LOW-TEMPERATURE ALKALI PEROXIDE PRETREATMENT OF LIGNOCELLULOSIC BIOMASS IN AQUEOUS-ORGANIC SOLVENT SYSTEMS

MS. JARUWAN DAMAURAI
ID: 55300700606

**A THESIS SUBMITTED AS A PART OF THE REQUIREMENTS
FOR THE DEGREE OF MASTER OF SCIENCE
IN ENERGY TECHNOLOGY AND MANAGEMENT**

**THE JOINT GRADUATE SCHOOL OF ENERGY AND ENVIRONMENT
AT KING MONGKUT'S UNIVERSITY OF TECHNOLOGY THONBURI**

2ND SEMESTER 2013

Low-temperature alkali peroxide pretreatment of lignocellulosic biomass in aqueous-organic solvent systems

Ms. Jaruwan Damaurai
ID 55300700606

A Thesis Submitted as a Part of the
Requirements for the Degree of Master
of Philosophy in energy technology

The Joint Graduate School of Energy and
Environment at King Mongkut's University of
Technology Thonburi

2nd Semester 2013

Thesis Committee

…….	
(Assoc. Prof. Dr. Navedol Laosiripojana)	Advisor
……..…	
(Dr. Verawat Champreda)	Co-Advisor
…….	
(Assoc. Prof. Dr. Worapon Kiatkittipong)	Member
…….	
(Dr. Navin Viriya-empikul)	Member
…….....	
(Dr. Vasimol Ruanglek)	External Examiner

Thesis Title: Low-temperature alkali peroxide pretreatment of lignocellulosic biomass in aqueous-organic solvent systems

Student's name, organization and telephone/fax numbers/email

Miss Jaruwan Damaurai

The Joint Graduate School of Energy and Environment (JGSEE)

King Mongkut's University of Technology Thonburi (KMUTT)

126 Pracha Uthit Rd., Bangmod, Tungkru, Bangkok 10140 Thailand

Telephone: 0-8704-95767

Email: jaruwan.damaurai@hotmail.com

Supervisor's name, organization and telephone/fax numbers/email

Assoc. Prof. Dr. Navadol Laosiripojana

The Joint Graduate School of Energy and Environment (JGSEE)

King Mongkut's University of Technology Thonburi (KMUTT)

126 Pracha Uthit Rd., Bangmod, Tungkru, Bangkok 10140 Thailand

Telephone: 0-2872-9014 or 02-470-8309-10 ext. 4146

Email: navadol_l@jgsee.kmutt.ac.th

Co-advisor's name, organization and telephone/fax numbers/email

Dr. Verawat Champreda

National Center for Genetic Engineering and Biotechnology

113 Phahonyothin Rd., Klong Luang, Pathumthani 12120, Thailand.

Telephone Number: 02-564-6700 ext. 3473

Email: verawat@biotec.or.th

Topic: Low-temperature alkali peroxide pretreatment of lignocellulosic biomass in aqueous-organic solvent systems

Name of student: Ms. Jaruwan Damaurai **Student ID**: 55300700606

Name of supervisor: Assoc. Prof. Dr. Navadol Laosiripojana

Name of co-supervisor: Dr. Verawat Champreda

ABSTRACT

Lignocellulosic biomass represents a promising renewable feedstock for conversion to biofuels and chemicals in biorefineries. Due to the recalcitrant nature of plant biomass, a pretreatment step is required in order to enhance the enzymatic hydrolysis efficiency of biomass in order to achieve a feasible level of fermentable sugars. Alkaline hydrogen peroxide (AHP) pretreatment is an effective low-energy chemical pretreatment method with the main effects on delignification of biomass and partial hydrolysis of hemicelluloses. In this study, the effects of key reaction parameters (solid loading, H_2O_2 concentration, temperature, and time) on efficiency and selectivity of AHP pretreatment of rice straw using the conventional water-based process was studied and modification of the process with the use of co-solvents and organic alkalines were further explored. The highest reducing sugar yield of 984.14± 8.17 mg/g was achieved from enzymatic of the pretreated rice straw under the optimized reaction for the conventional AHP process (5% w/v solid, 7.5% v/v H_2O_2 incubated at 35°C for 18 h), leading to 82% glucose recovery and 16% xylose recovery from the native biomass. The use of co-solvents (ethanol, isopropanol, and tert-butyl alcohol) and organic alkalines (ammonium hydroxide and triethylamine) resulted in lower glucose recovery (21-63%) and lower xylose recovery (8-30%) from the native rice straw compared to the conventional water-based/NaOH process. The modified AHP reaction using ammonium hydroxide as an alternative alkaline in aqueous reaction under the conditions containing 7.5% w/v solid, 2.5% w/v H_2O_2, incubated at 35°C for 24 h) led to reduction in reducing sugar yield, equivalent to 459.54±4.67 mg/g pretreated biomass and 41% glucose recovery. AHP pretreatment led to increasing enzyme accessibility to the cellulose microfibers and reduction in cellulose crystallinity as shown by scanning electron microscopy and X-ray diffraction analysis. The work demonstrates the potential of the developed process for pretreatment of rice straw for further conversion to value-added products in biorefineries.

