relationship with glucose rate that can be predicted over a wide range of selected waste fractions using the modified HCH-1 model.

As can be seen from the table 9.6 and table 9.7, for maximum adsorbed enzyme, the more lignin content one substrate has, the less maximum adsorbed enzyme one substrate has. This agrees with the experimental results as presented in Figures 9.2-9.8 (Section 9.3): substrates such as carrot peelings and grass with higher lignin content (1.0875 g/L and 1.0115 g/L respectively) have less amount of maximum E^a ($7.23 * 10^{-2}$ g/L and $5.11 * 10^{-2}$ g/L). In contrast, scrap paper and the combination of newspaper and scrap paper with the less amount of lignin content (0.407 g/L and

0.511 g/L)) have the higher amount of maximum E^a (10.36 * 10^{-2} g/L and 11.92 * 10^{-2} g/L). This finding supports the statement that the presence of lignin plays a significant role in maximum enzyme adsorption capacity, that is, the lignin is the main barrier preventing the enzyme getting contact with cellulose.

It is also obvious that the 2-h adsorbed enzyme is influenced not only by the lignin content but also by the cellulose crystallinity. The less lignin content and the more crystalline cellulose one substrate has, the greater the amount of adsorbed enzyme. For example, the higher greatest amount of adsorbed enzyme at 2 h found in scrap paper and the combination of newspaper and scrap paper where less lignin content (0.407 g/L and 0.511 g/L) and more crystalline cellulose (2.3373 g/L and 2.4482 g/L) were found. The obvious explanation for this finding is because crystalline cellulose required longer residence time to be hydrolysed compared to amorphous cellulose. This means that substrates with less crystalline cellulose but more amorphous celluloase are hydrolysed quick leading to quick release of adsorbed enzyme. Another reason is that the 2-h adsorbed enzyme is also affected by the amount of cellulose.

Lignin content and crystallinity play more significant roles than cellulose content in cellulose-cellulase adsorption. The presence of lignin has a greater effect on maximum enzyme adsorption capacity than cellulose content and crystallinity. Both lignin and crystallinity have greater on 2-h steady-state enzyme adsorption capacity than cellulose content. The lignin content as a layer of barrier prevents the enzyme getting contact with cellulose whereas crystalline cellulose contributed to the ultimate extent of biomass hydrolysis. This finding agrees with McMillan (1994) who stated that lignin interferes with hydrolysis by blocking access of cellulases to cellulose. However, the effects of lignin content, crystallinity and cellulose content on enzyme adsorption are, to some extent, interrelated (as can be seen from the model equation 9.1 and parameters presented in table 9.7).

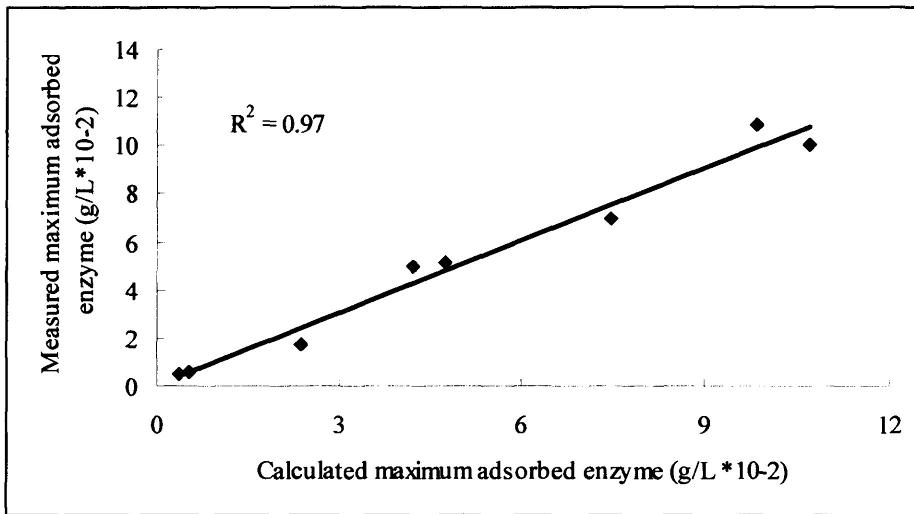


Figure 9.37 Correlations between the calculated maximum adsorbed enzyme from regression model and measured value from experimental results

