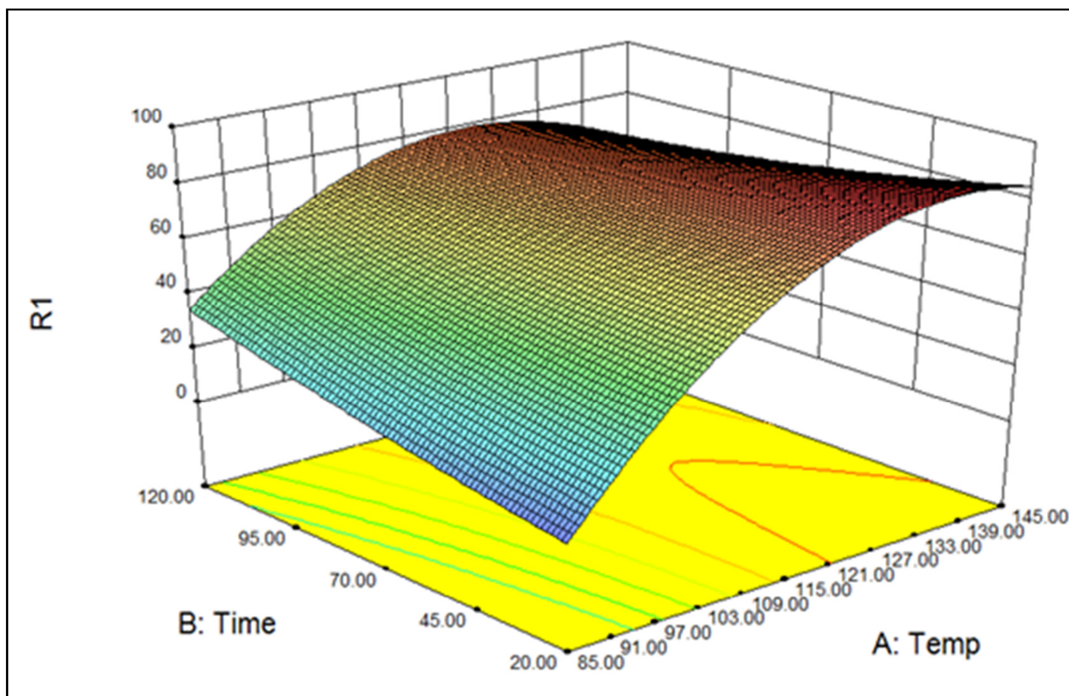
CHALMERS



Study on Dilute Acid- and Ionic Liquid Pretreatment of Agro Wastes

Potential Second Generation Bioethanol Production

Master of Science Thesis in the Master Degree Programme, Biotechnology

Yonis Ismail

Department of Chemical Reaction Engineering
Division of Bioprocessing Engineering
CHALMERS UNIVERSITY OF TECHNOLOGY
Göteborg, Sweden, 2011
Report No. xxxx

Title: Study on Dilute Acid- and Ionic Liquid Pretreatment of Agro Wastes
Subtitle: Potential Second Generation Bioethanol Production
Author: YONIS ISMAIL

© YONIS ISMAIL, 2011

Technical report no xxxx:xx
Department of Chemical Reaction Engineering
Chalmers University of Technology
SE-412 96 Göteborg
Sweden

Supervisors: Doctor Adeline Chua Seak May and Doctor Ngoh Gek Cheng
Department of Chemical Engineering
Faculty of Engineering
University of Malaya
50603, Kuala Lumpur
Malaysia

Examiner: Professor Claes Niklasson
Head of the Department of Chemical Reaction Engineering
Division of Bioprocessing Engineering
Chalmers University of Technology
SE-412 96 Göteborg
Sweden

Cover: Optimization results from this work where sago waste was pretreated with dilute sulfuric acid.

Göteborg, Sweden 2011
REPORT NO. XX/XX

Study on Dilute Acid- and Ionic Liquid Pretreatment of Agro Wastes

Potential Second Generation Bioethanol Production

YONIS ISMAIL

Department of Chemical Reaction Engineering
Chalmers University of Technology
Göteborg, Sweden

Title: Study on Dilute Acid- and Ionic Liquid Pretreatment of Agro Wastes.
Subtitle: Potential Second Generation Bioethanol Production

Yonis Ismail
Department of Chemical Reaction Engineering
Chalmers University of Technology

Abstract

Petroleum is today the dominating transportation fuel and provides the transportation sector with 97% of the total energy consumed. The escalating gasoline price, the non-renewable nature of fossil fuels, increased awareness of environmental effects, together with health and safety considerations all contribute to the effort of finding alternative fuel sources.

The most widely used biofuel for transportation is bioethanol made from biomass. Corn and sugarcane is not practical for bioethanol production as it competes with food and feed production on the agricultural land and its sustainability can be questioned. For large scale production of ethanol, it is desirable to use cheaper and more abundant substrates. Utilization of lignocellulosic material has potential for its low cost feedstock, its availability in large quantities and it is renewable. Additional advantages of second generation bioethanol are increased energy security, maximal economic benefits to farmers and rural communities and reduced greenhouse gas emissions. There are potentially $1*10^{10}$ metric tonne lignocellulose produced annually worldwide and lignocellulose is the most plentiful renewable biomass.

Pretreatment of biomass is considered to be one of the most expensive expenditures in the process of producing bioethanol. One aim of this work was to study and identify which of the two pretreatment and hydrolysis methods, dilute sulfuric acid and Ionic Liquid [EMIM]HSO_4, that generated the highest amount of reducing sugars from the three lignocellulosic substrates; sago waste, sugarcane bagasse and rice husk. Dilute sulfuric acid is the most widely studied pretreatment method while Ionic Liquids (ILs) is a new group that are potentially “green” due to minimal air emissions and no formation of explosive gases, and provides a new platform for utilization of cellulose resources.

Central Composite Design (CCD) was the experimental design method used as a statistical tool to study and optimize the processes. Miller’s method with DNS reagent was used to analyze the reducing sugar yield. The results showed that sago waste, a by-product from sago palm, generated close to maximum theoretical yield (97%) when pretreated with H_2SO_4. Sago waste is the cheapest and most available renewable natural polymer in Malaysia. The structural network appeared to be readily disrupted and cellulose was hydrolyzed in large quantities. However, the yield (46%) was not close to maximum when the reducing sugars was analyzed with high-performance liquid chromatography (HPLC), hence the results did not correspond for the two methods. The pretreatment and hydrolysis with IL did not result in satisfactory yield for any of the three substrates and the optimization experiments for this pretreatment method were reduced.

The structure of sago waste was studied with Fourier Transform Infrared (FTIR) spectroscopy, Scanning Electron Microscope (SEM) and X-ray Diffraction (XRD). It was studied after the three types of treatments had been conducted: physical pretreatment; physical and dilute sulfuric acid pretreatment; and physical and [EMIM]HSO_4 pretreatment. It appears that lignin was solubilized after both chemical pretreatments, but crystalline cellulose seemed to be relatively resistant to hydrolysis.

Key words: Lignocellulosic materials, lignocellulose, bioethanol, Second generation bioethanol, pretreatment, dilute sulfuric acid, Ionic Liquids, sago waste, sugarcane bagasse, rice husk.

Sammanfattning

Petroleum är i dagsläget det dominerande transportbränslet och förser transportsektorn med 97% av den totala konsumerade energin. Ökande oljepriser, fossila bränsles icke-förnybara samt begränsande natur, ökad förståelse för klimatförändringar, tillsammans med hälso- och säkerhetsbeaktande bidrar samtliga till satsningar att finna alternativa bränslen.

Bioetanol är det mest använda biobränslet inom transportsektorn och det görs på biomassa. Detta trots att majs och sockerrör inte är praktiska för bioetanolproduktion då de tävlar med odlingen av mat och föda och dess hållbarhet kan således ifrågasättas. Det är önskvärt att använda de billigare och mest överflödiga substraten för en storskalig bioetanolproduktion. Användning av lignocellulosa som råmaterial har därför enormt potential då detta är billigt, överflödigt och förnybart. Ytterligare fördelar med andra generationens bioetanol är ökad energisäkerhet, ökade intäkter för bönder och landsbygdssamhällen och minskade koldioxidutsläpp. Det produceras potentiellt $1*10^{10}$ ton lignocellulosa globalt årligen och lignocellulosa är alltså den rikligaste förnybara biomassan.

Förbehandlingen av biomassa brukar benämnas som den dyrare processen vid bioetanolproduktion. Ett av målen i detta arbete är att studera och identifiera vilken av de två förbehandling- och hydrolysmetoderna (utspädd svavelsyra och den joniska vätskan [EMIM]HSO_4) som kan generera högst mängd reducerat socker ifrån de tre olika lignocellulosa substraten sago avfall, sockerrörsblast och rishylsa. Utspädd svavelsyra är den mest välstuderade förbehandlingsmetoden medan joniska vätskor är en ny grupp som potentiellt är miljövänliga på grund av låga luftutsläpp och ingen bildning av explosiva gaser.

Central Composite Design (CCD) användes som ett statistiskt verktyg för att studera och optimera processerna. Millers metod med DNS reagens användes för att analysera utbytet av reducerat socker. Resultaten visade att sago avfall som är en biprodukt ifrån sago palm genererade nästintill maximalt teoretiskt utbyte (97%) när det förbehandlades med H_2SO_4. Sago avfall är det billigaste och den mest tillgängliga förnybara polymeren i Malaysia. Dess struktur tycktes bli helt rubbad och cellulosa hydrolys föreföll att ske hög grad. När mängden reducerat socker senare analyserades med högupplösande vätskekromatografi (HPLC) var utbytet dock inte högre än 46% och de två analysmetoderna korresponderade därför inte. Förbehandling och hydrolys med den joniska vätskan resulterade inte i tillräckligt utbyte för någon av de tre substraten så optimeringsexperimenten med denna förbehandlingsmetod minskades.

Sago avfallets struktur studerades med Fourier Transform Infrared (FTIR) spektroskopi, Scanning Electron Microscope (SEM) och pulverdiffraktion (XRD). Strukturen studerades efter de tre olika behandlingarna: fysisk förbehandling; fysisk- och utspädd svavelsyra förbehandling; samt fysisk och [EMIM]HSO_4 förbehandling. Det verkar som om lignin helt löstes upp efter båda kemiska förbehandlingar, men att kristallin cellulosa verkade vara relativt motståndskraftig mot hydrolys.

Acknowledgements

This Master's thesis has been conducted in Kuala Lumpur, Malaysia as a result of the collaboration between Chalmers University of Technology and the University of Malaya, through the Internationella Programkontoret's Linnaeus-Palme project. This exchange provides great opportunities for both students and teachers to share knowledge and also experience each other's cultures.

Through this exchange I have received immense knowledge not only about the ways of living in Kuala Lumpur, but for many parts of the Peninsular Malaysia region. The country is multicultural and it has been a thrill to have had the chance to be present for festivals, ceremonies and New Year's celebrations that regularly emerge. This exchange also gave me the possibility to experience, not only the metropolitan areas that exist in Southeast Asia, but also the tropical environment unique to this region.

I would therefore like to thank Professor Claes Niklasson for giving me this opportunity, but also for helping me throughout this Master's thesis journey and persistently providing feedback on this work.

The practical part has solely been accomplished in Kuala Lumpur from early January to the end of May 2011. By constantly engaging and helping me during my practical work and for receiving me very hospitable in Malaysia, I would like to thank Doctor Adeline Chua Seak May and Doctor Ngoh Gek Cheng. I would also like to thank the people in the Bioprocess Laboratory, at the Chemical Engineering Faculty, for helping me with everything on a daily basis in the lab, for showing me around the city and for making my stay very enjoyable.

Finally I would want to thank my family and friends!

Contents

Abstract c
Sammanfattning ..... d
Acknowledgements ..... e
Introduction 1
Aims and Objectives 2
Theoretical Background 3
Bioethanol as a fuel 3
  The four key arguments 4
  Combustion energy 5
  First Generation Bioethanol Vs. Second Generation Bioethanol 5
Lignocellulose and starch 6
Substrates 8
  Sago waste 8
  Sugarcane bagasse 9
  Rice husk 9
Production process of SGB 10
Pretreatment and hydrolysis process 12
  General overview of common pretreatment techniques 13
Theory on Methodology and Analysis Techniques 14
Acid pretreatment hydrolysis 14
Ionic Liquids 16
Factors studied 17
Response Surface Methodology & Central Composite Design, Optimization 18
Dinitrosalicylic acid Method for analysis of reducing sugars 20
Structural study of sago waste 21
Material and Methods 22
  Design of Experiments 22
  Pretreatment and hydrolysis 23
  DNS reagent 24
  Spectrophotometry 24
Composition determination 25
Structural analysis 27
Results and discussion 28
Initial experiments: A comparison of the three substrates 28
  Dilute sulfuric acid (H_2SO_4) pretreatment 29
  1-Ethyl-3-Methyl imidazolium hydrogensulfate ([EMIM]HSO_4) pretreatment 30
Sago waste and sugarcane bagasse comparison 31
  Dilute H_2SO_4 pretreatment 31
  [EMIM]HSO_4 pretreatment 32

Comments on the yields obtained ... 33
Further studies of sago waste with sulfuric acid pretreatment ... 35
Composition determination of sago waste ... 35
Optimization of the process with CCD ... 36
High-performance liquid chromatography of the optimal runs ... 38
Structural analysis of sago waste ... 40
Fourier Transform Infrared spectra ... 41
Results after powder X-ray Diffraction ... 45
Images from Scanning Electron Microscopy ... 47
Reliability of results ... 50
Conclusions ... 52
Future work ... 53
References ... 55

Introduction

Second generation bioethanol (SGB) has the possibility to overcome the difficulties of energy security and environmental degradation [1] that is presently encountered worldwide. The economies based on petroleum are facing obstacles with diminishing reserves, escalating fossil fuel prices, energy security, CO_2 emissions and so on [2]. There are several possible alternative fuels [3] and one category is the biofuels. These are made from biomass and presently the most widely used in the transportation sector is bioethanol. In fact, it has been reported [4] that ethanol is the most vital biotechnology product based on quantity and market share. Bioethanol produced from lignocellulosic materials is referred to as second-generation bioethanol [3]. Lignocellulose is natural, inexpensive and an abundant renewable bio resource [5, 6, 7, 8, 9, 10]. However, there are factors such as cost, technological knowledge, environmental issues and biomass availability that prevent full scale SGB production [1].

The key obstacle with SGB is the higher production cost in comparison with first-generation bioethanol (FGB) [1]. Given that FGB is produced from edible feedstock, such as corn or sugarcane [11], this ethanol production competes with food production on the already limited agricultural land [6, 12]. Another drawback of FGB is the raw material cost which can be as high as 70% of the total production cost [1]. The FGB production process is well-established [5] and although the ethanol is sold at competitive prices on the fuel market it cannot be produced in adequate amounts to meet the growing fuel demand [13]. By using inexpensive substrates such as lignocellulosic materials, the bioethanol production could be made more competitive with oil [5] in the longer run. Hence SGB has the potential to replace fossil fuels without affecting the food-supply, as lignocellulose is non-edible [11]. SGB can be found in agricultural wastes, industrial wastes, forestry residues, municipal solid wastes and so on [5].

Sago waste, sugarcane bagasse and rice husk are three types of residues that can be used to produce bioethanol and they are discussed in this work. Lignocellulosic material normally contains 20-50% cellulose, 20-35% hemicelluloses and 10-35% lignin [4, 14]. Cellulose and hemicelluloses are long-chain polysaccharides and when hydrolyzed the degradation products can be hexoses and pentoses [1, 12]. The key feedstock for the fermentation process during first- and second generation bioethanol production is glucose which is a hexose monosaccharide derived from for example cellulose, sugar and starch. In contrast to the FGB substrates the lignocellulosic materials has to be pretreated to increase the hydrolysis rate of cellulose. The additional process steps required results in SGB production being more expensive than FGB [1].

The pretreatment step can be the most costly process in SGB production [15, 16]. As of now this process (and the hydrolysis step) is not fully established, complex and requires energy [7]. By focusing further research on this process there is a possibility of increasing the efficiency and decreasing the expenses [15]. Aspects that affect the degradation of cellulose and retain the development of the process are for example cellulose cristallinity and the presence of lignin and hemicelluloses in substrate [17]. There are different pretreatment techniques and these can be categorized as: physical-; physico-chemical-; chemical- and biological processes [6].

This work presents studies on physical pretreatment such as grinding and sieving and chemical pretreatment and hydrolysis with dilute sulfuric acid and the Ionic Liquid 1-Ethyl-3-Methyl imidazolium hydrogensulfate([EMIM]HSO_4). Dilute acid pretreatment has been reported [17] as a prominent process and is presently under commercialization. According to literature [6] the cellulose hydrolysis rate can be considerably improved after dilute sulfuric acid pretreatment.

The Ionic Liquids (ILs) is a new and possibly environmentally friendly group of compounds used for the pretreatment of lignocellulose [18, 19]. It has been reported [19] that ILs dissolve high quantities of cellulose and are reusable. These qualities make the ILs attractive as a technique for pretreatment of lignocellulose [18, 19].

Aims and Objectives

The aims of this work are:

- To identify which of the two pretreatment methods; H_2SO_4 and [EMIM]HSO_4 that increases hydrolysis rate the most and generates highest yield of reducing sugars for the three lignocellulose substrates rice husk, sugarcane bagasse and sago waste.
- To study which of the three substrates that achieves highest yield of reducing sugars/total weight.
- To optimize the pretreatment methods individually for the substrate that generates the highest amount of reducing sugars/total weight for the specific method.
- To increase the knowledge regarding the structural changes the substrate goes through during pretreatment and hydrolysis.

The aims stated above are directly connected to one another. In order to identify the pretreatment method that is most efficient and effective an investigation of the variables affecting the pretreatment and hydrolysis is of need. The numerical variables that will be considered in this project are temperature, time, sulfuric acid concentration and solid loading (the amount of substrate/volume of pretreatment solution). A study on the variables and their interaction effects on the substrate pretreated will be done with design of experiment strategies to strengthen the analysis with proper tools.

Therefore the solution of the second aim will be attempted to be of analytical nature with design of experiments (Full Factorial Design and Central Composite Design). The substrates are to be treated as a categorical variable in the designs to decide its impact. Two separate paths of designs are to be set up. One for sulfuric acid pretreatment and one for [EMIM]HSO_4. To accomplish the first aim a more subjective approach is needed. The two pretreatment methods (paths of designs) will be compared in a subjective way. To statistically analyze and justly compare sulfuric acid with [EMIM]HSO_4 in regard to selection of the appropriate sulfuric acid concentration appears to be of difficulty.

Hence an approach to optimize the pretreatment methods individually for the substrate producing the highest yield for each will be attempted. The optimization process is likely to require several experimental designs to cover a broad range for the variables studied in order to discover a wide response (yield) space. Once the two pretreatments have been optimized a comparison between these can be accomplished.

Studies on the compositions and structural changes before and after chemical pretreatment will be done in order to attempt to increase the knowledge of the lignocellulosic residues during pretreatment and hydrolysis. The composition analysis will be accomplished with literature studies and also determined according to the methods suggested by the National Renewable Energy Laboratory [20, 21]. The structural changes will be analyzed with Fourier Transform Infrared (FTIR) spectroscopy, Powder X-ray Diffraction (XRD) and Scanning Electron Microscope (SEM).

Theoretical Background

The early parts of this topic provides information regarding: when the idea to use ethanol as a fuel arose; why bioethanol has the research devotion today; how ethanol combustion efficiency differentiates from gasoline and diesel fuels; and a comparison of first- and second generation bioethanol. The proceeding sections initially describes the structure of plant cell walls and goes on with molecular composition and information of the waste substrates studied in this research.

The later part of the topic outlines the production processes conducted from substrate based on lignocellulose to the end product ethanol. The final part emphasizes the main focus of this article; the pretreatment and hydrolysis process of lignocellulose to reducing sugars. A description of the structural changes that occurs throughout the pretreatment and hydrolysis process and also general information about commonly used pretreatment methods are provided.

Bioethanol as a fuel

The awareness of using ethanol as fuel for the automobile industry goes back to early in the 20th century owing to escalating gasoline prices amongst other factors. However, an investment opportunity for a large scale ethanol production was never presented in time due to a too late removal of excise duty on denatured ethanol [22]. Possible fuels were considered as a substitute for gasoline and Henry Ford reflected on using ethanol for his Model T in 1908 [22, 23]. More methods were developed such as Rudolf Diesel's engine which was based on cheap and readily available coal existing in Germany [22]. Nonetheless, between 1902 and 1912 the vehicles driving on American roads went from 20,000 to over 1 million and the huge increase for gasoline demand paved the way for a whole new market for J.D Rockefeller's oil companies [22].

Brazil has produced bioethanol for fuel as early as 1925 and the production and consumption were initially 70 times higher than petroleum [3]. However, the bioethanol production costs increased in comparison to petroleum and especially after World War II [24]. The petroleum production and consumption grew globally. Yet, after the oil crises in the 1970s there has been an increased interest for the previously ignored potential of bioethanol [3, 24, 25].

By providing the transportation sector with 97% of the total energy consumed [25], petroleum is today the dominating transportation fuel [3]. 60% of the oil consumption is designated for the transportation sector [3] and 2007 there were over 800 million cars and light trucks on the roads internationally [3]. This results in huge CO and CO_2 emissions [3]. The amount of vehicles is expected to be over 2 billion by year 2050 [3]. The escalating gasoline price, the non-renewable nature of fossil fuels, increased awareness of environmental effects, together with health and safety considerations all contribute to the effort of finding alternative fuel sources [3].