Keywords: Alkaline hydrogen peroxide; Biorefinery; Lignocellulose; Pretreatment; Rice straw

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation and sincere gratitude to my advisor, Assoc. Prof. Dr. Navadol Laosiripojana (the Joint Graduate School of Energy and Environment, JGSEE) and my co-advisor, Dr. Verawat Champreda (National Center for Genetic Engineering and Biotechnology, BIOTEC) for giving me an opportunity to be under their supervision. Without their encouragement, my graduate study and thesis would not have materialized. I would like to thank them for their kindness, motivation, understanding, patience and most importantly, their continuous supporting during my study. I also would like to thank them for teaching me numerous lessons, insightful guidance and suggestions. This valuable study would not been successful without the guidance and the kindly help. Their invaluable lessons, about both of academicals and livelihood, are really inspired, practical and helpful. I can use their guidance throughout my study, life, and further in my career.

I would like to thank my examining committee, Asst. Prof. Dr. Worapon Kiatkittipong, Department of Chemical Engineering, Faculty of Engineering, Silpakorn University, Dr. Nawin Viriya-empikul, National Nanotechnology Center (NANOTEC), National Science and Technology Development Agency (NSTDA) and the thesis external examiner, Dr. Vasimon Ruanglek from SCG Chemicals Co. Ltd. for their kindness, helpful discussions, comments and valuable suggestion.

I am also deeply thankful to my friends and especially, all members of Enzyme Technology Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC) for their assistance, guidance and importantly, their friendship during I have done the research there. It is the one of the best memory in my life.

My deepest appreciation and sincere gratitude go to my parents, who always love, encourage and also sharing the hard times with me.

Finally, I would like to express my gratitude to The Joint Graduate School of Energy and Environment (JGSEE), King Mongkut's University of Technology Thonburi under the JGSEE Research Scholar for the gratefully acknowledge the financial support.

CONTENTS

CONTENTS (Cont')

LIST OF TABLES

LIST OF TABLES (Cont')

LIST OF FIGURES

LIST OF FIGURES (Cont')

LIST OF FIGURES (Cont')

LIST OF FIGURES (Cont')

CHAPTER 1
INTRODUCTIONS

1.1. Rational/Problem Statement

Nowadays, the world faces an energy and environmental crisis due to the depletion of petroleum resources and the increasing of the oil price. These problems exist from overconsumption when global demand for energy grows rapidly as the expanding of human population and increasing of industrial prosperity in developing countries making the oil production cannot meet the projected demand. Since, the main energy is still supplied from conventional fossil fuels such as oil, coal and natural gas, the utilization of fossil fuels over the last century and following year has extremely increased the level of air pollution and greenhouse gases accumulation in the atmosphere [1]. Consequently, the global average near-surface atmospheric temperature has risen.