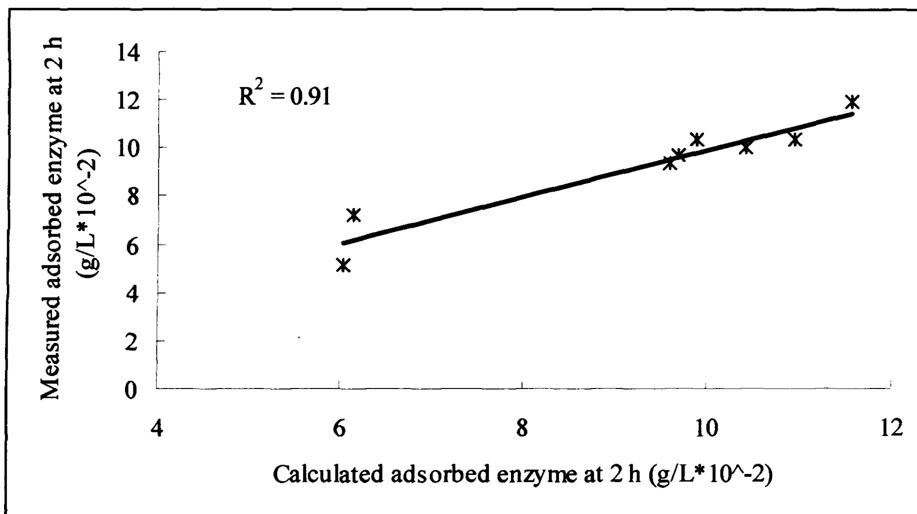


Figure 9.38 Correlations between the calculated maximum adsorbed enzyme from regression model and measured value from experimental results

9.7 Conclusions

It was shown in this chapter that the enzyme was adsorbed into free cellulose sites within the first 30 min of the reaction. The maximum adsorption happens within the first few minutes of reaction. Adsorbed cellulase and free cellulose forms

cellulase-cellulose complex. After that, enzyme starts to release from this complex. The adsorption and enzyme kinetics can be interpreted by HCH-1 model by finding new constants for the parameters, which respects to enzyme-cellulose adsorption and glucose produced over 2 hours of reaction time.

There is no obvious correlation between free cellulose site and crystalline cellulose. However, it is sure that crystalline cellulose does contribute to providing free cellulose sites. Moreover, the presence of lignin in the pre-treated biomass also affects the enzymatic hydrolysis by blocking the access of enzyme. This can lead to the decrease of the contacts between cellulase and cellulose.

The new model describes the effects of substrate features; it shows that lignin, crystallinity, cellulose content and their interaction have a significant influence on both maximum enzyme adsorption capacity and for 2-h (steady-state) enzyme adsorption. It is the first model to simulate cellulase-cellulose adsorption and substrate features for MSW biomass. It provides some understanding of hydrolysis mechanisms for the second generation biomass with more complex structures by starting to answer some of the fundamental questions around the role of substrate structures in enzyme adsorption. It also provides significant information for developing theoretical adsorption model for more complex biomass, such as lignocellulosic feedstock, in the future.

Chapter 10

Conclusions and future work

10.1 Conclusions

Bioethanol production from biodegradable municipal solid waste (BMSW) is a process that offers tremendous perspectives for many nations faced with the increasing demand of food and energy sources. Compared to existing or other proposed solutions, bioethanol production from BMSW has inherent advantages including the stability of source supply (BMSW), the existence of a suitable infrastructure for biomass collection and transportation; it also has a number of definite environmental advantages such as preventing biodegradable waste from landfill. For all these reasons, the production of second generation bioethanol is currently seen by many as one of the most promising alternatives to fossil fuel. However, the cost of this emerging process is still too high to make it a genuine contender. The yield of this emerging process that can be obtained with state of the art techniques and technologies is not sufficiently high to allow its exploitation on an industrial scale, mainly restricting its existence to the laboratory bench.