There are several possible alternative fuels such as biofuels, methanol, hydrogen, boron, natural gas, electricity, solar fuels and so on [3]. Important is that an alternative fuel must be technically possible, economical, environmentally tolerable and effortlessly accessible [26]. Biofuels are made from biomass and the most widely used for transportation is bioethanol. Other biofuels are biomethanol, biodiesel, biogas, vegetable oils, biohydrogen etcetera [3]. For the time being biofuels are the only appropriate and renewable energy sources for the transportation sector [6].

The four key arguments

Economies based on petroleum are facing obstacles such as diminishing reserves, escalating fossil fuel prices, energy security, CO_2 emissions amongst other factors [2]. According to literature [3] there are economic impacts (or benefits) using biofuels such as; augmented fuel variety, additional rural production contracts, higher income taxes, increased investments in facilities and tools, global competition and so on.

Table 1 The main impacts for biofuels are illustrated in the left column. It consists of the major groups economic-, environmental- and energy impacts. The benefits that influence the three groups are stated in the text in this section or can be read in Ref. 3. Ref. 22 illustrates the impacts and benefits in four main arguments and these are clarified below in the text and can be seen in the column to the right.

Economic Impacts	"Peak oil" Argument
Environmental Impacts	"Energy security" Argument
Energy Security	"Long-term environmental" Argument
-	"Acute climatic" Argument

According to Ref. 22 there are four key arguments in favor of bioethanol and other biofuels. The same arguments are illustrated in table 1 in the right column. Ref. 3 separates and rephrases the same arguments into three main impacts (and explained with several benefits for each impact) in favor for biofuels. The arguments (impacts) are:

- **The "peak oil" argument states that fossil energy is limited and may be depleted before 2050.**
  Estimates with varies methods have been done to quantify the amount of fossil fuels for both discovered and undiscovered reserves [27]. The global population growth and industrialization of more countries results in increased energy demand [6]. The prediction of this is a huge decrease, and eventually depletion [28] of oil by 2050 [27] and many nations' economies depend on oil [6].

- **The "energy security" argument emphasize that biofuels avoid dependence on oil imports.**
  Bioethanol production from lignocellulosic materials can represent a domestic energy source that is renewable [23]. The biomass is also biodegradable and readily available worldwide (domestic distribution) which may result in increased supply reliability [3].

- **The "long-term environmental" argument that considers a more efficient utilization of agricultural wastes for a sustainable development worldwide.**
  Ref. 3 illustrates that the land and water use will be developed and the agriculture will be improved. Some of economic impacts mentioned above, in this section, are related to this argument.

- **The "acute climatic" argument reflects on reduction of greenhouse gas and other pollutants.**
  CO_2 emissions from bioethanol are neutral in contrast to fossil fuels [23]. Lignocellulosic bioethanol lowers greenhouse gas emissions with almost 90% compared to gasoline while corn-based bioethanol lowers it with 18% [3].

Combustion energy

Ethanol has been reported [3, 22, 23] to have a higher density than gasoline and the energy capacity of a gallon ethanol therefore only contains approximately 70% of a gallon gasoline. The efficiency of ethanol combustion is however 15% higher than that of gasoline resulting in energy capacity difference of 80-85% [22, 23]. The increased efficiency is due to ethanol having a higher octane number and also possesses a better volume of combustion products per energy unit burned [3, 22, 23]. These arguments cannot be applied when ethanol is paralleled with diesel fuel and the energy of ethanol is only 60% of diesel [22]. All cars currently manufactured can utilize 10 or 85% ethanol blend [25] and biofuel programs are implemented in some nations [3].

First Generation Bioethanol Vs. Second Generation Bioethanol

Ethanol production from corn and sugarcane is directly competing with food and feed production on the agricultural land [6] and its sustainability is therefore questionable [9]. The production of corn is insufficient in the sense that if all the corn produced in the U.S were dedicated to first generation bioethanol (FGB) production only 12% of the gasoline consumption would be satisfied [29]. A third drawback of using FGB is that the raw material cost is expensive [12] and composes between 40-70% of the total production cost [30, 31].

For large scale bioethanol production it is consequently necessary to utilize the cheaper and abundant substrates [7]. Lignocellulosic materials are substrates that are potential for the low feedstock costs and availability in large quantities [6, 7, 8, 9]. Bioethanol from lignocellulosic substrates minimizes the food competition [2, 9], but not always feed [7]. There is between 1.3-2.3 billion tons of cellulosic biomass that supposedly can be utilized yearly and on a renewable basis for bioethanol production in the U.S. In theory this amount would correspond to 30-50% of the current gasoline consumption in U.S [23]. Second generation bioethanol (SGB) from lignocellulosic material is thus considered to be the most potential amongst the biofuels and can perhaps change the current necessity of gasoline in the transportation sector.

Ethanol from corn generates 20-30% more energy than the fossil fuel used during its production while both sugarcane and lignocellulosic bioethanol generates 9 times more energy than the fossil fuel consumed in the production of bioethanol from these substrates [32].

One main issue with SGB is that the production is too expensive [7]. Previous literature [1] compared corn with switchgrass. The article shows that the switchgrass feedstock is cheaper, but generates a lower yield of ethanol and has a higher processing cost resulting in higher total cost in comparison to corn. According to Ref. 18 the complete potential of utilizing cellulose has not been accomplished for the key reasons: the consumption of petroleum from the 1940s and onward; the involvement of non-environmentally-friendly methods to extract cellulose from substrates; the issue in altering structural properties of cellulose; and the restricted number of pretreatment solvents that can solubilize cellulose. Typical lignocellulose materials can be divided into six main groups of: crop residues; herbaceous biomass; hardwood; softwood; cellulose wastes; and municipal solid wastes [6, 7].

Bioethanol can be derived from the polysaccharides cellulose, hemicelluloses and starch [3]. Lignin is not a polysaccharide [3] and can therefore not be utilized for the purpose of producing ethanol. The next section provides further information about this and also the structure and composition of lignocellulosic materials and starch. An example of a substrate with a high amount of starch is corn.

Lignocellulose and starch

As lignocellulosic biomass exists in an ample quantity [33] (most plentiful biopolymer in the world) and on a renewable basis [34], it has the possibility to function as a cheap feedstock for SGB production [35, 36]. According to estimations biofuels can be manufactured from biomass in over a billion ton on a renewable basis in the U.S [37].

Lignocellulose mainly consists of cellulose, hemicelluloses and lignin [23, 38, 39] connected in a complex structure and natural lignocellulose is resilient to hydrolysis [39]. Cellulose and hemicelluloses are polysaccharides with the functionality to support the walls of plant cells [38]. The cell wall also comprise of lignin [39]. Although all plant cell walls have a similar layout, the proportion of the main components varies with the wall types and also with plant species [39]. The cell walls are divided into primary and secondary walls, where the thickest layer of the secondary wall contains the highest quota of cellulose compared to hemicelluloses and lignin [39].

Cellulose, the most abundant polymer on earth [5] is built up by repeating cellobiose (disaccharide) units and the glucose (monosaccharide) units are linked together by β-1,4-glucosidic bonds [38, 41] to form this disaccharide. This is shown in Fig. 1. The amount of monosaccharide units in one cellulose molecule varies and is measured as the degree of polymerization [38]. The role of the degree of polymerization is not entirely identified, but is supposed to influence cellulose hydrolysis [9]. The cellulose molecules are in turn linked together by a network of inter- and intra-molecular hydrogen bonds and van der Waals forces [19, 42, 43] creating microfibrils and cellulose do not exist as single molecules [38 ,39]. The microfibrils consist of well-ordered crystalline structures and other minor arranged non-crystalline structures also known as the amorphous regions [39]. As the density of cellulose molecules increases the crystalline regions arises [38]. This highly packed and ordered structure of the compound prevents even small molecules such as water to enter [2, 39] and it is highly resilient to hydrolysis [2, 43]. A protective layer exists around cellulose which consists of lignin and hemicelluloses linked together to create a hydrolysis insensitive complex [12, 38].

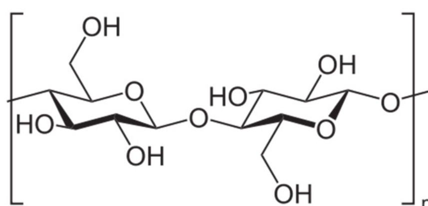


Figure 1 illustrates the cellulose structure where glucose units are linked together by β-1,4-glucosidic bonds.

Lignin is a highly complex aromatic and amorphous polymer [38, 44] built up by the three key monomer precursors; *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol [38] and these can be seen in Fig. 2. The monomers are connected by heterogeneous bonds of ether or carbon-carbon [38, 39]. The highest concentration of lignin is in the middle lamella of the cell, which almost only consists of lignin [38, 39]. However the highest amount of lignin exists in the cell wall [38]. This creates a three-dimensional network around cellulose and hemicelluloses [2]. The majority of the heterogeneous bonds in lignin are insusceptible to hydrolysis and lignin is water insoluble [39]. Although lignin is the second most abundant polymer in the world it is often burned and utilized as energy or discarded as waste [45, 46]. Research [9] on lignin is currently conducted to develop higher value products from it during a well-established bio refinery process.

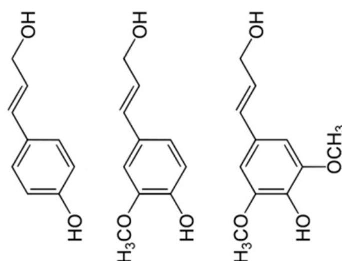


Figure 2 shows the three precursors: *p*-coumaryl alcohol (left); coniferyl alcohol (middle); and sinapyl alcohol (right).

Hemicelluloses is a group of short and branched polymers [3] that can consist of different sugars such as pentoses (D-xylose and L-arabinose), hexoses (D-glucose, D-mannose and D-galactose) and also different uronic acids [38]. In contrast to cellulose, hemicelluloses are completely amorphous [2, 3]. These traits make hemicelluloses easier to hydrolyze in comparison to cellulose [3]. Apart from the three main components lignocellulose also consists of extractives and ash. Extractives can be classified as terpenoids, steroids, fats, waxes and phenolic constituents [38].

Starch consists of a combination of amylose and amylopectine. The first polyglucan is linear and the latter branched, (see Fig. 3). The monosaccharides of amylose are glucose and maltose and enzymatic hydrolysis of the α-1,4 linkages of the polymer occurs in the presence of α-amylase. Since amylopectin is branched it can only be hydrolyzed at the α-1,4 linkages (by α-amylase) to dextrin, which are a mixture of maltose, glucose and other oligosaccharides connected with α-1,6 linkages. This results in a yield decrease of fermentable sugars (monosaccharides). Although starch and cellulose have similar molecular structure, starch is essentially a storage polymer, in for example corn, much unlike cellulose that supports the cell wall rigidity [22].

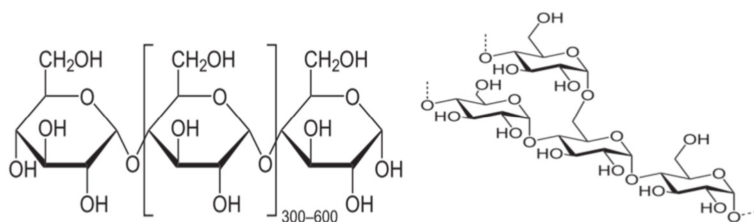


Figure 3 shows the molecules amylose (to the left) and amylopectin (to the right), that build up starch .

Substrates

The three substrates examined in the work are; sago waste, sugarcane bagasse and rice husk. This sub-topic supplies general information about the three substrates including their chemical composition, found in Table 2-4. Observe that compositional diversity exists even for the same type of substrate. Some factors that may be affecting this are for example that feedstock are residue products with different process efficiencies, that differences in plant genetics exist, environmental conditions, methods of harvesting and storage and lastly methods utilized for the compositional analyses [47].

Sago waste

Malaysias cheapest and most accessible renewable natural polymer is sago waste [48]. It is a by-product of sago palm (*Metroxylon sagu*) from the sago starch production [48]. In Sarawak, Malaysia, sago palms grow in swampy areas covering 1.5-2 million hectares of wild and 200 000 hectares of cultivated palms [48]. During photosynthesis the surplus of photosynthate is transferred from the palm leaves to the pith and stored as starch [49]. The palm can reach 7-15 meters in length where the pith normally contains about 250 kg of starch [49]. The sago starch is mainly used as an ingredient in different food types, but has other industrial uses such as processing glucose and fructose syrups [48]. Only 95% of the sago waste composition is illustrated in Table 2. The remaining components are yet to be determined.

Table 2 displays the sago waste composition.

Cellulose and Starch	78%
Moisture	10%
Acid insoluble lignin	6%
Ash	1 %
Acid soluble lignin	NA
Hemicellulose	0%

Sugarcane bagasse

The largest sugarcane (*Saccharum officinarum*) manufacturer in the world is Brazil [50] with sugarcane facilities generating sucrose and ethanol from the sugarcane juice [47]. The process involves crushing the sugarcane and extracting the juice [47, 51]. This generates one of the largest cellulosic agro-industrial by-product sugarcane bagasse which is normally burned for electricity [52], used for pulp and paper production or for production of fermentation products [51]. Circa 80 countries produce sugarcane in a quantity of around $5.4 \cdot 10^8$ dry tons annually with 1 ton generating approximately 280 kg of bagasse [7]. It is both efficient and economical to burn sugarcane bagasse for energy [51]. There is however potential of utilizing sugarcane bagasse to increase the bioethanol production and as an alternative burn leaves and tops for energy since these are left on the sugarcane fields [47]. The sugarcane bagasse composition can be found in table 3:

Table 3 contains the sugarcane bagasse composition. [47].

Component	Content
Cellulose	35-45%
Hemicelluloses	26-36%
Lignin	11-25%

Rice husk

Approximately 320 million tons of rice (*Oryza sativa*) was grown globally during 2009 of which Malaysian production constituted 2.2 million tons [53]. The by-product after dehusking at the rice mills is rice husk. About 230 kg rice husk can be generated for 1 ton rice processed and presently most of this is burned [54]. The composition of rice husk has been determined and is shown in Table 4:

Table 4 shows the rice husk composition. [5]

Component	Content
Cellulose	42%
Lignin	19%
Hemicellulose	18%
Ash	17%

Production process of SGB

An overview of the different production process routes of second generation bioethanol can be seen in Fig. 4. The key processes during the production are: pretreatment of the substrate; hydrolysis of cellulose and hemicelluloses; fermentation of the monosaccharides; separation of lignin; and ethanol retrieval and purification [9]. The steps mentioned above will be described briefly in this section to provide a complete outline of the production process (especially the enzymatic hydrolysis route), although this work mainly covers the total chemical pretreatment and hydrolysis process. This section is based extensively on Ref. 23.

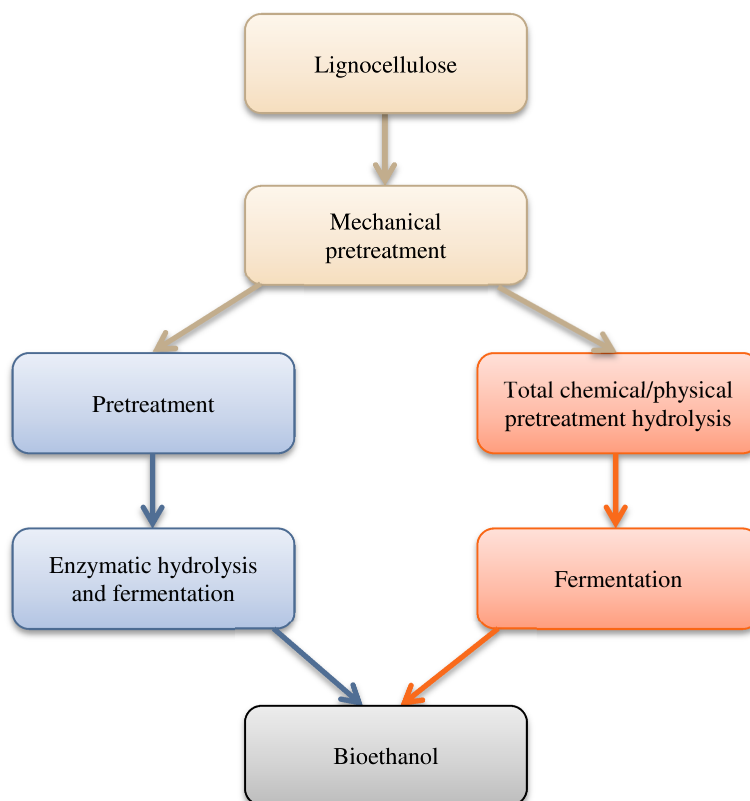


Figure 4 shows an overview of the different routes to choose for production of bioethanol from lignocellulosic materials. The main processes are pretreatment of the substrate; hydrolysis of cellulose and hemicelluloses; fermentation of the monosaccharides; separation of lignin; and ethanol retrieval and purification. There are two main routes, the total chemical/physical pretreatment hydrolysis route and the enzymatic hydrolysis route.

An initial step in the process is that the lignocellulosic material normally undergoes a mechanical pretreatment such as grinding and milling in order to increase the surface area of the substrate [6, 55]. To hydrolyze the cellulose and hemicelluloses to monosaccharides the mechanical pretreatment is followed by either a physical/chemical hydrolysis process (normally acid hydrolysis) or by pretreatment and then enzymatic hydrolysis [23, 56]. For these two process routes there are a broad variety of different methods to utilize and the selection depends on the type of raw material used, which organism to utilize for the fermentation and of course also on the cost [23].

The enzymatic hydrolysis can be divided into separate hydrolysis and fermentation (SHF), simultaneous saccharifiction and fermentation (SSF) and consolidated bioprocessing (CBP) [23]. For both SHF and SSF it is necessary to produce cellulases and hemicellulases. This is one major reason that hinders the enzymatic hydrolysis route to reach a commercial scale bioethanol production [23]. The enzyme production is expensive and should therefore be used in minimal amount, but the trade-off for the financial savings is a prolonged hydrolysis time [23]. However there is much space for improvement of this process [23, 57] and an example is to develop more

specific cellulases to reduce non-specific bindings to lignin and thus increase cellulose hydrolysis [57]. Minimization of distillation costs can also be done by striving to achieve optimal ethanol- and sugar concentrations during the production processes or to continuously remove ethanol to maintain non-inhibitory levels for the microorganisms [58].

The quantity of monosaccharides (pentoses and hexoses) generated is decided by which type of hydrolysis method and lignocellulosic material used [23]. Vital to the fermentation process is to strive for optimal ethanol yield from the wide diversity of available reducing sugars from the hydrolysis step in order for the process to be economical [23].

Formation of inhibitory molecules may occur during harsh hydrolysis conditions [23] from monosaccharides degradation [17, 59, 60, 61, 62, 63]. There are primary and secondary inhibitors. The primary inhibitors are compounds such as acetic acid (from the acetyl group bonds being broken during hemicelluloses decomposition), furfural (from pentose monosaccharides) and 5-hydroxymethyl furfural (5-HMF, from hexose monosaccharides) [62, 63]. If the severe hydrolysis conditions are further amplified secondary inhibitors like levulinic- or formic acids are produced [23] in the presence of water [64]. The inhibitors affect the microorganisms' performance considerably during the fermentation step. An extra detoxification step may therefore be employed to remove inhibitors prior to the fermentation by precipitation, extraction, chemically alter inhibitors to harmless forms or by addition of compounds that improves the fermentability of hydrolysates [65].

As the name states SSF achieves enzymatic hydrolysis and fermentation in one step while SHF does this in separate steps. The method may use one microorganism either naturally occurring or engineered to fit for this objective. This technique is often called CBP; however few microorganisms can directly convert lignocellulose to ethanol [23]. Another SSF technique uses specially engineered enzymes for enzymatic hydrolysis combined with an engineered microorganism that is able to produce ethanol from a wide variety of monosaccharides [23]. Although SSF normally involves fermentation of glucose, it may also include the pentoses fermentation and this can be denoted as simultaneous saccharification and co-fermentation (SSCF) [23].

The key benefit with SSF is that the monosaccharides can be fermented instantly to ethanol after the polysaccharides have been enzymatically hydrolyzed [23]. This leads to more efficient enzymatic activity since ethanol is less inhibitory to the enzymes than either glucose or cellobiose [6]. The SSF technique can consequently provide a more effective enzymatic hydrolysis in comparison to the SHF process and is predicted to cost less due to the need of only one equipment unit for the process [10, 23]. On an overall viewpoint SSF is considered to be more productive [23]. The major difficulty with the SSF process is that the optimal operating conditions vary for the hydrolysis and fermentation and this effects the efficiency. However the choice between SHF and SSF techniques depends on the lignocellulosic material and its properties [23]. In order for bioethanol to be a cheap product the process need to run in a non-sterile environment and contamination is therefore likely to be an issue [23]. Enzyme preparation is thought to be a possible cause for contamination problems [66]. According to literature [66] SSF is more inclined to contamination than SHF mainly due to the extended process times. Another reason why SHF is less liable to contamination is due to higher temperatures throughout the processes where few contaminants can survive [66].

Bioethanol production based on substrates containing the polysaccharide starch is a commercially feasible technique [23]. The starch is first pretreated by milling the substrate. After this it is mixed and cooked with water and finally hydrolyzed to dextrin with α-amylase [23]. After this the hydrolysis of dextrin to monosaccharides and fermentation is performed in a single process step [23]. A similar efficient routine, particularly during the final SSF process, is wanted for a production based on lignocellulosic materials [23].

Pretreatment and hydrolysis process

The pretreatment of lignocellulose is considered to be one of the most costly procedures when producing SGB [9]. The aim of pretreatment of lignocellulosic material is to make cellulose more accessible and increase digestibility for hydrolysis to take place by:

- Breaking up the cross-linked barrier of lignin and hemicelluloses, surrounding cellulose, in order to remove them.
- Increasing the porosity and surface area of the substrate.
- Decreasing the crystallinity of cellulose by disrupting the hydrogen bonds. [4, 6, 17, 23].

Key factors affecting enzymatic hydrolysis can be separated into the two categories, enzyme-related and structure-related factors [9]. Enzyme-related factors are investigated with the purpose of achieving total lignocellulosic degradation [9]. The development of enzyme mixtures is a priority today in order to significantly alter the complex structure of lignocellulose [67, 68]. Substrate-related factors affecting the hydrolysis are directly associated to the selected pretreatment method [9]. An efficient pretreatment method should be able to break the barriers of the lignocellulose [4] to solubilize and isolate one or more of the main components cellulose, hemicelluloses or lignin [39]. The remaining solid biomass can then be subjected to additional treatment such as chemical- and/or biological hydrolysis [39].

It has been observed [9] that less ordered (amorphous) regions of cellulose are more prone to hydrolysis while crystalline regions can be extremely resistant. The cristallinity is connected to the degree of polymerization [9]. Even though the role of the degree of polymerization is not totally understood it is thought to affect the hydrolysis [9]. Recent studies [9] have shown a more severe effect on cellulose chain length by xylan (hemicelluloses polysaccharide) removal than lignin removal. It is therefore important for a pretreatment to be able to decrease the crystallinity [69] and in turn reduce the degree of polymerization. It has however been reported [9] that these parameters alone do not affect the enzymatic hydrolysis rate.

As mentioned in the 'Lignocellulose and starch' section both lignin and hemicelluloses functions as a physical barrier and this prevents the cellulases access to cellulose for hydrolysis [70]. The presence of lignin may also function as a chemical barrier and decrease cellulose hydrolysis by binding to cellulases [9, 70]. Apart from cellulases binding to lignin, studies [69] have revealed that one key factor for decreased enzymatic hydrolysis is that cellulases are trapped in the pores of the substrate. Hemicellulose removal can increase the substrate's pore size which results in increased cellulase accessibility to cellulose [69]. Evidence has also revealed [6] that a reduction of the particle size of the substrate increases the surface area and therefore increases the cellulose hydrolysis. Cellulose access for the cellulases is affected by the available surface area and this is therefore considered an important factor influencing the hydrolysis rate [9]. Other than these factors the plant cell wall thickness affects the hydrolysis [9]. The knowledge of how the different pretreatment methods increases the hydrolysis rate of cellulose and hemicelluloses is off yet only limited [39].

There are numerous qualities to consider in order for a pretreatment method to be both effective and economically feasible [16]. The method should produce close to maximum sugar yield, with minimal amount of monosaccharide degradation, for a high variety of crops [9]. The process should run at minimum moisture content to reduce energy consumption and produce a sugar concentration above 10% to achieve a sufficient ethanol concentration after the fermentation process [6, 9]. It should preserve hemicelluloses to avoid sugar degradation as these compounds may inhibit the downstream hydrolysis and fermentation processes [6, 9, 71]. The lignin should also be recovered for possible conversion into valuable products [16]. The chemicals and the equipment used in the process should be of low-cost [72]. The reactors should be constructed with materials able to withstand the operational environment, with operating pressures held reasonable and the tank volume minimal [9]. Also, the waste from the pretreatment should not pollute, be toxic or of disposal challenge [9].

General overview of common pretreatment techniques

None of the existing pretreatment techniques can achieve all of the desired aims stated in the previous section, for every lignocellulosic material in an economically feasible way. This section provides information about some of the most common pretreatments studied to this day.

The different pretreatment methods can be broadly categorized as: physical-, chemical-, physico-chemical- and biologic pretreatment. Different combinations of the techniques have also been studied [9, 73].

Physical pretreatment consists of mechanical-, pyrolysis- and extrusion pretreatment [3, 9]. The mechanical pretreatment can be performed by a combination of chipping, grinding and/or milling [3, 9]. The power requirement of the pretreatment depends on wanted particle size and the substrate structure [3, 9]. The objective of mechanical pretreatment is to increase the surface area (by decreasing particle size) and decrease cristallinity (to reduce degree of polymerization) [9]. Mechanical pretreatment techniques take time, are energy intensive, and/or costly [3, 9] and should be avoided if possible.

Commonly used *chemical pretreatment* methods are acids, alkalines, ozonolysis, organosolvation and Ionic Liquids [3, 4, 9]. The latter is a relatively new lignocellulosic pretreatment method [9]. The acid and IL pretreatment will be explained in more detail in section 3.1 and 3.2.

Widely studied *physico-chemical pretreatment* methods are Steam Explosion (autohydrolysis), Ammonia Fiber Explosion (AFEX), Liquid Hot-Water pretreatment (LHW) and Wet Oxidation [3, 4, 9].

Biological pretreatment uses microorganisms such as brown-, white-, and soft-rot fungi that can decompose lignin and dissolve hemicelluloses [3, 9].

Every pretreatment technique has both benefits and drawbacks [39]. Some are more effective at disrupting part of the complex lignocellulosic structure and others work better in reducing cellulose cristallinity [39]. Dilute acid pretreatment is presently the most established as it may produce a small amount of inhibitors for the microorganisms during fermentation and can increase the hydrolysis rate considerably [39].

Theory on Methodology and Analysis Techniques

This section deals with the methodology and analysis techniques used in the work. A description of the two pretreatment methods (dilute acid and Ionic Liquid) is described in the first sections and is then followed by a short motivation of the variables studied in this work. The sequential sub-topic supplies theory for Central Composite Design (CCD), which is the tool used to optimize the pretreatment process based on the variables studied. The final section provides information about the methods used for the structural analyses.

Acid pretreatment hydrolysis

Acid pretreatment and hydrolysis usually generates a high amount of reducing sugars when used on lignocellulosic substrates and common acids are sulfuric-, nitric- and hydrochloric acids [3]. This pretreatment method commonly runs either under high temperatures together with a low acid concentration (called dilute acid pretreatment) or low temperatures with a high concentration (named concentrated acid pretreatment) [74]. The most common [4] and promising pretreatment technique is dilute sulfuric acid and it is under commercial development [17]. There are advantages in using sulfuric acid as it is effective and also cheaper in comparison to other acids [15]. Even though both sulfuric and hydrochloric acids are the most frequently utilized, phosphoric acid should not be neglected as it is less aggressive and the pretreatment can therefore result in a lower amount of formed inhibitors [63] which may disturb the fermentation process.

Concentrated acid hydrolysis normally operates at 30°C and results in yields approaching 100% of monosaccharides compared to total theoretical yield [2, 23, 75, 76] with a minimum degradation of monosaccharides [3, 23, 58]. An acid concentration above 60% w/V causes intracrystalline swelling of cellulose. The concentrated acid breaks the hydrogen bonds between the cellulose chains and alters the crystalline structure to totally amorphous [76]. When the acid concentration exceeds 75% w/V dissolution and decomposition of crystalline cellulose occurs [39].

Unlike concentrated acid the dilute acid pretreatment does not cause intracrystalline swelling of cellulose. The swelling that occurs is intercrystalline (similar to the effect of water) which is a requirement for any reactions to occur within the plant [39]. Dilute acid pretreatments can instead effectively improve enzymatic hydrolysis [77]. The pretreatment conditions with dilute acids can be divided into two groups: a temperature exceeding 160°C with a continuous-flow for solid loading in the range of 5-10% w/w; and temperatures below 160°C in a batch process for solid loading between 10-40% w/w [40]. The high temperature pretreatment leads to efficient removal of hemicelluloses and effectively catalyzes the hydrolysis of the polysaccharide to generate high yields of water soluble reducing sugars [40, 63, 78]. The lignocellulosic network consisting of covalent bonds, hydrogen bonds and van der Waals forces is disrupted [17]. The residue comprises of cellulose and lignin [40, 77].

The removal and hydrolysis of hemicelluloses therefore leads to cellulose being more accessible for enzymatic hydrolysis [78] due to bond disruptions and increased porosity of the substrate [39]. The lignin can be extracted with for example ethanol, butanol or formic acid [40]. If the extraction step is not performed the hydrolysis of cellulose will produce water-soluble reducing sugars together with insoluble residues consisting of lignin and unreacted substrate [40], as lignin may protect crystalline cellulose and preserve it [17, 80].

The harsh temperatures and the acidic conditions results in a major consequence. The conditions leads to rapid monosaccharide degradation and these compounds may inhibit microorganisms during the fermentation process [17, 56, 59, 60, 61, 62, 64, 63]. The different inhibitors have already been treated in the 'Production process of SGB'.

Drawbacks of the acid pretreatment hydrolysis technique are the energy demanding and costly recovery step for the acid [23, 40, 76]. It requires corrosion resistant equipment that is costly and it generates disposal wastes that pose environmental problems [19, 23, 39, 76]. Cellulose extraction and dissolution processes need to be both energetically and environmentally favorable and the traditional methods lack these requirements [18].

Ionic Liquids

Ionic Liquids (ILs) are organic salts that exist as liquids at room temperature to up too approximately 300-400°C [18, 19, 28, 81, 82]. Apart from being thermally and chemically stable they have a low volatility, low vapor pressure and unique solvent properties [18, 19, 83, 84, 85]. The physical and chemical properties are due to the arrangement of an anion and cation effecting the melting points, viscosity, hydrophobicity, hydrolysis stability and so on [2, 9, 86]. ILs normally consists of asymmetric and flexible ions, where the components are of varies sizes and shapes and a variety of dominating interactions [79].

Research results [87] have shown effective solubilization for the substrate switchgrass, where lignin and cellulose were dissolved and the polysaccharide could further be recovered. Articles [18, 81, 88, 89, 90, 91] report results from various ILs with dissolution of the components of lignocellulose and partial delignification for the substrates sugarcane bagasse, corn stalks, wheat, softwood and hardwood. Hydrophilic ILs containing chloride has been reported to break the hydrogen bonds of cellulose [18, 85]. The substrate's matrix is completely dissolved [85]. This efficient dissolution of cellulose leads to easy recovery of the polysaccharide (while lignin remains dissolved) during addition of an anti-solvent such as water, acetone, methanol or ethanol [18, 85, 87, 88, 92, 93, 94, 95] and 100% of the IL can be recovered to its original purity via flash distillation [19]. The degree of polymerization is the same as prior to the pretreatment, but both this and the crystallinity can be altered during the recovery [85]. The points stated above regarding a large amount of cellulose being able to be dissolved at rather moderate conditions and the fact that ILs can be recovered to the original cleanliness is what makes further studies on these solvents attractive for pretreatment of lignocellulose [19].

This new group of solvents is in theory "green", due to minimal air emissions and no formation of explosive gases [9, 19, 96], and has been reported [18] to offer an environmentally-friendly utilization of cellulose resources. Commercialization of ILs is progressing, but more extensive studies are required. The existing IL synthesis costs and total process cost data, together with physicochemical-, thermodynamic- and toxicology data (for both animal and marine life) need to be more thoroughly studied [18, 19, 28, 97]. Knowledge of the reaction mechanisms for the numerous lignocellulosic materials pretreated with the variety of ILs existing need to be further developed. The structural changes for the substrate together with the enzymatic hydrolysis step (to study possible cellulase inactivation) also need additional research [2, 16, 79, 98].

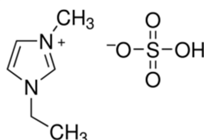


Figure 5 is a picture of the molecular structure of 1-Ethyl-3-Methyl imidazolium hydrogensulfate.

The structure of [EMIM]HSO_4 is illustrated in Fig. 5. There has not been found previous studies on pretreatment with this specific IL. [EMIM]HSO_4 is an acid with the pH of 1.

Factors studied

The two types of pretreatment hydrolysis methods studied in this work are sulfuric acid and the Ionic Liquid, 1-Ethyl-3-Methyl imidazolium hydrogensulfate ([EMIM]HSO_4). The variables varied for both these methods were: lignocellulosic substrate; temperature; time; and amount of substrate per liquid volume (solid loading).

The dilute acid pretreatment conditions stated in the 'Acid pretreatment hydrolysis' section was general. According to literature [7, 63] the sugarcane bagasse pretreatment has been conducted with conditions around 100-150°C with an acid concentration in the range of 1-10%. It has also been reported [99] that these operational conditions have led to formation of inhibitory compounds. The monosaccharide degradation rate is amplified by increased temperature and acid concentration [100].

An optimization experiment has been conducted with a 2^3 CCD with sulfuric acid as the pretreatment method followed by enzymatic hydrolysis on sugarcane bagasse. The independent variables were; temperature (112.5-157.5°C), time (5.0-35.0 minutes), sulfuric acid concentration (0.0-3.0% w/V) and the solid loading was a fixed parameter at 15% (w/w). The experiments were conducted in a volume of 200 mL. The highest hemicelluloses yield was 61.8% (w/w) of the maximum theoretical amount [47].

The pretreatment conditions (for dilute sulfuric acid) in this work has been varied from approximately 70-160°C, 5 min-2h, 0.5-10% w/V solid loading and 0.5-10% (w/V) acid concentration in many different CCDs. Previous articles with pretreatment conditions for sago waste and rice husk has not been acquired and no reports have been found on pretreatment of substrates with [EMIM]HSO_4. The IL was studied with two CCDs in the ranges of 70-140°C for 1-6h with the solid loading fixed at 4% w/V.

Response Surface Methodology & Central Composite Design, Optimization

The text written in this section is based on literature from the book 'Design and Analysis of Experiments' Ref. 101.

In a factorial design the variables in study is combined in all possible ways in one replicate of the experiment. There are typically 2 levels of each variable (low- and high point) in a 2^k design. These designs are suitable during the start of the experiments when the process is still relatively unexplored and several variables need to be examined. Its strength is that it consists of two levels of each variable and therefore requires the least amount of possible runs to combine the variables in all possible ways. The drawback of this method (in this work) is that it assumes a linear response (first-order model) from the variables in study.

Response surface methodology (RSM) composes of mathematical and statistical techniques for the purpose of modeling and analyzing problems. The aim is to optimize the response which is affected by variables with unknown relationship to both the response and each other. In this work the problem (or process) is the two pretreatments, the response is the achieved yield of reducing sugars per total weight and the variables are the substrates, temperature, time, solid loading and acid concentration. In other words RSM can find the optimum operating conditions (or suitable operating conditions) for the variables in the variable space.

The initial step in RSM is usually to search and find an appropriate estimation for the true functional relationship between the yield and the variables studied. If the yield lacks curvature (a linear function) in the variable space studied, the suitable estimated function is then the first-order model. This is normally experienced when the achieved yield is of distance to an optimum yield point. On the other hand, if curvature exists for the yield in the variable space this suggests presence of an optimum point and a second-order model may in this case be used to analyze and try to pinpoint the optimum point. The models work well as approximations for the true relationship of the variables in small regions, but rarely for the entire variable space.

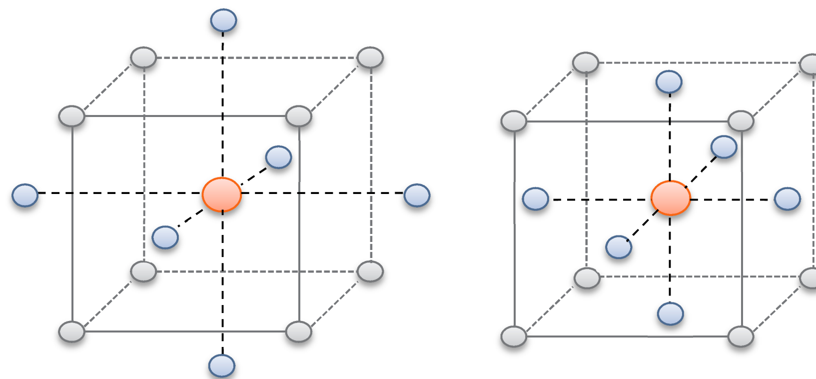


Figure 6 shows a spherical Central Composite Design to the left and a face-centered Central Composite Design to the right.

The Central Composite Design (CCD) is the most widely used design for fitting a second-order model. The design is typically employed when a 2^k design has revealed Lack of Fit for the first-order model. Usually the CCD is a 2^k factorial design (or fractional factorial design of resolution V) with n runs, $2k$ axial (or star) runs, and m center runs. The k is the number of variables studied. By adding axial points to the 2^k design with Lack of Fit the quadratic terms can be included and a second-order model employed. This is because the CCD has more independent runs than unknown parameters in contrast to the 2^k design (independent runs < unknown parameters) and the parameters can therefore be estimated for a second-order model.

Due to the fact that the optimum condition is unknown a design needs to provide equivalent accuracy of estimation in all directions of the variable space studied. The CCD is therefore made rotatable, that is, the variance of the predicted response is consistent at all points in the space that are at the same distance from the design center. The picture to the left in Fig. 6 illustrates a spherical CCD. Since the region of interest is normally spherical in a second-order model it makes sense to have rotatability as a design criterion to provide the equivalent accuracy of estimation. The amount of center runs is normally three to five. With these replications the variance can be monitored for the response.

If the region studied is cuboidal and not spherical the design can include axial points as in Fig. 6 to the right. This face-centered CCD needs less design- (three levels for the each variable) and center point runs compared to the spherical CCD. This may however give a worse approximation of the experimental error.

Dinitrosalicylic acid Method for analysis of reducing sugars

The reducing sugar content can be determined according to the method suggested in Ref. 102. Several parts of this section are written based on this reference.

The DNS reagent consists of dinitrosalicylic acid, phenol, sodium sulfite and sodium hydroxide. Dinitrosalicylic acid can react with reducing sugars and form 3-amino-5-nitrosalicylic acid in a 1:1 ratio. The amount of reducing sugars in a sample can be determined by studying the absorbance of 3-amino-5-nitrosalicylic acid at 540 nm with a spectrophotometer. The presence of phenol is to enhance the amount of color and sulfite to stabilize this color in presence of phenol. The role of sodium hydroxide is to reduce the action of the reducing sugar on dinitrosalicylic acid. After the sample has been treated with the reagent mixture and boiled in water, Rochelle salt (RS) is added to prevent the reagent from dissolving oxygen thus stabilizing the otherwise unstable color. The reagents in DNS interfere with the each other and this has proven to affect the absorbance of the sample studied. The optimal concentrations in regards to interference has been developed by Miller [102] and used in this research.

In the presence of the DNS reagent glucose loss has been experienced due to oxidation even though sodium sulfite was present in the reagent and the molecule has been shown to efficiently remove dissolved oxygen from liquids. The reason was due to interference caused by RS (and not observed in its absence), resulting in inefficient removal of dissolved oxygen and enhanced color intensity of the sample. RS has therefore been excluded from the traditional DNS reagent and is only added after the sample has been boiled and the removal of the dissolved oxygen and protection of the glucose has been ensured.

When the reagent contains a too high sodium hydroxide concentration both sugar degradation and increased color intensity has been observed. This method uses the concentration of sodium hydroxide showing optimum color intensity with minimal loss of glucose. The concentration of phenol (in the range studied in Ref. 102) does not affect glucose degradation, but absence of phenol gives significant decrease in color intensity. As for dinitrosalicylic acid concentration the maximum color intensity was achieved for the concentration used in this work and no glucose degradation could be observed.

This method can also be affected by molecules present in the substrate studied and this might cause interference such as glucose degradation and/or enhancement of the color intensity. As stated [102] the 3-amino-5-nitrosalicylic produced is not precisely corresponding to the sugar amount in the sample and different sugar types also appear to result in unlike color intensities. These factors, together with the presence of sodium hydroxide, complicate the analyses of the yield obtained with the DNS method.

The major strength with the DNS method lies in its convenience to study several tests in a short period of time. If the factors stated above are considered the chance of misinterpreting the results might be decreased.

Structural study of sago waste

In this section a short description regarding the structural analysis methods used to study sago waste will be provided. The analysis methods are Fourier Transform Infrared spectroscopy, X-ray Diffraction and Scanning Electron Microscope.

The equipment used in *Fourier Transform Infrared (FTIR) spectroscopy* is a single beam infrared unit, an interferometer and a detector. The spectrum is collected over the whole wavelength range and FTIR spectroscopy offers much faster data collection with increased spectral resolution in comparison to traditional spectrometers [103].

With this type of spectrophotometer it is possible to study the bonding mechanism as the vibrations of the molecules can be correlated to the molecules symmetry. In the IR wavelengths between 400 and 4000 cm^{-1} there are several essential molecular bonds that can be detected [103]. In this work the method is used to study the band intensities in the spectra both before and after the chemical pretreatments and make an attempt to investigate and compare the structural changes of the sago waste.

The *X-ray diffraction (XRD)* was used to study if the cellulose cristallinity changed after the chemical pretreatments of sago waste. The cristallinity index (CrI) has also been calculated in some articles [104, 105, 106] and is a measurement of the crystalline material in the sample. The CrI is reported as percentage and can be used to study the variation in cellulose cristallinity prior to and after pretreatment.