The study described in this document belongs to a body of work that aims at providing a greater understanding of the mechanisms involved in the bioconversion process, and at improving and developing new techniques to reduce its cost. This work was focused on the study of the conversion process of selected BMSW fractions to glucose for bio-ethanol production. It included (i) biomass resources analysis, (ii) BMSW characterisation, (iii) study of pre-hydrolysis treatment process and development of efficient methods, (iv) study and optimisation of the hydrolysis process and (5) study of cellulase-cellulose adsorption.

The scope of this study did not include the fermentation process which is a process

far more mature than the others. The investigation reported throughout nine previous chapters has enabled numerous conclusions to be drawn on the bioconversion of BMSW to glucose. They are presented here in consideration of the research objectives announced in Chapter 1.

Biomass resources analysis

In order to assess the potential of using MSW as biomass source, an estimate of the quantity of ethanol that can be produced from MSW in London by 2020 has been calculated. This estimation is based on three categories of MSW selected for this thesis: kitchen organics waste (KOW), green organics waste (GOW), and finally paper and card waste (PCW). The estimated values are as follow: a theoretical ethanol yield of (i) 363 L/dry tonne can be produced from KOW (accounting for 26% of total MSW; (ii) 420 L/dry tonne from GOW (accounting for 8% of total MSW) and (iii) 505 L/dry tonne from PCW (accounting for 23.6% of total MSW).

MSW data were collected mainly from the Department for Environment, Food and Rural Affairs (DEFRA) and WasteCapitalFacts and concerns the period 2002 – 2005. Assumption is made that the entire potentially availble BMSW is used for ethanol production. In this case this potential MSW-based ethanol could be used to replace as much as 14.6% of the London 2004 petrol-cars' needs by 2020.

This study also considers the requirement of EU Landfill Directives (99/31/EC), UK National Waste Strategy (2003) and London's Waste Management Strategy (2003). Conversion of MSW to ethanol provides an alternative solution of preventing BMSW to landfill required by these legislations. Based on economic estimation and requirements to comply with BMSW disposal legislations, it is suggested that the amount BMSW available is sufficiently large to consider BMSW as a credible bioethanol source.

This work also reviewed the complex structures of lignocellulosic materials (such as crystalline cellulose and the presence of lignin). In order to open the structure and provide maximum contact of cellulose with catalyst, characterisation of the substrate features and pre-hydrolysis are both necessary in order to provide better

understanding of the influence of substrate features.

Based on the results of this estimation, it is concluded that MSW, as one of the promising biomass sources, has the potential of replacing primary biomass sources. As the future use of MSW as biomass source is considered to be economically viable and to have numerous environmental benefits, further study of the conversion process appears to be justified not only from a scientific perpective but also from socio-economic and environmental ones.

BMSW characterisation

In order to measure the performance of the process and understand how they can be improved, the chemical composition and structural features of eight selected types of feedstock, i.e. BMSW fractions, have been characterised. The analytical methods used for this study were inspired from the standard ethanol analytical procedures published by the US National Renewable Energy Laboratory (NREL). The experimental results of this chemical analysis show that:

(i) Scrap paper has the highest *cellulose content* (63.76%) followed by newspaper (44.21%) and carrot peelings (42.41%), while grass (22.50%) and potato peelings (21.84%) have the lowerst cellulose content;

(ii) Carrot peeling and newspaper have the highest *lignin amount* (25.78% and 25.74%), followed by grass (23.85%), potato peeling (20.72%), and scrap paper (14.08%);

(iii) Potato peeling has the highest *hemicellulose* content (42.78%), followed by grass (39.59%), newspaper (24.36%) and carrot peelings (23.30%) and far more than scrap paper (6.83%).