Scanning Electron Microscope (SEM) gives both objective and subjective information. Its power lies in creating an image resembling the macroscopic world of micro-sized samples when the samples studied are complex and problematic to analyze by completely objective approaches. [107]. In this work SEM images have been created in different magnifications in order to visualize the structural effects of the chemical pretreatments.

Material and Methods

A typical experiment during the planning and work is described in more detail below. In short the experiments consisted of the following steps: set-up of the experimental design with the ranges of the variables studied; preparation of the samples; pretreatment and hydrolysis of the samples; treatment with DNS reagent; and finally analyze the samples with spectrophotometry. Final experiments were studied with High-Performance Liquid Chromatography (HPLC) instead of using the DNS method with spectrophotometry.

Besides studying the optimization of the response (yield), the composition of sago waste was also decided and can be seen in Table 3. Sago waste's structural changes were studied before and after chemical pretreatment with FTIR spectroscopy, XRD and SEM. This section provides a description of the material and methods used to study this.

Design of Experiments

Design of Experiments with variables and ranges were initially decided based on literature data and later based on the experiments conducted. By using the software Design Expert the experimental design was generated and the run order was randomized. Temperature, time, solid loading (amount of substrate/volume liquid) and liquid concentration were four numerical factors studied in this work.

Pretreatment and hydrolysis

Sago waste, sugarcane bagasse and rice husk were ground and sieved (physical pretreatment) to achieve a particle size of 250-500 µm for sugarcane and 500 µm for sago and rice.

Sago waste, sugarcane bagasse or rice husk was weighed and emptied into a 10 mL glass test tube. Sulfuric acid or ionic liquid of 1 mL were added to the test tube. The addition of either acid or IL was fixed to 1 mL while the amount of substrate varied depending on which percentage of the solid loading that was the set point. This is illustrated in the image to the left in Fig. 7. The samples were then placed in a water- or oil bath at the certain temperature and time according to the experimental design (the picture in the middle in Fig. 7). No randomization was performed at this step due to the different ranges of temperatures studied and samples were heated in order of ascending temperatures.

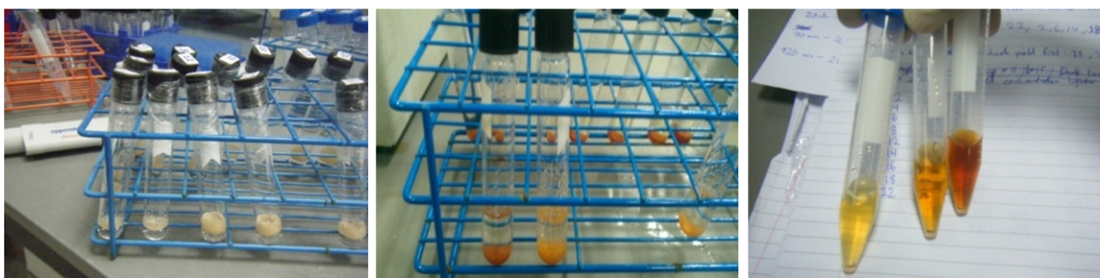


Figure 7 shows experiments prior to the heat treatment when the substrate sago waste had been soaked in dilute sulfuric acid (left). Samples after the heat treatment are illustrated in the middle picture and the picture to the left shows samples neutralized with sodium hydroxide and prepared for analysis with spectrophotometry.

After the heat treatment the sample was cooled down in tap water and diluted with 2 mL ultra pure water (dilution 1:3). It was centrifuged at 3500 rpm for 20 minutes at 25°C to separate the supernatant from the fluid. The liquid phase was transferred to a 14 mL falcon tube with a glass pipette. Sodium hydroxide with a concentration of 5M was added to the sample to neutralize it from acidic- to alkaline condition. The image to the right in Fig. 7 shows samples that had been neutralized. The amount of sodium hydroxide drops added varied depending on if the test contained IL or dilute acid and also on the acid concentration of the sample. This additional dilution was not considered although the sample volume increased. The calculated response (yield) will therefore be slightly lower than the actual (or true) response. The neutralization of the sample is important in order to have a color development of the sample when it is treated with DNS reagent (see ‘Dinitrosalicylic acid Method for analysis of reducing sugars’ section).

DNS reagent

The dinitrosalicylic acid reagent was prepared according to the Miller method [102]. The reagent was prepared in a batch of 1L with the composition according to Table 5. The batch was prepared without sodium sulfite, wrapped in folia and stored in 4°C. If sodium sulfite is present in the batch and stored over time oxidation of the molecule may occur. Sodium sulfite was instead added just prior to experimental runs.

Table 5 shows the recipe for DNS reagent used in this work.

Compound	Amount
Dinitrosalicylic acid	1% w/V
Phenol	0.2% w/V
Sodium sulfite	0.05% w/V
Sodium hydroxide	1% w/V

After the sample was neutralized with addition of sodium hydroxide, 0.5 mL of the sample in the falcon tube was transferred to a glass test tube containing 1 mL ultra pure water. This was a second 1:3 dilution of the sample. The DNS reagent was added in a volume of 3 mL to the glass test tube (another 1:3 dilution) and boiled for 5 minutes. Fig. 8 shows a picture of four boiled samples containing DNS reagent. The sample was cooled down in water bath and 1 mL of 40% Rochelle salt (potassium sodium tartrate) was added to stabilize and maintain the color change.

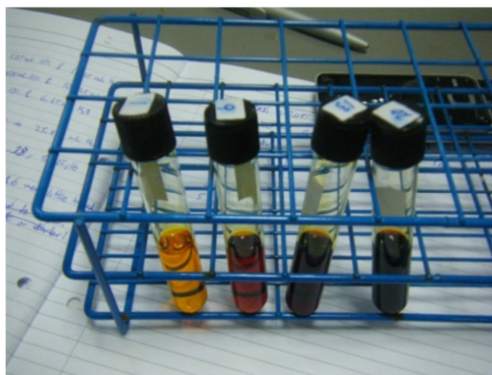


Figure 8 displays four boiled samples with DNS solution.

The procedure above was also used on standard samples where the sugar (glucose) concentrations were known. This was done to create a glucose standard curve. From this curve a first order function was calculated. With this function a quantification of reducing sugars in the samples, that had undergone pretreatment and hydrolysis, was possible. However the dilutions of the standard samples were different from the test samples. Instead of transferring 0.5 mL sample to 1 mL ultra pure water in a falcon tube, 1.5 mL of standard sample was transferred to an empty falcon tube. Beside this the following procedure for the standard samples was the same as for the test samples. Once RS was added and the sample had cooled down spectrophotometry was used to study the absorbance of the sample.

Spectrophotometry

The wavelength of the spectrophotometer was set to 540 nm and blanked with ultra pure water. 0.2 mL of the sample, which had been treated with DNS reagent, was transferred to a glass cuvette containing 2.5 mL ultra pure water. The absorbance was read and noted. If the absorbance was out of range of the glucose standard curve the dilution of the sample was adjusted accordingly. With knowledge of the dilution factor and absorbance of the sample it was possible to quantify the amount of reducing sugars by usage of the standard curve function.

Composition determination

Sago waste composition was characterized according to the methods in “Determination of Structural Carbohydrates and Lignin in Biomass” and “Determination of Total Solids in Biomass and Total Dissolved Solids in Liquid Process Samples” [20, 21].

The moisture content was determined according to [20]. Three physically pretreated substrates of 50 mg sago waste was dried at 105°C on three pre-dried aluminum foil boats until constant weight was achieved. The moisture content was calculated with Eq. 1. The mean of the three samples was then calculated.

$$\%Moisture = 100 - \frac{Weight_{dry\ alum.\ boat+sample} - Weight_{dry\ alum.\ boat}}{Weight_{sample}} * 100 \quad (1)$$

The procedure included addition of 3 mL of 72% (w/w) sulfuric acid added to five samples of 300 mg physically pretreated sago waste in 300 mL Erlenmeyer flasks and placed in a water bath for one hour at 30°C. Every 5 minutes the samples were shaken manually without removal from the water bath. After 60 minutes the samples were diluted with 84 mL deionized water and mixed thoroughly. A set of five sugar recovery standards with known sugar concentrations were prepared of D-(+) glucose, D-(+)xylose, D-(+)galactose, L-(+)arabinose and D-(+)mannose. These were prepared as a correction for sugar hydrolysis of the samples during dilute acid hydrolysis. The standards were prepared with the same acid concentration as for the samples. The Erlenmeyer flask tops were covered with paper towel and autoclaved with the sugar recovery standards for one hour at 121°C. The filtrate was separated from the residue by vacuum filtration. The acid insoluble lignin and ash content were determined with the residue by rinsing the solid with minimum of 50 mL deionized water. The residue was dried in already weighted crucibles at 105°C until constant weight. The crucible and residue was further dried at 550°C until constant weight. Equation 2 calculates ash content while equation 3 is the calculation for acid insoluble lignin (AIL):

$$\%Ash = \frac{Weight_{crucible+AIR} - Weight_{crucible}}{ODW_{sample}} * 100 \quad (2)$$

$$\%AIL = \frac{(Weight_{cruc.+AIR} - Weight_{cruc.}) - (Weight_{cruc.+ash} - Weight_{cruc.})}{ODW_{sample}} * 100 \quad (3)$$

AIR in the equation above is short for acid insoluble residue which is the weight of the sample after drying at 105°C.

The filtrates from after the autoclave were transferred to 50 mL Erlenmeyer flasks and neutralized to approximately pH 7 with calcium carbonate. A calibration curve were prepared with the concentrations 0.5, 1, 2, 3 and 4 g/L of the six sugars D-cellobiose, D-(+)glucose, D-(+)xylose, D-(+)galactose, L-(+)arabinose, D-(+)mannose. The neutralized samples, calibration curve sugars and sugar recovery standards (CVS) were filtered through a 0.2 µm filter into an autosampler vial. The samples were analyzed with Waters HPLC with the conditions in Table 6.

Table 6 Consists of the HPLC conditions used to analyze the composition of sago waste.

Injection volume	10 µL
Mobile phase	Filtered deionized water
Flow rate	0.6 mL/min
Column temperature	75°C
Upper/lower temperature	70°C/80°C
Internal temperature	35°C
Detector	Differential Refractometer

The equations 4-8 were used to calculate the sugar percentage.

$$\%CVS\ recovery = \frac{conc.\ detected\ by\ HPLC, mg/mL}{known\ conc.\ of\ standard, mg/mL} * 100 \qquad (4)$$

In order to calculate the amount of sugar lost during autoclave the sugar recovery standard values were used and a mean value could be calculated.

$$\%R_{Sugar} = \frac{conc.\ detected\ by\ HPLC, mg/mL}{known\ conc.\ of\ sugar\ before\ hydrolysis, mg/mL} * 100 \quad (5)$$

$$C_x = \frac{C_{HPLC} * dilution\ factor}{\%R_{avg.sugar}/100} \qquad (6)$$

The dilution factor in the formula was any dilution made prior to the HPLC run. C_x can later be corrected by calculation of the average of each sample to get C_{corr}.

$$C_{anhydro} = C_{corr} * Anhydro\ correction \qquad (7)$$

The anhydro correction factor is 0.9 (as proposed in Ref. 20) for glucose and was used to translate the concentration from monomer sugars to polymeric sugars.

$$\%Sugar_{ext\ free} = \frac{C_{anhydro} * V_{filtrate} * \frac{1g}{1000mg}}{ODW_{sample}} * 100 \quad (8)$$

$V_{filtrate}$ is the total volume of the sample and in this case 87 mL.

Structural analysis

The FTIR-FIR spectrometer was a Perkin Elmer (Massachusetts, USA). The samples were mixed with potassium bromide (KBr) and the mixture was pressed into a disc and scanned in the range of 4000-450 cm^{-1} with a resolution of 4 cm^{-1}.

The XRD diffractogram were acquired using D8 Advance X-Ray Diffractometer (Bruker AXS, USA). The scanning range were 10-80° (2θ) with a step size of 0.02° and step time of 1 s. The scans were done at 40kV and 40mA under ambient temperature.

The scanning electron microscope were of the type Quanta 200 FESEM, FEI, (USA). The samples were mounted to aluminum sample stubs with double sided carbon tape and images were obtained with 10kV acceleration voltage.

The samples were sent to the Technical Service Unit of Combicat Laboratory for Institute of Postgraduate Studies, University of Malaya for the analysis of the sago waste.

Results and discussion

In this topic the results of the experiments conducted will be treated and discussed. The results are presented in chronological order of the practical work.

Initial experiments: A comparison of the three substrates

In the initial experiments the setup consisted of a comparison between the three different substrates sago waste, sugarcane bagasse and rice husk for two separate experimental designs of dilute sulfuric acid and 1-Ethyl-3-Methyl imidazolium hygrogensulfate. The procedures of the experiments were performed according to the description in the Materials and Methods topic.

The standard glucose curve can be observed in Fig. 9. This curve has been used throughout this whole work to measure the amount of reducing sugars of the samples. The y and x in the equation (in the top left corner Fig. 9) corresponds to the absorbance and concentration, respectively. After the absorbance has been determined for the sample, with spectrophotometry, the unknown concentration (x) can be solved from the equation. By considering the dilution factor the total amount of reducing sugars in the sample can be determined.

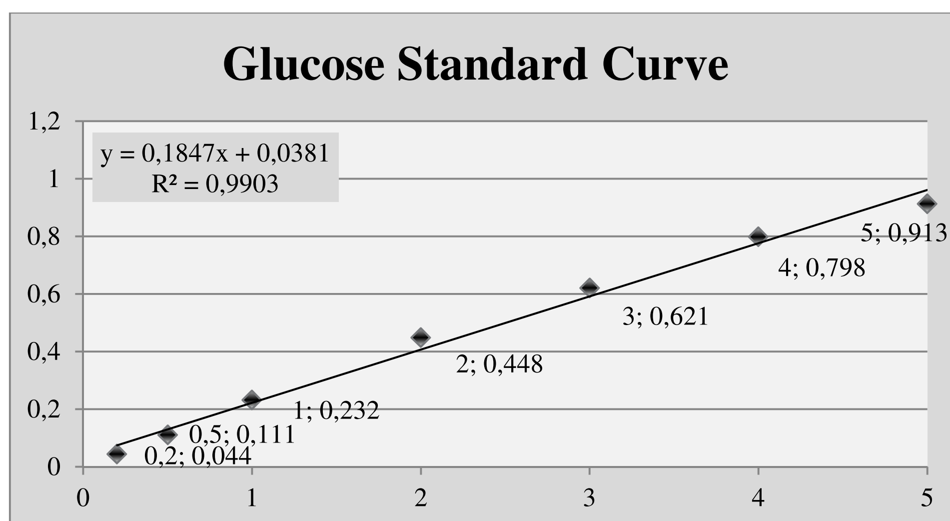


Figure 9 shows the graph of the glucose standard curve used extensively throughout this work. The pre-determined concentration of the glucose ladder can be seen on the x-axis. On the y-axis the absorbance results are illustrated after the standards had been treated with DNS reagent and boiled for five minutes. In the top left corner the equation and the R^2 generated from the curve can be seen.

Dilute sulfuric acid (H_2SO_4) pretreatment

The variables and ranges studied in the first sulfuric acid pretreatment experiment were temperature (70-130°C), time (0.5-1.5h), substrate (sago waste, sugarcane bagasse and rice husk) and sulfuric acid concentration (2-5%) in a 2^3*3^1 mixed full factorial design (see Table 7). The solid loading was fixed at 4% w/V i.e. 40 mg substrate/1 mL sulfuric acid. The setup did not include replicates of the samples except for 9 center points.

Table 7 includes the 2^3*3^1 experimental design with dilute sulfuric acid pretreatment. Temperature, time and acid concentration were varied in two levels while the substrate was varied in three levels.

Sulfuric acid pretreatment (H_2SO_4)			
Variables/Levels	**Low (-)**	**High (+)**	
Temperature	70°C	130°C	-
Time	0.5h	1.5h	-
Substrate	Sugarcane bagasse	Sago waste	Rice husk
Conc. H_2SO_4	2%	5%	-

The results after the sulfuric acid pretreatment showed unrealistically high yields for individual experimental runs. The Analysis Of Variance-table (ANOVA) with calculations of significance for the treatments was therefore not used. The high yields were potentially due to experimental errors during the dilution of the samples. From the model conclusions could still be made regarding ranges of variables and which substrate that potentially may generate highest yield.

The center point runs with sago waste pretreated for 1h with the temperature of 100°C and an acid concentration of 3.5% gave the three following yields of 111%, 79% and 132% reducing sugars/total weight with the latter being the highest one in this design. Other high yield experiments were with the conditions of 130°C for 30 min with 2% acid concentration for sago waste resulting in 96.5% yield. The trend was that sago waste experiments gave higher yields than sugarcane bagasse and sugarcane bagasse generally resulted in higher yields than rice husk. Rice husk experiments had a low yield overall with the highest at 23.7% at one of the center points.

Although no statistical analysis and calculation were possible for the first design, conclusions regarding ranges of the variables for the second design could be decided. Rice husk was not further studied in the research with sulfuric acid pretreatment due to the lower yields achieved compared to both sago waste and sugarcane bagasse. Sago waste showed high yields (even if they were unrealistic) at two different range conditions. The first range condition is described above with a temperature of 100°C and at a time around 60 minutes with acid concentration of 3.5%. The second was for 130°C, for 30 min with acid concentration of 2%. The variables seemed to correlate with one another where lower temperatures required longer hydrolysis time and higher acid concentration and vice versa. The sulfuric acid pretreatment of sago waste and sugarcane bagasse would be further studied at the two conditions stated above.

1-Ethyl-3-Methyl imidazolium hydrogensulfate ([EMIM]HSO_4) pretreatment

The experimental plan for the Ionic Liquid, 1-Ethyl-3-Methyl imidazolium hydrogensulfate, was a 2^2*3^1 mixed full factorial design and can be seen in Table 8. The substrate loading was fixed at 4% w/V and the design was conducted with one replication.

Table 8 includes the 2^2*3^1 experimental design with [EMIM]HSO_4 pretreatment. Temperature and time were varied in two levels while the substrate was varied in three levels.

1-Ethyl-3-Methyl imidazolium hydrogensulfate ([EMIM]HSO_4)			
Variables/Levels	**Low (-)**	**High (+)**	
Time	2h	6h	-
Temperature	80°C	140°C	-
Substrate type	Sugarcane bagasse	Sago waste	Rice husk

The 1-Ethyl-3-Methyl imidazolium hydrogensulfate ([EMIM]HSO_4) pretreatment showed small difference between the different pretreatment conditions. There was no clear trend regarding time, temperature or the substrate and the yields were uniform. The variation between the replicates was also high considering the low yields obtained. The highest yield obtained was 17.4% with the replicate at 14.3% at 80°C for 2h with sugarcane bagasse. Second best experiment was for sago waste at 140°C for 2h with 16.6% yield with the replicate at 14.1%. The yields for the rice husk experiments were all below 4%. Due to the low yields of the experiments with rice husk no further studies were conducted for the substrate during Ionic Liquid pretreatments.

The variable ranges time and temperature would be shorter and lower for the second [EMIM]HSO_4 pretreatment experimental design. As mentioned in the section 'Production process of SGB' high temperatures for longer times (harsh hydrolysis conditions) may result in monosaccharide degradation.

Sago waste and sugarcane bagasse comparison

Pretreatment of rice husk gave a low yield of reducing sugars and further research on the substrate was not conducted. The following experiments were performed according to Central Composite Designs (CCDs) to fit second-order models and not according to full factorial experiments used in the initial experiments. Two new experimental set-ups were executed for both dilute sulfuric acid and [EMIM]HSO_4 and the substrates of interest were sago waste and sugarcane bagasse.

Dilute H_2SO_4 pretreatment

The second experiment with sulfuric acid pretreatment was performed with response surface methodology (RSM) and central composite design (CCD) with the studied factors in Table 9. The solid loading and acid concentration was fixed at 4% w/V and 3.5% w/w respectively.

Table 9 The central Composite Design for dilute sulfuric acid was executed for the three variables temperature, time and substrate.

Sulfuric acid pretreatment (H_2SO_4)		
Variables/Levels	**Low (-)**	**High (+)**
Temperature	85°C	115°C
Time	0.75h	1.75h
Substrate	Sugar	Sago

The Analysis Of Variance-table (Table 10) shows that all the three main treatments have an effect on the yield.

Table 20 The ANOVA-table shows the significance of the model, variables and Lack of Fit in this experiment.

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	7262.134	5	1452.427	30.14842	< 0.0001	significant
Temp	826.1664	1	826.1664	17.14896	0.0005	
Time	286.8066	1	286.8066	5.953323	0.0241	
Subst.	4183.87	1	4183.87	86.84574	< 0.0001	
A^2	1143.117	1	1143.117	23.72799	< 0.0001	
AC	822.1741	1	822.1741	17.06609	0.0005	
Residual	963.5176	20	48.17588			
Lack Of Fit	880.1142	12	73.34285	7.035002	0.0050	significant
Pure Error	83.40336	8	10.42542			
Cor Total	8225.652	25				

The top five predicted optimizations were the following (Table 11):

Table 3 The predicted optimal experiments based on the results obtained are illustrated below. It appears that a higher temperature for longer times with sago waste will generate the highest response (yield).

Number	Temp	Time	Substrate	Response	Desirability
1	113.25	102.86	Sago	76.6109	1.000
2	108.21	96.35	Sago	75.1889	1.000
3	108.75	97.34	Sago	75.4794	1.000
4	108.22	100.77	Sago	75.8158	1.000
5	113.22	96.16	Sago	75.6695	1.000

The higher yields are achieved with higher temperatures for longer time with sago waste. However this model has a significant Lack Of Fit which shows that the quadratic model does not fit properly.