Besides, study of their structural features shows that:

(iv) Newspaper has the highest *crystallinity* (70.8%), shortly followed by scrap paper (67.4%), then grass (37.3%), potato peelings (21.2%) and finally carrot peelings (16.8%).

Ethanol is indirectly obtained from cellulose via glucose; the cellulose content determines the maximum theoretical ethanol yield. In theory, scrap paper with the highest amount of cellulose content should produce the highest glucose yield.

However, the conversion efficiency is influenced by the structure properties. This is due to the fact that cellulose is surrounded by hemicellulose and lignin, forming a layer of barriers preventing the catalyst to have access to the cellulose. Hence, pre-hydrolysis with the purpose of breaking down these structural barriers is essential in order to obtain maximum hydrolysis conversion rate.

From these findings, it is clear that the conversion efficiency is not only affected by chemical compositions but by cellulose structures too. It is still unclear though what substrate can provide the best product yield.The substrate composition indicates the amount of cellulose, lignin, and hemicellulose of each model waste. The cellulose content indicates the potential glucose/ethanol product yield. Other chemical composition such as lignin and hemicellulose indicate the level of difficulty for the catalyst to access the cellulose. Cellulose crystallinity indicates the level of difficulty for the cellulose to be converted to glucose. All these contents are important information that must be taken into account when selecting pre-treatment methods, especially since different pre-hydrolysis processes have different principal impact such as removing lignin and hemicellulose and affecting cellulose crystallinity.

Pre-hydrolysis treatment process for MSW feedstock

An efficient pre-treatment process (acid impregnated steam treatment) for MSW-feedstock has been developed. The efficiency is measured based on how much lignin and hemicelluloses has been removed, how much glucose has been produced and the change of cellulose crystallinity. This process removes more than 80% of lignin and hemicellulose content for the selected BMSW fractions under the following conditions: Temperature 121 °C, acid concentration 1% and residential time 60 min. This process is inspired from existing processes for primary biomass feedstock such as corns and energy crops, but its originaility comes from its relatively low temperature (121°C) compared to other techniques.

The selection of pre-treatment methods depends on the composition of selected biomass sources. In this work, different promising pre-treatment methods (acid treatment, steam treatment, microwave treatment and combination of two-step

treatment) were tested on the selected biodegradable waste fraction. Among all the selected pre-hydrolysis methods, sulphuric acid-impregnated steam treatment is the most effective methods for the selected BMSW fractions as it leads to higher glucose yield for the following enzymatic hydrolysis process. This thesis also indicates the importance of considering the effects on cellulose crystallinity since it is increased by some pre-treatment methods (e.g. high temperature).

The adopted method, in this case, acid-impregnated steam treatment, was further analysed with ANOVA technique. Residence time has a greater impact on the lignin/hemicellulose removal than the other two factors, acid concentration and temperature. With the conditions of temperature 121 °C, acid concentration 1% and residence time 60 min, this method can remove more than 80% of lignin and hemicellulose for all the study biomass. The optimal conditions of this pre-treatment method were published and used for further hydrolysis study.

The cellulose crystallites structure increases after dilute-acid pre-treatment and steam treatment. A small increase of CrI has been observed in any methods that involve heat treatment (e.g. steam treatment and acid-impregnated steam treatment). The increase of CrI after acid treatment may be due to the oven drying process (105 °C) before the compositional analysis and to the reduction of amorphous cellulose which has been hydrolysed during the process. It is concluded that that the heat increases the crystallites of the materials studied. Hence, the relative low temperature (121 °C) process explored in this work has more advantages compared to the studies available in the literature since since it does not causes a large increase of crystalline cellulose content which increases the level of difficulty for the cellulose to be converted to glucose.

Hydrolysis and its optimisation

The main factors influencing the hydrolysis process performance have been identified; they are threefold: substrate concentration, pH and temperature. In particular, it is concluded that substrate concentration and pH are key variables for most of the substrates such as CP, GR, SP and mixed BMSW fractions; apart from substrate concentration, controlling parameters (e.g. the interaction of particle size

and cellulase concentration and interaction of particle size, cellulase concentration and temperature) are not the same for potato peeling and newspaper. The introduction of fractional experimental design and ANOVA analysis has helped to identify the interactions between factors.

Studying process conditions helped to identity the factors that contribute most significantly to the process yield, and was used for optimising it and minimising the cost associated with non-significant factors such as the enzyme concentration. With the final goal of maximising the glucose yield from MSW-feedstock, it is important to control the key factors (the substrate concentration, pH value, and temperature) within the study range. After the key factors were identified, the optimal conditions for each type of selected waste fractions or combination were given. The experimental results obtained with the suggested conditions shows very good correlations with the predicted values generated from the ANOVA model. This confirms the significance of the key parameters identified in this work: substrate concentration, pH and temperature.

It is also important to note that the samples used in this hydrolysis process have undergone the pre-treatment process developed in this work. Based on the study of two most commonly used enzymes (*Trichoderma virid* and *Trichoderma seerei*), it is also concluded that enzyme *T. virid* leads to higher performance on hydrolysis than *T. seerei*. The selected cellulase (*T. virid*) is used for all the enzymatic hydrolysis involved in this work.

Cellulase-cellulose adsorption

A model has been developed to simulate the effects of substrate features on cellulase-cellulose adsorption. The model supports our understanding of how the substrate features influence the adsorption process when using MSW-feedstock. The study showed that lignin, crystallinity, cellulose content and their interactions have an important influence on maximum and 2-h enzyme adsorption. It is concluded that lignin content and crystallinity play a greater role than cellulose content in cellulose-cellulase adsorption. The presence of lignin and crystallinity have a greater effect than cellulose content on 2-h steady-state enzyme adsorption capacity.

The presence of lignin has a greater effect than crystallinity and cellulose content on the maximum enzyme adsorption capacity. The lignin content behaves as a layer of barriers that prevents the enzyme from getting in contact with the cellulose whereas crystalline cellulose contributes to biomass hydrolysis.

It was shown in this thesis that the enzyme was adsorbed into free cellulose sites within the first 30 min of the reaction. The maximum adsorption occurs within the first few minutes of reaction, typically within 5 min. Adsorbed cellulase and free cellulose forms a cellulase-cellulose complex. After that, enzyme starts to escape from this complex. The adsorption and enzyme kinetics can be interpreted by HCH-1 model (initially developed for pure cellulose) by finding new constants for the parameters. This interpretation is valid within 2 hours of reaction time. This work extends the understanding of this model by fitting the experimental data from selected BMSW fractions to the development of new parameters. It indicates that the cellulose-cellulase adsorption for second generation biomass abides the similar mechanism as the first generation biomass.

The modelling of the effects of substrate features on cellulase-cellulose adsorption with the extended understanding of the hydrolysis mechanism for MSW-feedstock added knowledge of enzymatic hydrolysis mechanism for lignocellulosic materials with more complex structures. It shall provide important information and references for further investigation of theoretical model for the second-generation biomass process.

10.2 Dissemination

The work described in this thesis resulted in the publication of three articles in peer reviewed journals [1-3] and four presentations at international conferences [4-7]:

[1] Li, A., and Khraisheh, M. (2008) Bioconversion of municipal solid waste to ethanol and its related environmental issues. *International Journal of Soil, Sediments and Water: Documenting the Cutting Edge of Environmental Stewardship* **1,** 5-10.

[2] Li, A., and Khraisheh, M. (2008) Rubbish or Resources: an investigation of converting municipal solid waste to bio-ethanol production. *Transaction in Ecology and the Environment* **109**, 115-122.

[3] Li, A., Antizar-Ladislao, B., and Khraisheh, M. (2007) Bioconversion of municipal solid waste to glucose for bio-ethanol production. *Bioprocess and Biosystems Engineering* **30** (3), 189-196.

[4] Li, A., and Khraisheh, M. (2008) An integrated system approach to study Biodegradable Municipal Solid Waste (BMSW) as biomass The second International Symposium on energy from biomass and waste, Italy, 2008.