[EMIM]HSO_4 pretreatment

For pretreatment with 1-Ethyl-3-Methyl imidazolium hydrogensulfate the setup is shown in Table 12. The experimental design was a CCD with the substrate loading fixed at 4%.

Table 4 The CCD for the Ionic Liquid pretretreatment can be seen below where temperature, time and substrate were variables varied.

1-Ethyl-3-Methyl imidazolium hydrogensulfate ([EMIM]HSO_4)		
Variables/Levels	**Low (-)**	**High (+)**
Temperature	70°C	120°C
Time	1h	4h
Substrate	Sugar	Sago

The ANOVA-table (Table 13) follows below:

Table 5 ANOVA-table for pretreatment results with [EMIM]HSO_4 follows below.

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	143.5283	6	23.92138	15.0823	< 0.0001	significant
Temp	11.07908	1	11.07908	6.985299	0.0160	
Time	4.809385	1	4.809385	3.032292	0.0978	
Subst.	109.9254	1	109.9254	69.30738	< 0.0001	
AB	0.676048	1	0.676048	0.426245	0.5217	
AC	15.12953	1	15.12953	9.539086	0.0060	
BC	1.908837	1	1.908837	1.203512	0.2863	
Residual	30.13507	19	1.586056			
Lack of Fit	29.28488	11	2.662262	25.05121	< 0.0001	significant
Pure Error	0.850182	8	0.106273			
Cor Total	173.6633	25				

The temperature and time treatments were significant for the experiment and the software suggested the top three optimizations which follow below in Table 14.

Table 6 The predicted optimal experiments generated from the software Design Expert based on the experiments conducted in the CCD.

Number	Temp	Time	Substrate	Response	Desirability
1	70.00	240.00	Sugar	7.1177	0.868
2	120.00	60.00	Sago	3.57308	0.415
3	120.00	231.99	Sago	3.40522	0.394

With the low yield generated this particular Ionic Liquid appears to be a non-appropriate pretreatment hydrolysis method and further optimizations with CCD appears to be ineffective.

Comments on the yields obtained

Although sago waste generated higher yield when compared with both sugarcane bagasse and rice husk when analyzed with the DNS method the result can still be discussed. The yield was calculated based on weight of reducing sugars divided by total weight of the substrate. This can be considered as an unfair comparison. The sago waste is much richer in cellulose and starch, based on the compositional determination performed in this research (See 'Composition determination' and Table 3), compared to both sugarcane bagasse and rice husk. The sugarcane bagasse and rice husk composition (illustrated in Table 4 & 5) was not determined in this research and instead taken from literature.

Sago waste also contains a lower amount of lignin and as already described (in 'First Generation Bioethanol Vs. Second Generation Bioethanol' section) lignin is not hydrolyzed to monosaccharides as it is not a polysaccharide. Since both lignin and hemicelluloses works as protective layers for cellulose against hydrolysis it can be speculated that the higher concentrations of these components the harder it becomes to pretreat and hydrolyze the substrate. It also depends on other factors and some of these factors have been treated in this work, for example in the 'Pretreatment and hydrolysis process' part.

Reports state that hemicelluloses are easier to hydrolyze than cellulose and therefore hemicelluloses content should also be considered when calculations of the yield are made. Therefore a more fair yield calculation than in the two previous sections can be done based only on cellulose and hemicelluloses content. This was not done after the experiments had been analyzed and a new experiment was set up only based on total substrate weight. This was first done after all experiments in this research had been finished.

As stated the composition of sugarcane bagasse and rice husk was not determined in this work. As compositional differences exist between the same substrate, depending on several factors already discussed in 'Substrates' section the correct way would be to do the composition determination of the two substrates as well. Another reason why determination of composition is important is that DNS does not discriminate between different types of reducing sugars present in the sample. Although different types of reducing sugars may cause different color intensities when treated with DNS reagent (and be an error to the yield calculation in that sense) it is probably impossible to understand which types of reducing sugars that exist in the mixed sample based solely on the absorbance.

Since the experiments where rice husk was studied had dilution errors a calculation of rice husk based on reducing sugars per theoretical amount of reducing sugars will not be attempted. However an attempt will be made for the sugarcane bagasse and sago waste yields with experiments based on the experimental design described in the previous section.

From the Design of Experiment in the previous section the highest yield of sugarcane bagasse was 46.97% reducing sugars/total weight and 74.98% for sago waste. Sago waste composes of 78% cellulose while sugarcane bagasse has been reported to compose of 35-45% cellulose and 26-36% hemicelluloses. The cellulose degradation to glucose has to consider an anhydro factor which is set 0.9 [20]. The anhydro factor varies depending on the monosaccharide type and hemicelluloses compose of a variety of monosaccharides, but for simplicity the glucose anhydro correction factor of 0.9 is used. The lowest reported cellulose and hemicelluloses content of sugarcane bagasse will be used in this yield comparison from Table 4. Eq. 7 was used to translate the concentration of reducing sugars (glucose) to percentage of cellulose and hemicelluloses for both sago waste and sugarcane bagasse:

$$C_{SCB\ cell.+hemicell.} = C_{SCB\ red.sugars} * Anhydro\ correction = 19.022\ \frac{g}{L} * 0.9 = 17.12\frac{g}{L}$$

By assuming same properties of all components in the substrate the cellulose and hemicelluloses can be based on the composition percentage.

$$C_{Theoretical\ SCB} = Conc._{Substrate} * \left(cellulose_{percent.} + hemicellulose_{percent.}\right) = 40.2\frac{g}{L} * (0.35 + 0.262) = 26.6\ \frac{g}{L}$$

SCB is short for sugarcane bagasse. By dividing $C_{SCB\ cell.+hemicell.}$, with $C_{Theoretical\ SCB}$ the yield after dilute sulfuric acid pretreatment of sugarcane bagasse is calculated to 64%.

The same calculations as above can be done for sago waste and the yield will be 86.5% of hydrolyzed polysaccharides (cellulose) per total amount of polysaccharides. Hence, further studies on the optimization of sago waste are strengthened by basing the calculations on total amount of reducing sugars as well as on total weight.

However as already stated this calculation has several flaws including unknown sugarcane bagasse composition of this specific substrate, assumption of same density of every component in the substrate and assumption on the anhydro correction factor based only on glucose as a reducing sugar for sugarcane bagasse.

Calculation of the yield based on total weight may not be fair as substrates vary in the amount of possible reducing sugars. But it may also not be fair to only base calculations on total theoretical amount of reducing sugars when a substrate rich in cellulose and hemicelluloses may generate twice as much amount of monosaccharides and in the final process double the amount of bioethanol compared to a substrate with low amount of cellulose and hemicelluloses but with a similar yield based on theoretical amount of reducing sugars.

Further studies of sago waste with sulfuric acid pretreatment

Although the previous models for both the sulfuric acid and Ionic Liquid pretreatment showed a significant Lack Of Fit it can be concluded that sago waste gave highest amount of reducing sugars per total weight with the sulfuric acid pretreatment. Further studies on this pretreatment and substrate were conducted. By looking at the second experiment's predicted optimal conditions (see 'Dilute H_2SO_4 pretreatment' part) the highest yields are achieved during pretreatment at the range around 110°C for 100 minutes and this was further studied. The objective was to study the yield in detail and how the variables affected it around this space. From experiment one the conditions 130°C for 30 min with 2% acid concentration gave a high yield and further study around this space were needed.

Therefore the continued studies with sago waste would be split up between two different ranges. Several CCDs were set-up and ran and the variables temperature, time, acid concentration and solid loading were varied to cover a wide range in order to improve knowledge of the wide yield space. With the knowledge gained from these experiments a final set-up could be performed to optimize the yield and achieve a significant model with a non-significant Lack Of Fit.

Composition determination of sago waste

In the section 'Composition determination' the procedure is described on how to decide which components sago waste consists of. The composition determination was done in 4 replicates and has already been provided in Table 3. In the subsequent section (see Table 18) the yield is also calculated based on total theoretical amount of cellulose and could be provided because the compositional analysis had been done. Two yields is therefore reported in Table 18 (column 6 and 8) based on reducing sugars per total weight and reducing sugars per total theoretical amount of glucose in sago waste.

The amount of acid soluble lignin was not calculated as the formula required the absorptivity of sago waste. The absorptivity has not been found during literature studies. However, total lignin percentage does not appear to exceed 11%, even if the remaining undecided portion (5%) of sago waste is assumed to be acid soluble lignin (Table 3).

Optimization of the process with CCD

In Table 15 the CCD with face-centered axial points with 6 center points were performed.

Table 7 This table shows the variables and ranges varied for the face-centered axial CCD for pretreatment of sago waste with dilute sulfuric acid.

Sulfuric acid pretreatment (H_2SO_4)		
Variables/Levels	**Low (-)**	**High (+)**
Temperature	85°C	145°C
Time	20 min	120 min
Acid concentration	1.25%	5.25%
Substrate loading	1.5%	6.5%

Analysis Of Variance Table for the face-centered CCD can be seen in Table 16.

Table 8 illustrates the ANOVA for the face-centered CCD.

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	16715.93	14	1193.995	26.79587	< 0.0001	significant
Temp	4057.977	1	4057.977	91.06989	< 0.0001	
Time	0.359194	1	0.359194	0.008061	0.9296	
Acid	71.53886	1	71.53886	1.605488	0.2244	
Subst.	24.36474	1	24.36474	0.546798	0.4710	
A^2	2219.242	1	2219.242	49.80464	< 0.0001	
B^2	9.818624	1	9.818624	0.220351	0.6455	
C^2	39.68142	1	39.68142	0.890538	0.3603	
D^2	17.73711	1	17.73711	0.39806	0.5376	
AB	2703.146	1	2703.146	60.66452	< 0.0001	
AC	2016.077	1	2016.077	45.24519	< 0.0001	
AD	46.27816	1	46.27816	1.038583	0.3243	
BC	57.34464	1	57.34464	1.286939	0.2744	
BD	9.093888	1	9.093888	0.204087	0.6579	
CD	6.421901	1	6.421901	0.144122	0.7095	
Residual	668.384	15	44.55893			
Lack of Fit	577.6138	10	57.76138	3.181736	0.1067	not significant
Pure Error	90.77022	5	18.15404			
Cor Total	17384.32	29				

The ‘Pred R-Squared’ of 0.7870 is in reasonable agreement with the ‘Adj R-Squared’ of 0.9257. From the ANOVA it can be seen that the temperature has a significant effect on the yield in the studied space.

The 3D graph of the response space can be seen in Fig. 10. The slope has the shape of a saddle-point. By looking at the yield from reducing sugars per theoretical amount of reducing sugars the model slope is close to maximum possible yield.

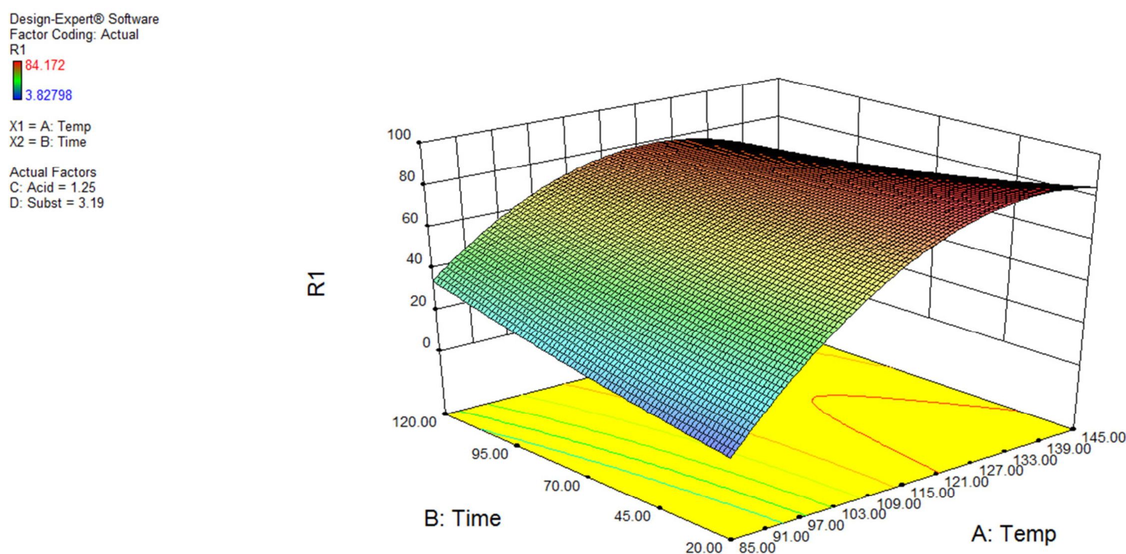


Figure 10 illustrates the response curve for the results from face-centered CCD

The predicted optimal runs can be seen in Table 17. Note that these yields are predicted from the results from the CCD illustrated above (in Table 15 and 16) by the software Design Expert and not conducted practically.

Table 9 This table consists of information of predicted optimal experiments based on the model above. The result of executing experiments with these variable conditions is predicted to be those listed in the column below Red. sug / tot. w. which is short for reducing sugars/total weight. That response was translated to reducing sugars/theoretically total glucose (see column to the far right).

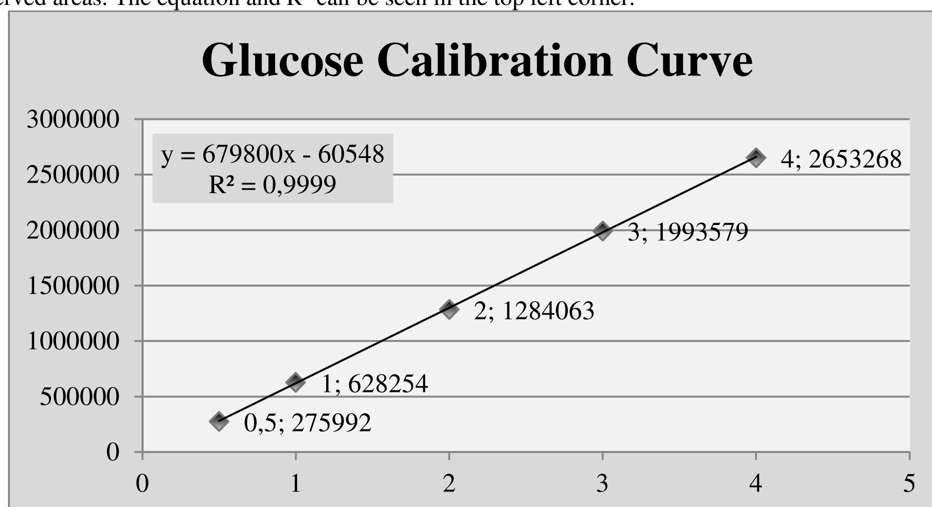
Number	Temperature	Time	Acid conc.	Subst. Load	Red. sug /tot. w.	Desirab.	Red sug/theo. Tot. Glu.
1	131.20	26.59	2.81	4.02	84.4911	1.000	97.43021
2	134.9	24.34	1.44	2.7	87.0424	1.000	100.3722
3	141.30	24.61	1.82	3.4	84.9351	1.000	97.9422
4	125.00	20.63	3.03	3.52	84.3029	1.000	97.21319
5	137.3	25.33	2.25	3.97	85.347	1.000	98.41718
6	120.00	120	1.82	4.56	85.162	1.000	98.20385
7	119.94	120.00	1.83	4.57	80.427	0.953	92.74373
8	119.84	120.00	1.83	4.60	80.4266	0.953	92.74326
9	119.56	120.00	1.92	4.47	80.4132	0.953	92.72781
10	119.63	120.00	1.80	4.25	80.3791	0.953	92.68849

The optimal yields are all very high in comparison to total theoretical amount of cellulose illustrated in the column to the right in Table 17. The variable ranges are considered moderate for a pretreatment and to be as economical as possible the temperature and acid concentration should be kept as low as possible, the time should be as short as possible and the substrate loading as high as possible. Therefore the optimal yield might not be optimal in an economical perspective. This will however not be further studied. The ten predicted optimal conditions in Table 18 were practically conducted, but instead of analyzing the results with the DNS method a High-Performance Liquid Chromatography (HPLC) was used to confirm the optimization of the process. The results are described in the subsequent section.

High-performance liquid chromatography of the optimal runs

The glucose calibration curve used to calculate the concentrations obtained from the HPLC analysis is illustrated in Fig. 11. A glucose ladder had been analyzed with the HPLC. This generated the function which can be seen in the left corner of the figure. The y and x in the function is the peak area and concentration (in mg/mL). When the experiments were analyzed with the HPLC the area was obtained and from this the concentration (x) could be calculated. By considering the dilution factor the amount of reducing sugars for the total volume of the sample could be calculated. The yield was then calculated by division of the reducing sugars by total weight.

Figure 11 shows Glucose Calibration Curve where the areas are read in the HPLC based on a standard ladder with known glucose concentration. The concentration is illustrated on the x-axis and on the y-axis is the observed areas. The equation and R^2 can be seen in the top left corner.



Six of the predicted optimal conditions were pretreated in duplicates and the results can be seen in Table 18.

Table 10 Below are the results from the HPLC analysis. The pretreatment conditions are also listed. The column to the far right shows the yields.

Sample Number	Temperature (℃)	Time (min)	Acid conc. (w/V)	Subst. load (w/V)	%Reducing sugars/total weight	Mean of yield %
1	131.20	26.59	2.81	4.02	47.34	45.45
					43.56	
2	134.9	24.34	1.44	2.7	46.47	46.82
					47.18	
3	141.30	24.61	1.82	3.4	16.59	16.94
					17.29	
4	125.00	20.63	3.03	3.52	41.62	41.20
					40.78	
5	137.3	25.33	2.25	3.97	12.59	21.85
					31.12	
6	120.00	120	1.82	4.56	31.09	30.77
					30.45	

The results illustrated in Table 18 and the predicted optimal conditions generated by the software Design Expert are equivocal. The DNS method yields nearly double the amount of the HPLC. The cause of this is of yet uncertain. These results will further be discussed in both the conclusions and future work sections regarding continued studies of this to get a clearer picture.

What is noticeable is the differences in yields obtained for the repetitions in sample number 1 and 5. The conditions for the different pretreatments are not varying from each other in a great extent. The fact that sample number 3 is so different from 1,2 and 4 appears strange when the conditions are quite a like.

Structural analysis of sago waste

The structural changes of sago waste was studied after sulfuric acid and [EMIM]HSO_4 pretreatment with Scanning Electron Microscope, Fourier Transformation Infrared spectroscopy and powder X-ray Diffraction and the results can be seen below. The structure was studied after sulfuric acid pretreatment under the conditions of 115°C for 65 minutes with acid concentration of 3.25% and a solid loading of 4%. The conditions for [EMIM]HSO_4 pretreatment were 125°C for 3 hours with the solid loading of 5%. The experimental conditions had previously been conducted and the yield for H_2SO_4 was 87.2% and for [EMIM]HSO_4 7.0% reducing sugars per total weight. Both were calculated from the absorbance (DNS method). The sample called untreated in this analysis has actually been mechanically pretreated to a particle size of 500 µm. The samples were dried at 37°C for several days prior to the structural studies.

Fourier Transform Infrared spectra

The results from the FTIR spectroscopy are illustrated in Fig. 12. The FTIR spectra are discussed below and have been analyzed based on previous studies. The conclusions are therefore frequently referenced. The physically pretreated (called untreated below) sample is the grey spectra, the sulfuric acid (and physically) pretreated sample is illustrated in red and the [EMIM]HSO_4 (and physically) pretreated sample is the blue spectra.

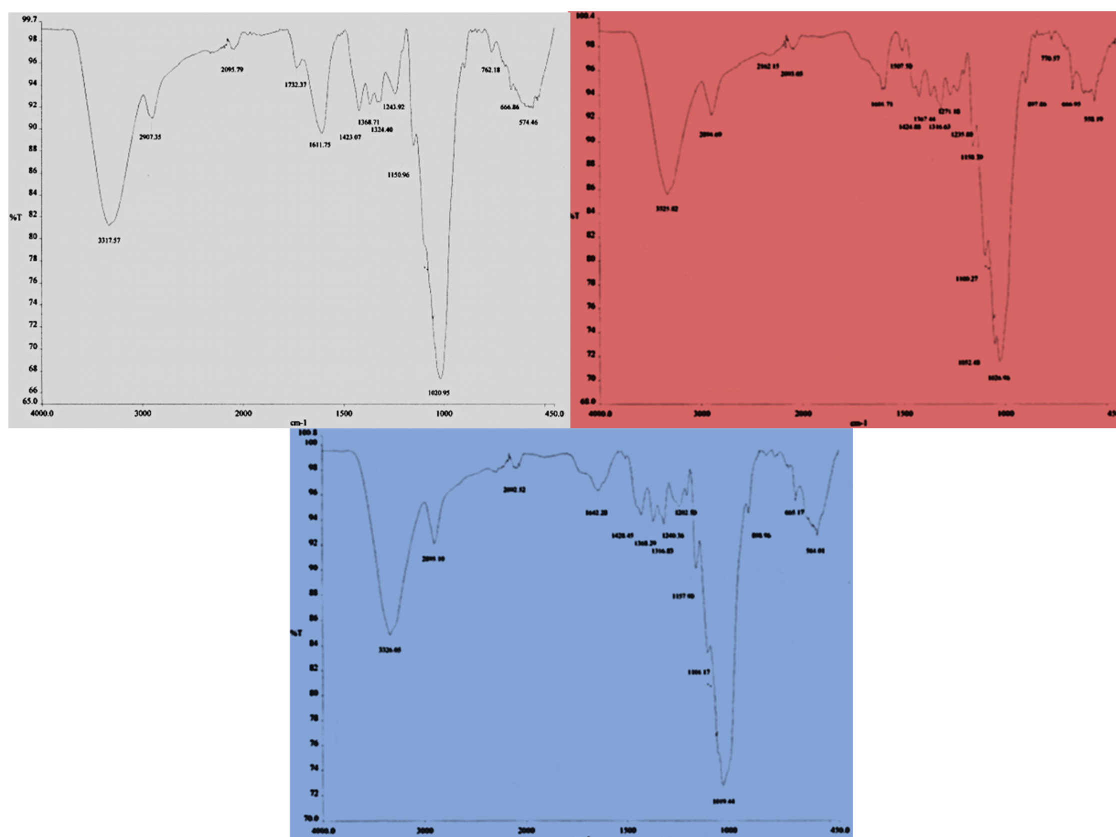


Figure 12 shows the FTIR spectra for sago waste before and after chemical pretreatment. The gray colored spectra is before chemical pretreatment, the red is after dilute sulfuric acid pretreatment and the blue is after IL pretreatment.

It can be seen that the band intensities at 3317 cm^{-1} and 2907 cm^{-1} appears to decrease after pretreatment with both H_2SO_4 and [EMIM]HSO_4 (see Fig. 13). According to literature [104, 108, 109, 110] the first band (3317 cm^{-1}) can be assigned to the O-H stretching of hydrogen bonds of cellulose (and between lignin and polysaccharides [109]) and the second band to the C-H bond stretching vibration within the methylene of cellulose [110]. It has been reported [105, 110] that a decrease in the peak assigned to O-H insinuates destruction of hydrogen bonds and disruption of crystalline cellulose. The decrease appears to be small and publications [110] state that dilute acid cannot significantly disrupt the hydrogen bonds of crystalline cellulose, but this was for the substrate corn stover. The decrease of the band at 2907 cm^{-1} has been suggested to be disruption of the methyl and methylene portions of cellulose [105].

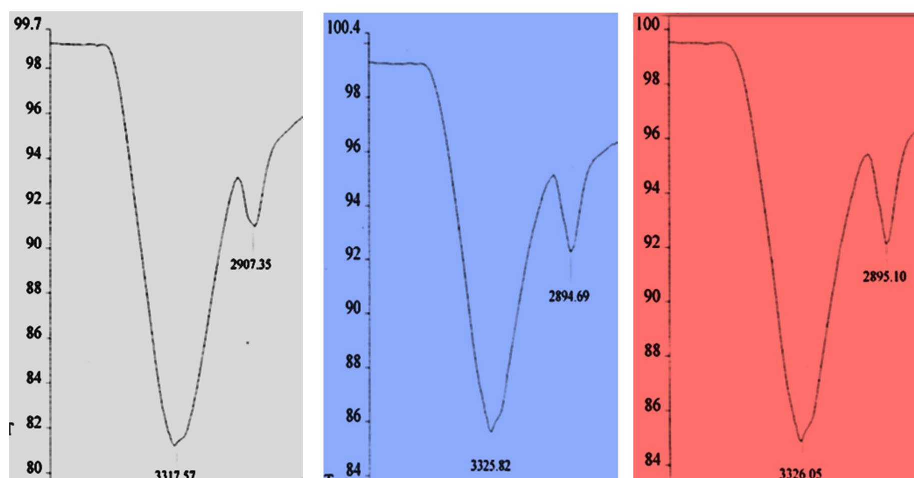


Figure 13 shows the range between 4000-2800 cm^{-1}. The gray colored spectra is before chemical pretreatment, the red is after dilute sulfuric acid pretreatment and the blue is after IL pretreatment.

Bands at the spectra around 1600 cm^{-1} have been assigned [104, 105, 109] to the aromatic skeletal vibration in lignin. The band observed in this spectra at 1612 cm^{-1} appears to be decreased for H_2SO_4 and even more so for [EMIM]HSO_4 (see Fig. 14). It has been suggested [105, 109] to be a result due to condensation reactions and/or cleavage of lignin's aliphatic side chains. There appears to be a peak at 1508 cm^{-1} for H_2SO_4 spectra and not for the untreated sample and [EMIM]HSO_4. The two peaks at 1600 and 1508 cm^{-1} has previously been assigned to lignin [104, 109, 110]. The peak at 1508 cm^{-1} may be explained to be due to higher amount of guaiacyl units (trans-coniferyl alcohol) [111].

The reduction of peak intensity at 1732 cm^{-1} after both dilute acid and IL pretreatment has previously been observed [108] and might be associated with carbonyl C=O stretching (reported to have been observed at 1745 cm^{-1} [105]). It is explained as cleavage of lignin side chains [105]. This peak (reported at the band 1740, 1732 and 1720 cm^{-1}) has also been assigned different hemicelluloses bonds [104, 105, 109, 110, 111]. The composition determination (see Table. 3) in this work showed no evidence of hemicelluloses present in sago waste and the band is therefore assumed to be associated to lignin. A decrease at the peaks 1369 cm^{-1} and 1324 cm^{-1} has also been reported [110] to be adsorptions generated by cleavage of ether bonds within the lignin, but in these spectra it is hard to determine if a decrease has occurred after the pretreatments.

The band at 1244 cm^{-1} has been reported [105] to be associated to acetyl groups (related to hemicelluloses) and although the peaks might have decreased for dilute acid and more so for the [EMIM]HSO_4 spectra the sago waste composition determination showed no result of hemicelluloses in the substrate.

A doublet peak at 1324 cm^{-1} appears in the untreated sample and can be assigned to the cellulose and reported [111] as a relationship between the content of crystallized I and amorphous regions. Authors' state [111] that a doublet peak only appears in cellulose with high

crystallized cellulose I content. In the same article it says that a decrease in the ratio between the doublet (in that article the doublet bands are 1335 and 1316 cm^{-1} with the ratio of the peaks 1335/1316) signifies an increase in crystallinity. The ratio is not calculated in this work. In these spectra it appears that this doublet is altered with a clearer defined peak at 1316 cm^{-1} (therefore indicating a decrease in the ratio) after both IL and dilute acid pretreatment. Similar results were previously obtained [111] and the conclusion was that a more intense degradation of the amorphous region had occurred than on crystallized cellulose I after pretreatments. The authors also write that this peak is progressively converted to a clear doublet when crystallized cellulose I and/or II content are increased for the sample (due to removal of amorphous regions content). This doublet does not seem to become clearer in this work and instead the peak at 1316 cm^{-1} appears increased after the pretreatments and conclusions that the crystalline regions are not altered cannot be made.

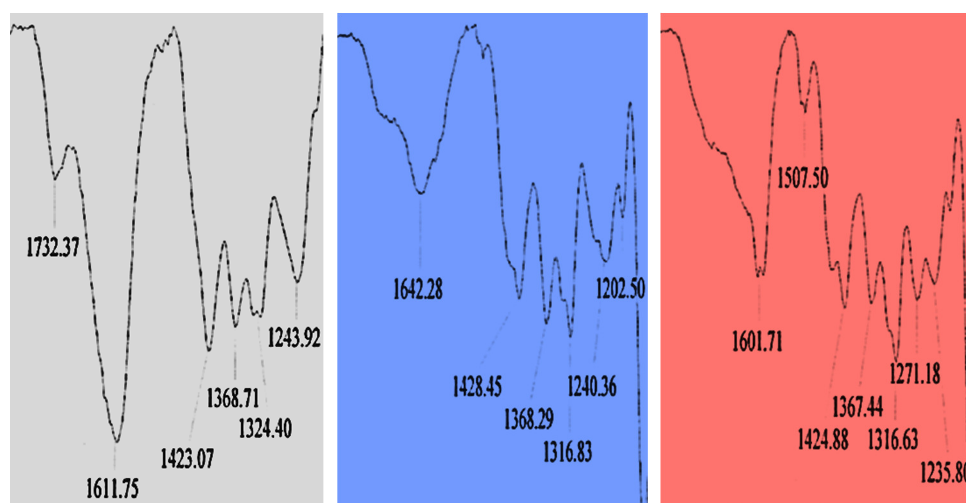


Figure 14 illustrates the range of 1800-1200 cm^{-1}. The gray colored spectra is before chemical pretreatment, the red is after dilute sulfuric acid pretreatment and the blue is after IL pretreatment.

The bands at 1423, 1369, 1151, 1100, 1021 and 898 cm^{-1} appears to correspond to the peaks reported in previous studies [104, 109, 111] and can be seen in Fig. 14-15. The first peak (at 1423 cm^{-1}) was assigned to C-H_2 bending vibrations, followed by the peaks assigned to C-H symmetric deformation, C-O-C asymmetric vibration, glucose ring asymmetric stretching, C-O stretching and C-O-C stretching at glycosidic linkages between glucose, in this order.

The peak at 1423 cm^{-1} in the untreated sample is assigned (as previously in [109, 111, 112]) to CH_2 scissoring motion in the mixture of crystallized cellulose I and amorphous cellulose. It has been reported [112] that this band appears strong around 1430 cm^{-1} for type I crystalline (cellulose I) and weak at 1420 cm^{-1} when there is high presence of type II crystalline (cellulose II) and amorphous cellulose. In these spectra the band seems to be altered for both the pretreatments with bands at 1428 cm^{-1} for IL and 1425 cm^{-1} for dilute acid. This shift in band towards 1430 cm^{-1} is explained [111] to be due to higher presence of crystallised cellulose I indicating that the amorphous area of the cellulosic component is more affected by degradation. This opinion is strengthened when combined with the previous discussion regarding the doublet ratio band. The band at 1369 appears to decrease after the pretreatments and have been assigned to -CH bending from the methoxyl group [109, 111].

In the spectral region assigned to cellulose C-O-C asymmetric vibration [109] there appears a band located at 1150 cm^{-1} for untreated sago waste shifted to 1158 for pretreatments with both H_2SO_4 and [EMIM]HSO_4. It has earlier been reported [111] that a band at 1156 cm^{-1} can be related to amorphous cellulose and a peak at 1163 cm^{-1} is related to crystallized cellulose. A shift may therefore be the due to increased crystalline content after the pretreatments (as a result of hydrolysis of amorphous regions).

The bands at 1021 cm^{-1} appears to decrease for both pretreatments and they have previously [109] been assigned to the stretching vibrations of C-O ether, methoxyl and β-O-4 bonds. The peak at 897 cm^{-1} is attributed to the C-O-C stretching at glycosidic linkages between glucose. The intensity of this band appears to increase for both the pretreatments spectra. This band has been observed [17] to become weaker after dilute acid pretreatment due to hydrolysis of amorphous regions and intensified after Ionic Liquid pretreatment. This appears to be due to the disruption of hydrogen bonds in crystalline cellulose. It might indicate that in the pretreatment of sago waste with both techniques lead to disruption of hydrogen bonds of crystalline cellulose. The peak intensity at 1100 cm^{-1} appears to increase (or are at least more apparent) for both dilute acid and IL and has been assigned to an increase of crystalline cellulose content (due to less changes in crystalline structure). An increase in the band around 1100 cm^{-1} and a decrease at 897 cm^{-1} has been observed [17] for dilute acid pretreatment but not for the untreated sample and IL pretreated switchgrass substrate. These authors reported that after the IL pretreatment the band was missing at 1100 cm^{-1} and intensified at 900 cm^{-1} due to disruption of hydrogen bonds of crystalline cellulose indicating increased content of amorphous regions.

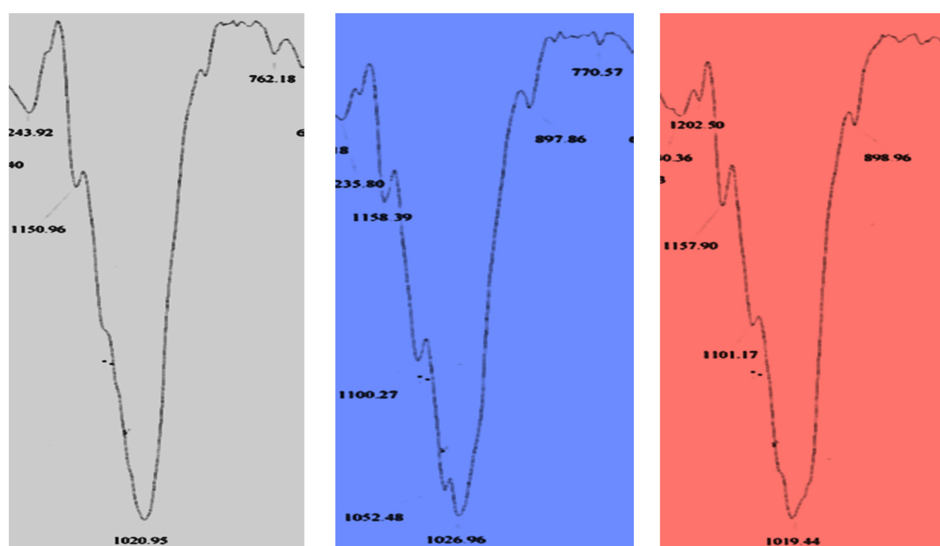


Figure 15 shows the range of 1250-750 cm^{-1}. The gray colored spectra is before chemical pretreatment, the red is after dilute sulfuric acid pretreatment and the blue is after IL pretreatment.

Results after powder X-ray Diffraction

The powder X-ray diffraction curve for sago waste is illustrated in Fig. 16. This figure shows untreated sago waste in black, dilute sulfuric acid pretreatment of sago waste in red and [EMIM]HSO_4 pretreatment of sago waste in blue color. Important to note is that the sample denoted as 'untreated' actually has been physically pretreated. The structure of the "untreated" sample has therefore already been modified. See the section "The different pretreatment categories".

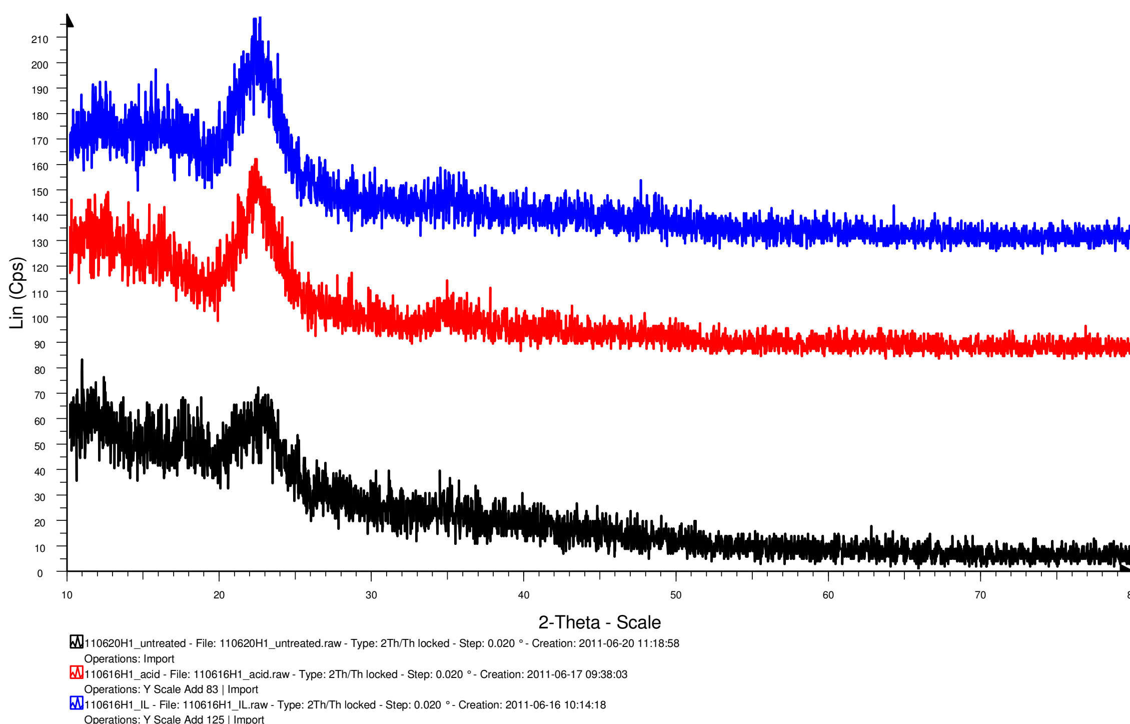


Figure 16 shows the results from XRD before and after chemical pretreatment of sago waste. The black curve is the result before chemical pretreatment, the red curve is the result after H_2SO_4 pretreatment and the blue curve is the result after IL pretreatment.

It has been reported [17] that the crystallinity effects the enzymatic (and chemical) hydrolysis rate of cellulose. One of the purposes of pretreatment is to disorder the inter- and intra-molecular hydrogen bonds of cellulose fibrils [17] to alter the crystalline structure to less ordered amorphous regions which increases cellulose surface accessibility and promotes a more efficient hydrolysis of cellulose [17].

The peak broadness at around 22.5° appears to be quite similar for all three samples suggesting that the pretreatment methods might have been unable to disorder the hydrogen bonds in the cellulose fibrils in a great extent [17]. Results have been published [17] where the sample treated with dilute acid demonstrated minor change in crystallinity compared to the untreated sample, but the IL displayed a much broader diffraction peak.

Slight transformation of crystalline- to amorphous cellulose have been reported [106] where the XRD results showed a double peak at around 21.5° and 20° and a small peak around 12° suggesting structural disorder in cellulose [17]. Such a shift is not observed in this XRD diffraction graph. The only noticeable difference in the graph might be the peak around 17° for the untreated sago waste and appears to decrease for both H_2SO_4 and [EMIM]HSO_4 pretreatment. The peak's height is perhaps almost as high as for the peak at around 22.5° for the untreated sample. Other than this observation, it is difficult to discuss the graph without further normalization of the three curves.

CrI can be used as a measure of crystalline cellulose in the total sago waste solid. Therefore it might be considered normal that the CrI increases after dilute acid pretreatment. According to literature [17, 104] this is due to partial removal of lignin, hemicelluloses (both are amorphous) and amorphous cellulose and the pretreated samples therefore contain higher content of crystalline cellulose (more problematic to disrupt) compared to untreated sago waste. Reports [105] state that lower acid concentrations breaks down more of amorphous regions while higher acid concentrations can possibly disrupt more of the cellulose crystalline structure. The Ionic Liquids have the potential to disrupt the hydrogen bonds of crystalline cellulose and theoretically [17] increase the surface accessibility resulting in increased cellulose hydrolysis rate.

Images from Scanning Electron Microscopy

SEM images have been created with different magnifications for sago waste before and after chemical pretreatment. The magnifications are illustrated in the images. The untreated sago waste can be seen in Fig. 17, a-i to a-iiii, the dilute sulfuric acid pretreatment is observed in Fig. 18, b-i to b-iii and the IL pretreatment is from c-i to c-iii.

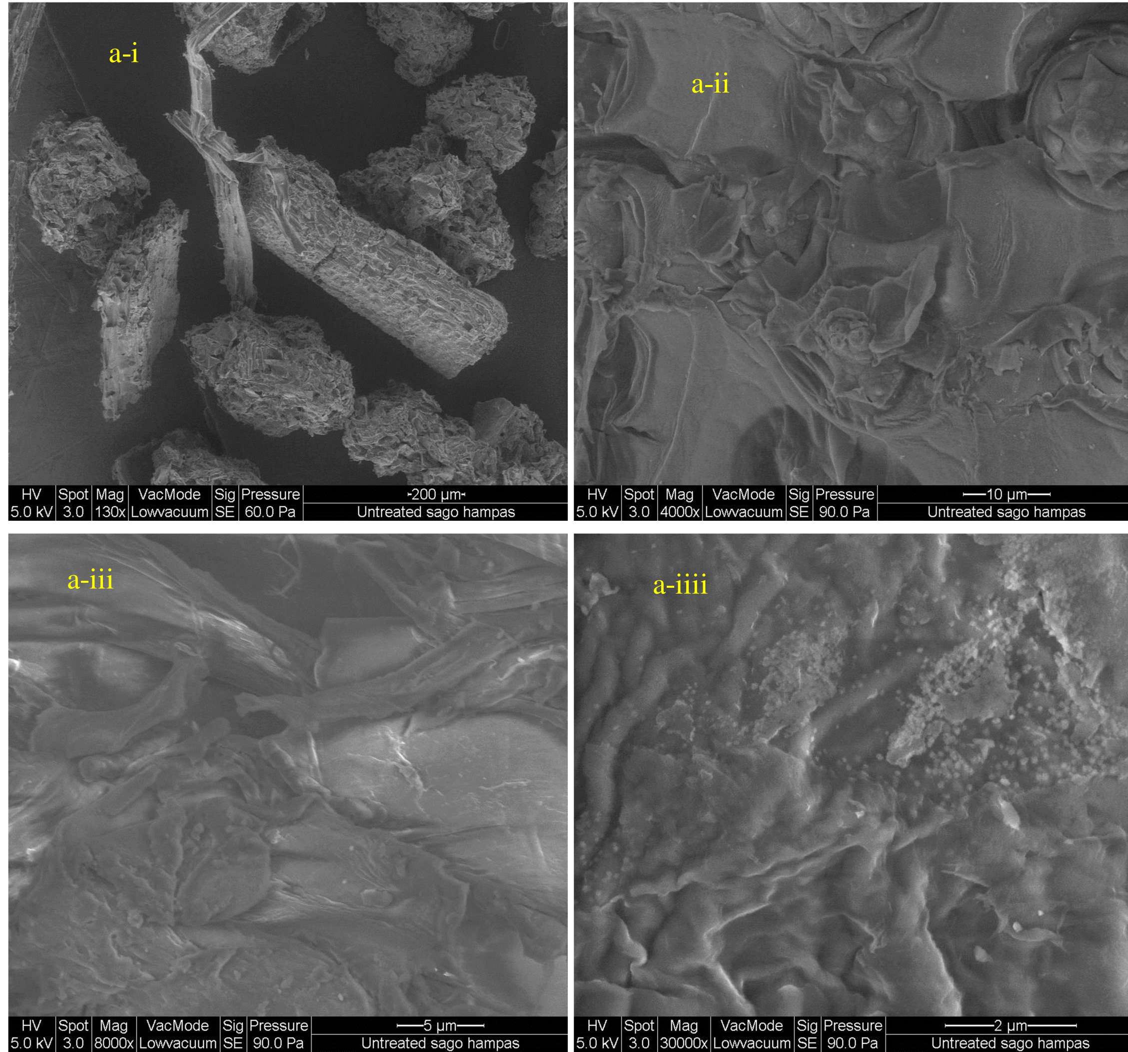


Figure 17 shows SEM images for untreated (physically treated) sago waste.