[5] Li, A., and Khraisheh, M. (2008). Rubbish or Resources: an investigation of converting municipal solid waste to bio-ethanol production. Waste management, Granada, Spain, 2008.

[5] Li, A, and Khraisheh, M. (2007). Municipal solid waste used as bio-ethanol resources and its related Environmental Impacts. The 23rd Annual Conference in Water, Soil and Sediments, University of Massachusetts Amherst, USA, 2007.

[7] Li, A., and Khraisheh, M., Antizar-Ladislao, B., Simons, S. (2006). Bioethanol production from municipal solid waste. Bioenergy - I: From Concept to Commercial Processes, Tomar, Portugal, 2006.

In addition to the above, four papers are in the process of being submitted to peer reviewed journals including:

- Li, A., and Khraisheh, M. (in preparation) Modelling the effects of substrate features from MSW-feedstock on cellulase-cellulose adsorption during hydrolysis processA. *Biotechnology Advances.*
- Li, A., and Khraisheh, M. (in preparation) Optimisation of enzymatic hydrolysis process from MSW-feedstock by introducing fractional experimental design. *Biotechnology and Bioengineering.*
- Li, A., and Khraisheh, M. (in preparation) Acid catalysed steam treatment process for MSW-to-ethanol conversion. *Bioresources Technology.*
- Li, A., and Khraisheh, M. (in preparation) An alternative sustainable waste management approach: bioconversion of MSW to ethanol. *Environmental Science & Technology.*

10.3 Suggestions for future work

From the list of above publications, it is humbly perceived that this work will have enhanced our understanding of conversion of BMSW to bioethanol, and brought a tangible contribution to improve the process efficiency. It is clear to the author that further progress must be achieved in order for the scientific and industrial community to design a process with the level of efficiency demanded by the market. It is in this context, that the findings from this study have been used to draw a number of recommendations with respect to the directions further work should take; the following points of interest shall assist in understanding and approaching new aspects of the vast topic that is conversion of BMSW to bioethanol:

- After analysing the potential of using BMSW as biomass sources in this thesis, it would be relevant to investigate how different types of biodegradable waste can be separated at the source to provide feedstock in a commercialisation scale.
- As pointed out in Chapter 7 (pre-hydrolysis treatment process), applying a microwave treatment to selected BMSW fractions lower the glucose yield. It was suspected that although microwave treatment may be more efficient in removing hemicellulose and lignin present in the selected BMSW fractions, it also may have facilitated glucose degradation. Future research is required to test this interpretation and provide with a more rigorous explanation for the observed result.
- In this thesis, as the main focus was hydrolysis and its product (glucose), hemicellulose was removed during pre-treatment process. However, as hemicellulose was broken down into different sugars (xylose, mannose, galactose, and arabinose), future research can be carried out on fermenting these hemicellulose sugars to by-products. Additional economic benefits could be obtained by studying the potential of co-fermenting these sugars.
- As mentioned in Chapter 4, the mechanism of hydrolysis depends on substrate features, process conditions as well as the enzyme system. As the main focus of this thesis is substrate features and process conditions, it will be necessary to explore how the enzyme system impacts on the cellulase-cellulose adsorption. However, this investigation will require the research on protein structure to make further progress.

- This thesis has improved the fundamental understanding of cellulase-cellulose adsorption at the mechanism level by extending the understanding of pure-cellulose models and introducing regression model to simulate the effects of substrate features. It is recommended that further study should aim at developing a theoretical model underpinned by the results in this thesis.
- This thesis has selected the representatives of each category of waste. With the optimal conditions provided from this work, it will now be relevant to test the process conditions with BMSW directly collected from source. This requires the BMSW fractions to be separated in order to avoid the contamination from other chemicals.
- The author has started to assess the environmental impacts of this conversion process which was published in the International Journal of Soil, Water and Sediments, as shown in the list of publications above. However, full life-cycle analysis will be necessary in order to fully understand the sustainability of the process and support its commercialisation in the future.

Chapter 11

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