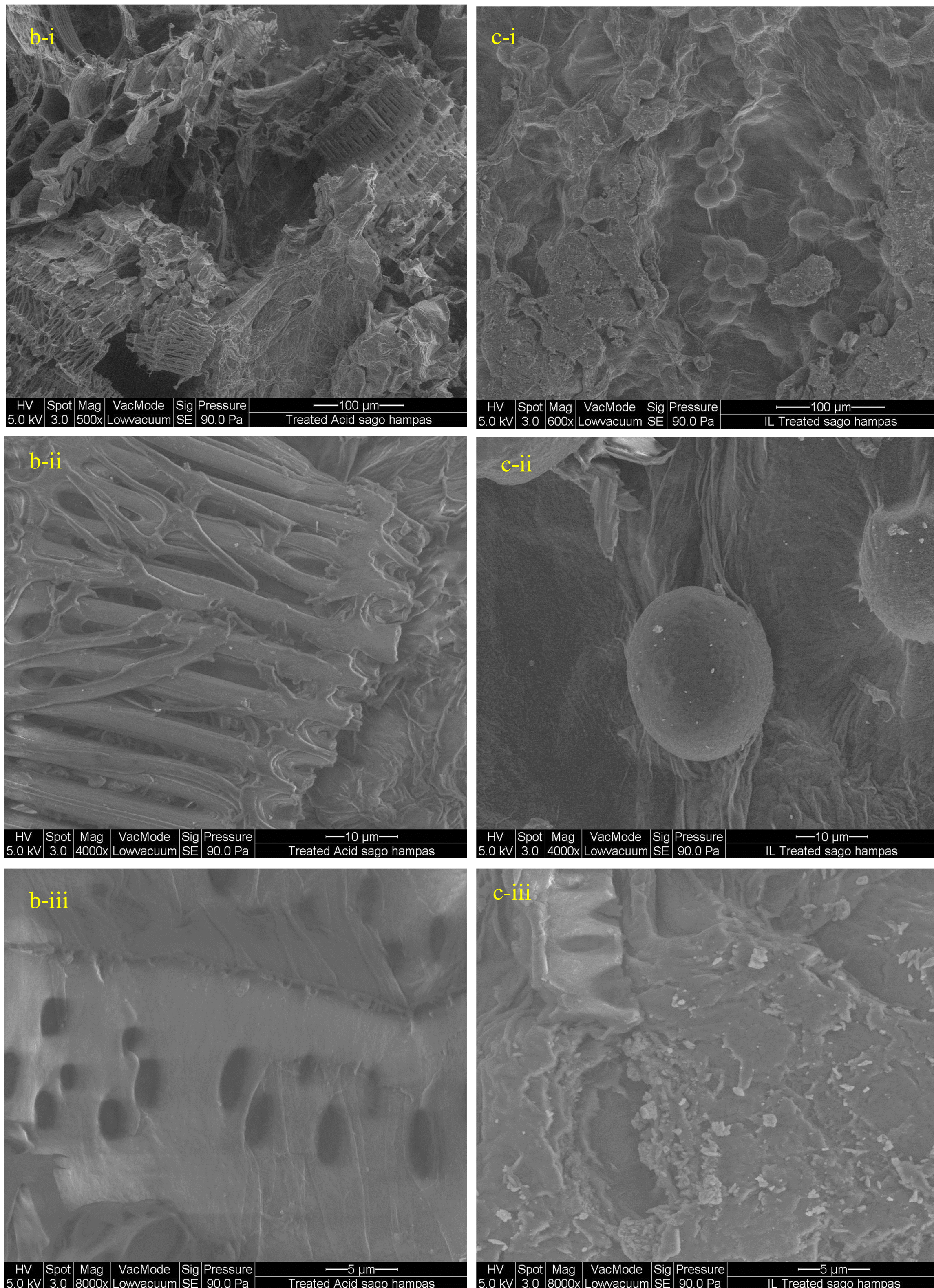


Figure 18 displays SEM images of sago wasted that has been chemically pretreated with H_2SO_4 (b-i to b-iii) and [EMIM]HSO_4 (c-i to c-iii).

The SEM images may respectively reveal changes in the sago waste prior and subsequent to chemical pretreatment. It can be observed that after H_2SO_4 pretreatment the structure of the biomass appears to go through changes where pores and holes are created on the surface when compared to the untreated sample, which seems to have a compact structure. Similar holes have been detected and explained in literature [105] as disorder of the substrate structure due to lignin and hemicelluloses removal. The article discusses that the dilute acid pretreatment resulted in a

reduction in fiber length and total disorder of the substrate network. The pores and holes may indicate that the surface accessibility has been dramatically increased.

For the IL pretreatment droplets is present in the image. Droplets have been discovered and described [105] as lignin that has been agglomerated on the surface of the biomass after ammonia fiber explosion pretreatment. However these droplets are small and visible in the 10 µm images [105] while the droplets from these SEM images are clearly visible in the 100 µm SEM images and clustered together. The reasons for the bigger droplets in the images are unclear and other explanations for their existence in the sample are unknown. Pores and holes observed after dilute sulfuric acid pretreatment are not observed after the IL pretreatment.

Reliability of results

A large number of experiments have been executed in this work and experimental errors appear to exist, such as high variations for samples treated in same conditions (for example center points in the designs). Additional potential problems have been observed which may be a result of the small scale of the experiments. Small scale experiments consisting of 1 mL is extremely hard to control and repeat with good accuracy. The dilute sulfuric acid pretreatment had a tendency to sometimes completely or partly evaporate and the substrate was burned even if the cap was tightly screwed on the tube (see the image to the left in Fig. 19). After the final experiment was conducted one third of the samples were completely burned. For such small scale this explains the high variation. Temperatures of around 120-130°C are sufficient to cause this. If the sample is completely burned a repetition of the sample is easily perform, however if it is partly burned it can be hard to notice. As the analysis also involved keeping track of dilution steps the partial evaporation may affect the calculated yield significantly.

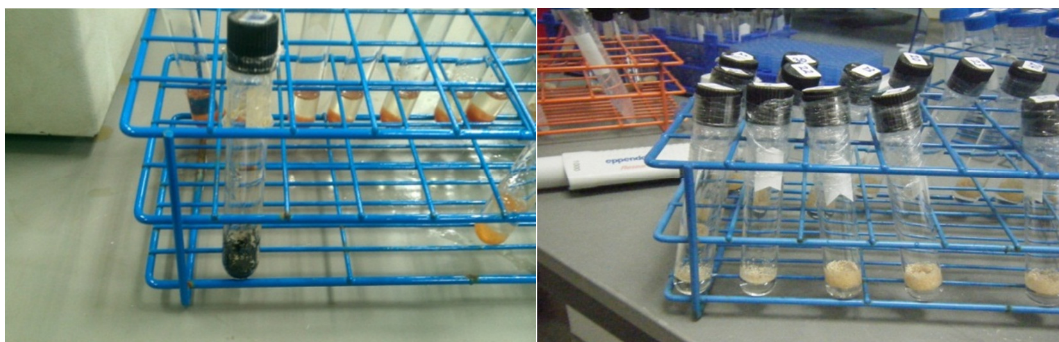


Figure 19 displays two typical experimental issues during this work. The sulfuric acid often evaporated causing the sample to burn and this is illustrated in the image to the left. The substrate attached to the sides after the dilute acid was added to the sample and this is shown in the picture to the right.

Another potential problem with small scale experiments is that after addition of the liquid to the substrate in the glass tube a visible amount of substrate got attached to sides of the test tube and remained there throughout the pretreatment. This problem is illustrated in the picture to the right in Fig. 19. This may seem as an insignificant amount of substrate being attached to the side of the test tube, but at a small scale of 40 mg even 1 mg of substrate attached to the surface of the glass would result in 2.5% of the substrate being unreacted. This will of course have an effect on the final yield. Samples were tested with as low as 15 mg substrate and here the unreacted substrate would be 6.7% if it is assumed that 1 mg is attached to the glass sides. This could not be avoided as the slightest movement where a solid is mixed with a liquid result in the substrate attaching itself to the sides of the tube.

As this research has been almost entirely based on analysis with DNS reagent and spectrophotometry the results obtained from the HPLC changed the view regarding almost complete cellulose hydrolysis of sago waste. It is still unclear why and how the HPLC analysis indicates lower yields than the DNS method in this work. As have been mentioned (in the section 'Dinitrosalicylic acid Method for analysis of reducing sugars') the intensity of the color change is sensitive to the concentration in each of the reagent compound in DNS. The DNS reagent was prepared in a large batch prior to the experiments and never re-made. It could instead have been made in one replicate for comparison in differences from batch to batch. The HPLC results may also be misleading as they show differences in yield for the duplicates. The pretreatment conditions were also relatively similar, yet they generated a high variety of yields.

Another potential explanation why the DNS method may have resulted in higher yields than the HPLC analysis can be due to the neutralization of the sample prior to addition of the DNS reagent. The sample was made alkaline with sodium hydroxide far over pH 7 after the pretreatment. Degradation of glucose may be a result under alkaline conditions, but authors stated that the sample is usually fine for up to two weeks. However, sago waste was easily pretreated with dilute sulfuric acid and high yields were observed after short time during

moderate temperatures. The heat-treatment with DNS reagent for five minutes in alkaline conditions may therefore have a significant effect on the substrate as a kind of second step pretreatment and this was not considered.

From Miller's article [102] regarding DNS reagent concentrations the protocol for optimal concentrations and steps for optimal color intensity was described. Sodium hydroxide is one of the reagents in the DNS solution and it was reported that 1% NaOH gives optimal color intensity without glucose degradation [102]. The article also emphasize that higher concentrations may lead to increased color intensity and at the same time a decrease in reducing sugars [102]. The DNS reagent batch consisted of 1% w/w of sodium hydroxide, so when the DNS is mixed with the already alkaline sample the sodium hydroxide concentration will be over 1%. This may have resulted in increased color intensity of the sample. Apart from this, the substrate contains a high variety of molecules that can cause interference with the DNS reagent.

Another possible explanation for the differences in yield between DNS and HPLC can be due to the glucose degradation during the pretreatment. Although glucose is normally degraded to compounds such as HMF and furfural only under very harsh heat treatments it may occur when sago waste is treated at around 120-130 °C. As the previous potential explanations refer to the DNS practice being the error in these experiments it may also be caused during the HPLC analysis. If the pretreatment conditions were harsh enough for formation of glucose degradation products these have been reported to have a retention time of 30 minutes in the HPLC and therefore would not be detected during the HPLC analysis [113]. This is because the HPLC analysis of the samples only lasted for 15 minutes in this research, which is enough time to detect monosaccharides and disaccharides, but not enough to detect peaks of glucose degradation compounds.

Even though both the methods used to study the yield can be further evaluated the results from the FTIR and XRD experiments suggests that not all the cellulose have been degraded during the dilute sulfuric acid pretreatment.

Conclusions

This work is a study of the commonly used pretreatment dilute sulfuric acid and a novel pretreatment of the potentially green ionic liquid [EMIM]HSO_4. Sago waste is less studied than the other two substrates. The fact that sago waste consists of a high amount of cellulose and starch (almost 80% of total composition) it can theoretically produce much bioethanol in the end of the process. When the glucose yield during the hydrolysis process has been confirmed to correspond for both DNS method and HPLC analysis the fermentation process can possibly be quite straight forward with use of a traditional microorganism. A substrate generating a high variety of hexoses and pentoses will need a versatile and specific microorganism for fermentation in order to have an efficient process.

Even though the methods used in this research showed results from the DNS and HPLC analysis which contradict one another, further studies of sago waste should be conducted. The main contradictions will be presented and a possible way of getting results more corresponding to one another will be presented in the 'Future work' section.

The fact that the research has been based on the DNS method and the practice of the method has been conducted without extensive caution during the neutralization the results generated can be questioned. However since the practice of the samples has been conducted in a uniform way throughout the practical work the comparison of the three substrates could still be correct, with sago waste generating higher yield than sugarcane and sugarcane bagasse generates higher yield than rice husk, in the range of the studied variables.

The amount of reducing sugars produced is of course directly connected to the amount cellulose and hemicelluloses in the substrate and therefore a composition determination of the substrate under study is good to conduct. As have been mentioned, both lignin and hemicelluloses and the network between these and cellulose affect the hydrolysis rate. Since the composition determination showed no evidence of hemicelluloses present in sago waste the disruption of the substrate network could potentially be achieved during milder conditions than for a substrate rich in lignin and hemicelluloses. As have been reported [39] the higher amount of lignin present in the substrate the lower is the cellulose degradation. Xylan removal has also shown [9] to have higher impact on cellulose length than lignin removal. Perhaps it is possible to draw preliminary conclusions regarding the potential of the substrate, in the sense of cellulose hydrolysis, based on its composition and structural analysis.

Since the experiments with the DNS method has been conducted in a uniform way it may also be that the optimization of sago waste may be correct, but perhaps with enhanced color intensity.

Based on the structure analysis, mainly FTIR and XRD, it appears that [EMIM]HSO_4 potentially works in solubilizing lignin in sago waste just as good or perhaps better than H_2SO_4. Normally Ionic Liquids are used in a double step method where the IL alters the network and an acid or enzyme is added for hydrolysis of the cellulose and/or hemicelluloses.

The efficiency of dilute sulfuric acid in pretreatment and hydrolysis is widely studied and its effect well known. Although the process may not have been optimized with a yield corresponding for both DNS and HPLC the dilute sulfuric acid may be able to pretreat and hydrolyze sago waste in an economically beneficial perspective with its relatively mild process conditions.

The main focus in this work has been on sago waste due to the relatively easy cellulose hydrolysis, but also due to the limited research of the substrate for second generation bioethanol production. Sago waste has a lot of potential as a possible waste for the bioethanol production industry but further research on the substrate is of need.

Future work

Future work in the area covered in this study can be to elaborate on the results achieved. By producing two batches of DNS reagent and then re-run the suggested optimized conditions from the experimental model one can study if there is difference in the color intensities between two batches. The neutralization step with addition of sodium hydroxide is assumed to cause interference in the color intensity and perhaps degradation of glucose in the sample so this should be done with caution until pH 7 is achieved. The calculated yields based on color intensity from DNS should be confirmed by a longer HPLC analysis time of the sample to monitor if glucose has been degraded to other products such as HMF.

If the two batches of DNS correspond with each other and with the HPLC analysis the experimental plan in this research can be repeated to study this optimized process. The sample could be scaled up to avoid common difficulties encountered when working with very small scales, as have been described in the 'Reliability of results' section. The substrate attached to the side of the test tube would not matter as much as during large scale experiments as it does for the experiments that have been conducted in this work. Stirring could also possibly be applied for the samples as an improvement to maintain circulation of the liquid to avoid solids being stuck to the glass tube throughout the whole pretreatment. This could lead to a more homogenous solution. Stirring could also increase the solid surface area exposed to liquid contact by having it surrounded by liquid instead of other substrate particles. This may be vital for particles that are in the bottom of the test tube and never is completely exposed to the liquid. The stirring rate can be treated as a variable in an experimental design.

The small scale pretreatment is valuable for optimization of the process and as a study of the substrate, but not for bioethanol production. Therefore a gradual increase of volume of dilute sulfuric acid and substrate can be done and optimization of the gradual scale up could be conducted.

One thing that could be done in order to thoroughly study the structural changes of sago waste is to get data from the sample that was treated with concentrated sulfuric acid when the composition of sago waste was determined. The solid residue structure should be completely disrupted and SEM images, XRD results and FTIR spectra could help in further understanding how severely the structure has been altered during this dilute sulfuric- and [EMIM]HSO_4 pretreatment. What is sometimes referred to as untreated sago waste in this report is actually physically treated sago waste to a particle size of 500 µm. It is therefore not correct to assume a completely natural structure since it has been damaged. Sago waste could be studied when it is less physically pretreated (bigger particle sizes) to get an understanding of the effect of the size. The particle size of sago waste could therefore be treated as a variable in Design of Experiments to study its significance. This could provide knowledge of the structural changes of sago waste from almost natural structure to when it is completely hydrolyzed.

It appears that [EMIM]HSO_4 lack the ability to hydrolyze the cellulose effectively and studies on whether an acid or enzyme such as cellulase can be added after the IL treatment to enhance the hydrolysis can be evaluated. The SEM images revealed holes on the substrate structure after the dilute sulfuric pretreatment, but this was not observed for [EMIM]HSO_4. It could be of interest to study the liquid of the sample after the IL pretreatment to determine which components this phase contains. The FTIR spectroscopy, XRD diffraction and SEM images only observe the solid (non-dissolved) components and no further study on the liquid phase was conducted as for the dilute sulfuric acid with HPLC analysis.

There are also many acids which can be studied as a substitute for sulfuric acid. Although sulfuric acid is the cheapest acid its use poses a problem as a non-disposable waste. An interesting acid is phosphoric acid which is a weaker one, but is also effective in pretreatment of lignocellulose and provides an environmentally friendly solution [114, 63]. During mild conditions it causes a very low production of the toxic byproducts, like HMF, for

microorganisms [114], and therefore does not require an extra detoxification step prior to the fermentation. As acids need to be neutralized prior to fermentation by addition of an alkali, the formed salt compounds need to be filtered to clean the liquid [63]. These extra steps leads to additional costs, but with phosphoric acid the toxic byproduct formation is minimal and when neutralized with NaOH, sodium phosphate salt is formed which can be used as a nutrient by microorganisms [63]. This also decreases the needed amount of this nutrient during fermentation [63]. This acid is well worth further investigation of in order to compare it with sulfuric acid.

Apart from acids there are several other pretreatments that can be further studied for sago waste pretreatment and hydrolysis. Articles state that acid pretreatments remove hemicelluloses components and exposes cellulose for enzymatic digestion [78]. However sago waste lacks hemicelluloses and another approach to the hydrolysis of cellulose may be to use a method that solubilizes the lignin or cellulose and potentially also decrystallizes cellulose. A pretreatment used prior to enzymatic hydrolysis such as Organosolv has shown ability to remove lignin with a mix of organic liquids and water. The mixture is heated with the substrate and dissolves lignin and parts of hemicelluloses [4]. Other pretreatment methods that have been reported [9] to disorder lignin-polysaccharide bonds and decrease the lignin content are alkaline based techniques such as ammonia fiber explosion (AFEX).

When and if an efficient pretreatment and hydrolysis method has been developed and optimized for sago waste in larger scales the fermentation process must be studied as well. Since only glucose has been received after the pretreatment of sago waste in this research the fermentation can potentially make use of a common microorganism for bioethanol production. This research has not focused on the fermentation process and therefore lacks information on how future work could be conducted for this process. However the sago waste differs from lignocellulosic wastes in its presence of cellulose and starch compared to sugarcane bagasse and rice husk that contains cellulose and hemicelluloses. While the degradation of sago waste generates one type of reducing sugar the others generate a wide variety. The wide variety of reducing sugars also increases the difficulty of the fermentation. That is why a common microorganism in theory can ferment the reducing sugars derived from sago waste. It has been reported that *Saccharomyces cerevisiae* can produce ethanol close to the theoretical maximal yield of 0.51 g ethanol * g glucose^{-1} and can use all hexoses obtained from lignocellulose as a nutrient, but cannot use pentoses [23]. Therefore, if sago waste can be completely hydrolyzed to glucose during moderate conditions the fermentation could in theory be equally efficient as without having to use additional metabolic engineering of the microorganism strain.

If the process using sago waste as a substrate for second generation bioethanol production can be proven to be price worthy, this waste can be of value when it elsewise would be unused. It could potentially work as an addition to the bioethanol industry, but the amount produced is not in the quantity of say sugarcane bagasse or rice husk. If it can be shown to be feasible an extension of the growth territory of sago waste may have an impact on the wide diversity of species existing in the tropical region of Malaysia. However, although it is produced in smaller quantity continued research should be conducted to try to develop the process and improve knowledge regarding if and how the wastes' generated in much larger quantities can be made price valuable. Studies on the bioethanol production of a wide range of lignocellulosic materials, regardless of the quantity produced globally, can only help with increasing knowledge of the process and can work as a guide for making substrates such as sugarcane bagasse and rice husk feasible and for second generation bioethanol to be a serious substitute for gasoline.

References

1.Tan KT, Lee KT, Mohamed AR. Role of energy policy in renewable energy accomplishment: the case of second-generation bioethanol. Energy Policy. 2008;36:3360–3365.

2. Zavrel M, Bross D, Funke M, Buchs J, Spiess AC. High-throughput screening for ionic liquids dissolving (ligno-)cellulose. Bioresoure Technology. 2009;100:2580–2587.

3. Balat M. Production of bioethanol from lignocellulosic materials via the biochemical pathway: A review. Energy Conversion and Management. 2011;52:858-875

4. Taher-Taherzadeh MJ, Karimi K. Enzymatic-based hydrolysis processes for ethanol from lignocellulosic materials: A review. BioResources. 2007;2:707-738.

5. Zaldivar J, Nielsen J, Olsson L. Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. Appl Microbiol Biotechnol. 2001;56:17-34

6. Sun Y, Cheng J. Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresource Technology. 2002;83:1-11

7. Cardona CA, Quintero JA, Paz IC. Production of bioethanol from sugarcane bagasse: Status and perspectives. Bioresource Technology. 2010;101:4754-4766

8. Cheng KK, Cai BY, Zhang JA, Ling HZ, Zhou YJ, Ge JP, Xu JM. Sugarcane bagasse hemicellulose hydrolysate for ethanol production by acid recovery process. Biochemical Engineering Journal. 2008;38:105-109.

9. Alvira P, Pejó ET, Ballesteros M, Negro MJ. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. Bioresource Technology. 2010;101:4581-4861.

10. Karimi K, Emtiazi G, Taherzadeh MJ. Ethanol production from dilute-acid pretreated rice straw by simultaneous saccharification and fermentation with *Mucor indicus, Rhizopus oryzae, and Saccharomyces cerevisiae.* Enzyme and Microbial Technology. 2006;40:138-144

11. Goh CS, Tan KT, Lee KT, Bhatia Subhash. Bio-ethanol from lignocellulose: Status, perspectives and challenges in Malaysia. Bioresource Technology. 2010;101:4834-4841.

12. Taherzadeh MJ, Karimi K. Acid-based hydrolysis processes for ethanol from lignocellulosic materials: A review. BioResources. 2007;2:472-499.

13. Hahn-Hägerdal B, Galbe M, Gorwa-Grauslund MF, Lidén G, Zacchi G. Bio-ethanol – the fuel of tomorrow from the residues of today. TRENDS in Biotechnology. 2006;24:549-556.

14. Kumar S, Singh SP, Mishra IM, Adhikari DK. Recent advances in production of bioethanol from lignocellulosic biomass. Chem. Eng. Technol. 2009;32:517-526

15. Kumar P, Barrett DM, Delwiche MJ, Stroeve P. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Ind. Eng. Chem. Res. 2009;48:3713-3729

16. Yang B, Wyman CE. Pretreatment: the key to unlocking low-cost cellulosic ethanol. Biofuels, Bioprod. Bioref. 2008;2:26-40

17. Li C, Knierim B, Manisseri C, Arora R, Scheller HV, Auer M, Vogel KP, Simmons BA, Singh S. Comparison of dilute acid and ionic liquid pretreatment of switchgrass: Biomass recalcitrance, delignification and enzymatic saccharification. Bioresource Technology. 2010;101:4900-4906.

18. Zhu S, Wu Y, Chen Q, Yu Z, Wang C, Jin S, Ding Y, Wu G. Dissolution of cellulose with ionic liquids and its application: a mini-review. Green Chemistry. 2006;8:325-327.

19. Dadi AP, Varanasi S, Schall CA. Enhancement of cellulose saccharification kinetics using an ionic liquid pretreatment step. Biotechnology and Bioengineering. 2006;95:904-910.

20. Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D, Crocker D. Determination of structural carbohydrates and lignin in biomass. National Renewable Energy Laboratory. 2008.

21. Sluiter A, Hames B, Hyman D, Payne C, Ruiz R, Scarlata C, Sluiter J, Templeton D, Wolfe J. Determination of total solids in biomass and total dissolved solids in liquid process samples. National Renewable Energy Laboratory. 2008

22. Mousdale DM. Biofuels. Biotechnology, chemistry, and sustainable development. 1st ed. CRC Press; 2008

23. Olsson L, Jørgensen H, Krogh KBR, Roca C. Dumitriu S, editor. Polysaccharides. Structural diversity and functional versatility. 2nd ed. CRC Press; 2004

24. Demirbas MF, Balat M, Balat H. Potential contribution of biomass to the sustainable energy development. Energy Conversion and Management. 2009;50:1746-1760

25. Mielenz JR. Ethanol production from biomass: technology and commercialization status. Current Opinion in Microbiology. 2001;4:324-329.

26. Meher LC, Sagar DV, Naik SN. Technical aspects of biodiesel production by transesterification – a review. Renewable and Sustainable Energy Reviews. 2006;10:248-268.

27. Campbell CJ, Laherrere JH. The end of cheap oil. Science America. 1998;3:78-83.

28. Nguyen TAD, Kyoung-Rok K, Jong Han S, Cho HY, Kim JW, Park SM, Park JC, Sim SJ. Pretreatment of rice straw with ammonia and ionic liquid for lignocellulose conversion to fermentable sugars. Bioresource Technology. 2010;101:7432-7438.

29. Hill J, Nelson Erik, Tilman D, Polasky S, Tiffany D. Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. Proceedings of the National Academy of Sciences of the United States of America. 2006;103:11206-11210.

30. Sendelius J. Steam pretreatment optimization for sugarcane bagasse in bioethanol production. Department of chemical engineering. Lund university. 2005.

31. Quintero JA, Montoya MI, Sánchez OJ, Giraldo OH, Cardona CA. Fuel ethanol production from sugarcane and corn: Comparative analysis for a Colombian case. Energy. 2008;33:385-399.

32. Philippidis G. The potential of biofuels in the Americas. Center for hemispheric policy.2008. University of Miami.

33. Kim S, Dale BE. Global potential bioethanol production from wasted crops and crop residues. Biomass and Bioenergy. 2004;26:361-375.

34. Sánchez OJ, Cardona CA. Trends in biotechnology production of fuel ethanol from different feedstocks. Bioresource Technology. 2008;99:5270-5295.

35. Gomez LD, Steele-King CG, McQueen-Mason SJ. Sustainable liquid biofuels from biomass: the writing´s on the wall. New Phytologist. 2008;178:473-485.

36. Wyman CE, Dale BE, Elander RT, Holtzapple M, Ladisch MR, Lee YY. Comparative sugar recovery data from laboratory scale application of leading pretreatment technologies to corn stover. Bioresource Technology. 2005;96:2026-2032.

37. Simmons BA. Loque D, Blanch HW. Next-generation biomass feedstocks for biofuel production. Genome Biology. 2008;9:

38. Alriksson B. Ethanol from lignocellulose: Alkali detoxification of dilute-acid spruce hydrolysates. Karlstad University Studies. 2006:30:1-46.

39. Laxman RS, Lachke AH. Bioethanol from lignocellulosic biomass. In : Pandey A, editor. Handbook of plant-based biofuels. CRC Press. 2009.

40.Hayashi K, Saha BC,editors. Lignocellulose biodegradation. Washington, DC: American chemical society; 2004

41. Lutzen NW, Nielsen MH, Oxenboell KM, Schulein M, Stentebjerg-Olesen B. Cellulases and their application in the conversion of lignocellulose to fermentable sugars. Industrial and Diagnostic Enzymes. 1983;300:283-291.

42. Li C, Zhao ZK. Efficient acid-catalyzed hydrolysis of cellulose in ionic liquid. Adv. Synth. Catal. 2007;349:1847-1850.

43. Zhbankov RG. Hydrogen bonds and structure of carbohydrates. Journal of Molecular Structure. 1992;270:523-539.

44. Demirbas A. Heavy metal adsorption onto agro-based waste materials: A review. Journal of Hazardous Materials. 2008;157:200-229.

45. Mora-Pale M, Meli L, Doherty TV, Linhardt RJ, Dordick JS. Room temperature ionic liquids as emerging solvents for the pretreatment of lignocellulosic biomass. Biotechnology and Bioengineering. 2011;108:1229-1245.

46. Thielemans W, Can E, Morye SS, Wool RP. Novel applications of lignin in composite materials. Journal of Applied Polymer Science. 2002;83:323-331.

47. Canilha L, Santos VTO, Rocha GJM, Silva JBA, Giulietti M, Silva SS, Felipe MGA, Ferraz A, Milagres MF, Carvalho W. A study on the pretreatment of a sugarcane bagasse sample with dilute sulfuric acid. J Ind Microbiol Biotechnol. 2011;38:1467-1475.

48. Pushpamalar V, Langford SJ, Ahmad M, Lim YY. Optimization of reaction conditions for preparing carboxymethyl cellulose from sago waste. Carbohydrate Polymers. 2006;64:312-318.

49. Pei-Lang AT, Mohamed AMD, Karim AA. Sago starch and composition of associated components in palms of different growth stages. Carbohydrate Polymers. 2006;63:283-286.

50. Luo L, van det Voet E, Huppes G. Life cycle assessment and life cycle costing of bioethanol from sugarcane in Brazil. Renewable and Sustainable Energy Reviews. 2009;13:1613-1619.

51. Pandey A, Soccol CR, Nigam P, Soccol VT. Biotechnological potential of agro-industrial residues. I:sugarcane bagasse. Bioresource Technology. 2000;74:69-80.

52. Chisti Y. Biodiesel from microalgae beats bioethanol. Trends in Biotechnology. 2008;26:126-131.

53. Malekmohammadi I, Hosseini SJF, Yazdani S, Shadfar S. Analytical globalized state intervention model in rice production development. African Journal of Agricultural Research. 2011;6:2015-2025.

54. Kumar U, Bandyopadhyay. Sorption of cadmium from aqueous solution using pretreated rice husk. Bioresource Technology. 2006;97:104-109.

55. Sousa LdC, Chundawat SPS, Balan V, Dale BE. ‘Cradle-to-grave’ assessment of existing lignocellulose pretreatment technologies. Current Opinion in Biotechnology. 2009;20:339-347.

56. Taherzadeh MJ, Eklund R, Gustafsson L, Niklasson C, Lidén G. Characterization and fermentation of dilute-acid hydrolyzates from wood. Ind. Eng. Chem. Res. 1997;36:4659-4665.

57. Sheehan J, Himmel M. Enzymes, energy, and the environment: A strategic perspective on the U.S department of energy’s research and development activities for bioethanol. Biotechnol. Prog. 1999;15:817:827.

58. Duff SJB, Murray WD. Bioconversion of forest products industry waste cellulosics to fuel ethanol: a review. Bioresource Technology. 1996;55:1-33.

59. Ramos LP. The chemistry involved in the steam treatment of lignocellulosic materials. Quimica Nova. 2003;26:863–871.

60. Larsson S, Palmqvist E, Hahn-Hägerdal B, Tengborg C, Stenberg K, Zacchi G, Nilvebrant NO. The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. Enzyme and Microbial Technology. 1999;24:151-159.

61. Sreenath HK, Jeffries TW. Production of ethanol from wood hydrolyzate by yeasts. Bioresource Technology. 2000;72:253-260.

62. Taherzadeh MJ, Niklasson C, Lidén G. Conversion of dilute-acid hydrolyzates of spruce and birch to ethanol by fed-batch fermentation. 1999;69:59-66.

63. Lenihan P, Orozco A, O´Neill E, Ahmad MNM, Rooney DW, Walker GM. Dilute acid hydrolysis of lignocellulosic biomass. Chemical Engineering Journal. 2010;156:395-403.

64. Mosier NS, Ladisch CM, Ladisch MR. Characterization of acid catalytic domains for cellulose hydrolysis and glucose degradation. Biotechnol Bioeng. 2002;79:610-618.

65. Palmqvist E, Hahn-Hägerdal B. Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification. Bioresource Technology. 2000;74:17-24.

66. Stenberg K, Galbe M, Zacchi G. The influence of lactic acid formation on the simultaneous saccharification and fermentation (SSF) of softwood to ethanol. Enzyme and Microbial Technology. 2000;26:71-79.

67. García-Aparicio MP, Ballesteros M, Manzanares P, Ballesteros I, Gonzáles A, Negro MJ. Xylanase contribution to the efficiency of cellulose enzymatic hydrolysis of barley straw. Applied Biochemistry and Biotechnology. 2007;136-140:353-365.

68. Merino ST, Cherry J. Progress and challenges in enzyme development for biomass utilization. Adv Biochem Engin/Biotechnol. 2007;108:95-120.

69. Olsson L, editor. Biofuels. Adv Biochem Engin/Biotechnol. 2007;108:1-372

70. Mansfield SD, Mooney C, Saddler JN. Substrate and enzyme characteristics that limit cellulose hydrolysis. 1999;15:804-816.

71. Laser M, Schulman D, Allen SG, Lichwa J, Antal Jr. MJ, Lynd LR. A comparison of liquid hot water and steam pretreatments of sugar cane bagasse for bioconversion to ethanol. Bioresource Technology. 2002;81:33-44.

72. Martín C, Klinke HB, Thomsen AB. Wet oxidation as a pretreatment method for enhancing the enzymatic convertibility of sugarcane bagasse. Enzyme and Microbial Technology. 2007;40:426-432.

73. Mosier N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M, Ladisch M. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresource Technology. 2005;96:673-686.

74. Taherzadeh MJ, Karimi K. Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: a review. Int. J. Mol. Sci. 2008;9:1621-1651.

75. Galbe M, Zacchi G. A review of the production of ethanol from softwood. Appl.Microbiol Biotechnol. 2002;59:618-628.

76. Yu Y, Lou X, Wu H. Some recent advances in hydrolysis of biomass in hot-compressed water and its comparisons with other hydrolysis methods. Energy & Fuels. 2008;22:46-60.

77. Keshwani DR, Cheng JJ. Switchgrass for bioethanol and other value-added applications: A review. Biological Systems Engineering: Papers and Publications. 2009. University of Nebraska.

78. Silverstein RA, Chen Y, Sharma-Shivappa RR, Boyette MD, Osborne J. A comparison of chemical pretreatment methods for improving saccharification of cotton stalks. Bioresource Technology. 2007;98:3000-3011.

79. Olivier-Bourbigou H, Magna L, Morvan D. Ionic liquids and catalysis: Recent progress from knowledge to applications. Applied Catalysis A: General. 2010;373:1-56.

80. Zhu Z, Sathitsuksanoh N, Vinzant T, Schell DJ, McMillan JD, Zhang YHP. Comparative study of corn stover pretreated by dilute acid and cellulose solvent-based

lignocellulose fractionation: enzymatic hydrolysis, supramolecular structure, and substrate accessibility. 2009;103:715-724.

81. Lee SH, Doherty TV, Linhardt RJ, Dordick JS. Ionic liquid-mediated selective extraction of lignin from wood leading to enhanced enzymatic cellulose hydrolysis. Biotechnology and Bionengineering. 2009;102:1368-1376.

82.Wasserscheid P, Keim W. Ionic liquids – New “solutions” for transition metal catalysis. Angew. Chem. Int. Ed. 2000;39:3772-3789.

83. Brennecke JF, Maginn EJ. Ionic liquids. Innovative fluids for chemical processing. AIChE Journal. 2001;47:2384-2389.

84. Weingärtner H. Understanding ionic liquids at the molecular level: Facts, problems, and controversies. Angew. Chem. Ins. Ed. 2008;47:654-670.

85. Hayes DJ. An examination of biorefining processes, catalysts and challenges. Catalysis Today. 2009;145:138-151.

86. Huddleston JG, Visser AE, Reichert WM, Willauer HD, Broker GA, Rogers RD. Characterization and comparison of hydrophilic and hydrophobic room temperature ionic liquids incorporating the imidazolium cation. Green Chemistry. 2001;3:156-164.

87. Singh S, Simmons BA, Vogel KP. Visualization of biomass solubilization and cellulose regeneration during ionic liquid pretreatment of switchgrass. Biotechnology and Bioengineering. 2009;104:68-75.

88. Sun N, Rahman M, Qin Y, Maxim ML, Rodríguez H, Rogers RD. Complete dissolution and partial delignification of wood in the ionic liquid 1-ethyl-3methylimidazolium acetate. Green Chem. 2009;11:646-655.

89. Tan SSY, MacFarlane DR, Upfal J, Edye LA, Doherty WOS, Patti AF, Pringle JF, Scott JL. Extraction of lignin from lignocellulose at atmospheric pressure using alkylbenzenesulfonate ionic liquid. Green Chem. 2009;11:339-345.

90. Dadi AP, Schall CA, Varanasi S. Mitigation of cellulose recalcitrance to enzymatic hydrolysis by ionic liquid. Applied Biochemistry and Biotechnology. 2007;136-140:407-421.

91. Li C, Wang Q, Zhao ZK. Acid in ionic liquid: An efficient system for hydrolysis of lignocellulose. Green Chem. 2008;10:177-182.

92. Li Q, He YC, Xian M, Jun G, Xu X, Yang JM, Li LZ. Improving enzymatic hydrolysis of wheat straw using ionic liquid 1-ethyl-3-methyl imidazolium diethyl phosphate pretreatment. Bioresource Technology. 2009;100:3570-3575.

93. Zhao H, Jones CL, Baker GA, Xia S, Olubajo O, Person VN. Regenerating cellulose from ionic liquids for an accelerated enzymatic hydrolysis. Journal of biotechnology. 2009;139:47-54.

94. Laus G, Bentivoglio G, Schottenberger H, Kahlenberg V, Kopacka H, Röder T, Sixta H. Ionic liquids: Current developments, potential and drawbacks for industrial applications. Lenzinger Berichte. 2005;84:71-85.

95. Swatloski RP, Spear SK, Holbrey JD, Rogers RD. Dissolution of cellulose with ionic liquids. J. AM. CHEM. SOC. 2002;124:4974-4975.

96. Anderson JL, Ding J, Welton T, Armstrong DW. Characterizing ionic liquids on the basis of multiple solvation interactions. J. AM. CHEM. SOC. 2002;124:14247-14254

97. Joglekar HG, Rahman I, Kulkarni BD. The path ahead for ionic liquids. Chem. Eng. Technol. 2007;30:819-828.

98. Zhao H, Jones CL, Baker GA, Xia S, Olubajo O, Person VN. Regenerating cellulose from ionic liquids for an accelerated enzymatic hydrolysis. Journal of biotechnology. 2009;139:47-54.

99. Gámez S, González-Cabriales JJ, Ramírez JA, Garrote G, Vázquez M. Study of the hydrolysis of sugar cane bagasse using phosphoric acid.

100. Hamelinck CN, Hooijdonk GV, Faaij AP. Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle- and long-term. Biomass and Bioenergy. 2005;28:384-410.

101. Montgomery DC. Welter J editor. Design and analysis of experiments. United States: John Wiley & Sons Inc; 2005.

102. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry. 1959;31:426-428.

103. Peak D. Fourier transform infrared spectroscopy. Elsevier Ltd. 2004:1-6.

104. Zhao X, Zhang L, Liu D. Pretreatment of siam weed stem by several chemical methods for increasing the enzymatic digestibility. Biotechnol. J. 2010;5:493-504.

105. Kumar R, Mago G, Balan V, Wyman CE. Physical and chemical characterizations of corn stover and poplar resulting from leading pretreatment technologies. Bioresource Technology. 2009;100:3948-3962.

106. Oh SY, Yoo DI, Shin Y, Kim HC, Kim HY, Chung YS, Park WH, Youk JH. Crystalline structure analysis of cellulose treated with sodium hydroxide and carbon dioxide by means of X-ray diffraction and FTIR spectroscopy. Carbohydrate Research. 2005;340:2376-2391.

107. Hayes TL. Scanning electron microscope. In AccessScience. McGraw-Hill Companies; c2008.[Cited 2011 Oct 26]. Available from http://www.accessscience.com

108. Yang H, Yan R, Chen H, Lee DH, Zheng C. Characteristics of hemicellulose, cellulose and lignin pyrolysis. Fuel. 2007;86:1781-1788.

109. Adel AM, Abd El-Wahab ZH, Ibrahim AA, Al-Shemy MT. Characterization of microcrystalline cellulose prepared from lignocellulose materials. Part I. Acid catalyzed hydrolysis. Bioresource Technology. 2010;101:4446-4455.

110. Hsu TC, Guo GL, Chen WH, Hwang WS. Effect of dilute acid pretreatment of rice straw on structural properties and enzymatic hydrolysis. Bioresource Technology. 2010;101:4907-4913.

111. Colom X, Carillo F, Noguéz F, Garriga P. Structural analysis of photodegraded wood by means of FTIR spectroscopy. Polymer Degradation and Stability. 2003;80:543-549.

112. Kuo CH, Lee CK. Enhancement of enzymatic saccharification of cellulose by cellulose dissolution pretreatments. Carbohydrate Polymers. 2009;77:41-46.

113. Su Y, Brown HM, Huang X, Zhou XD, Amonette JE, Zhang ZC. Single-step conversion of cellulose to 5-hydroxymethylfurfural (HMF), a versatile platform chemical. Applied Catalysis A: General. 2009;361:117-122.

114. Kim JW, Mazza G. Optimization of phosphoric acid catalyzed fractionation and enzymatic digestibility of flax shives. Industrial Crops and Production. 2008;28:346-355.