There have been a number of researchers focusing on both adequacy of energy supply long-term and also the environmental implications. A lot of efforts have been carried out to develop clean sustainable renewable energy to be an alternative fuel for the current energy instead of conventional fuels. There are several strategies to minimize energy and environmental problems. One of the current interesting solutions is initiation of new alternative fuels for replacing gasoline from renewable feedstocks [2]. Several countries have been attempting to develop bioethanol which is the most practical biofuels. Although bioethanol production has been greatly improved by new technologies, there are still challenges that need further investigations [3]. The utilization of lignocellulosic materials as renewable resources for ethanol production motivates a great deal of interest because it is one of the most abundant carbon sources and non-food bio-feedstock does not compete with food supply. A variety of pathways can be applied to convert cellulosic biomass fuels and value added chemicals. An overview of energy that can be produced from lignocellulosic residues by thermochemical or biochemical processing including liquid fuels such as ethanol or pyrolysis oil, gaseous fuels such as biogas (methane) and electricity is shown in Fig. 1.1. Among various local lignocellulosic materials, a variety of agricultural residues are considered to be patent feedstock for bioethanol production in Thailand (Table 1.1). Lignocellulosic materials can also be converted to a range of various chemical and fuel production. Moreover, the uses of these biomasses have the benefits on disposal of

problematic solid wastes providing power opportunity to improve energy security, reducing the trade deficit, dramatically reducing greenhouse gas emissions, and improving agricultural products [4].

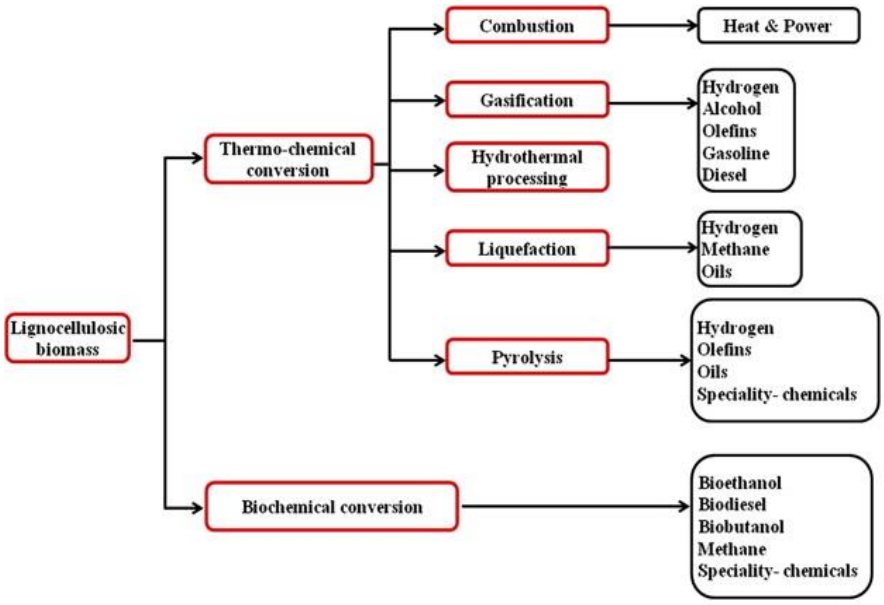


Fig. 1.1. Thermochemical and biological processing of lignocellulosic biomass [5]

Table 1.1 Biomass residues from rice, sugar cane, and palm oil in Thailand, 2004 [6]

(Unit: 1,000 tons per year)

Types	Production	Agricultural residues	Residues
Sugar cane	70,101	Bagasse	20,399
		Trash	21,171
Rice	26,841	Rice husk	6,173
		Rice straw	11,998
Palm Oil	4,903	Empty fruit bunches	1,226
		Fiber	721
		Shells	240
		Fronds	12,767
Total			74,695

Lignocellulosic biomass, which is the most economical and highly renewable natural resource on earth, is considered to be a promising alternative energy resource for Thailand. These include a variety of agricultural wastes, wood wastes, and forestry residues [7]. Lignocellulosic materials have a complex structure consisting of three primary chemical fractions or precursors: (a) cellulose, a linear homopolymer of glucose molecules linked together in long parallel chains that are hydrogen-bonded to one another in a crystalline structure to form into highly organized microfibers, (b) hemicellulose, a branched heteropolymer of pentoses and hexoses, and (c) lignin, a complex polymer of phenolic subunits, shielding plants from external stresses and microbial attacks [8]. In addition to other minor components such as ash, and a small amount of proteins which do not participate significantly in forming the structure of the biomass. There is a significant variation of the lignin, hemicellulose, and cellulose content which highly depends on its source. This complex structure results in recalcitrance of biomass to enzymatic hydrolysis. Table 1.2 shows the composition of various lignocellulosic feedstocks. Conversion of cellulose and hemicellulose materials into fermentable sugars is an important process because these fermentable sugars extracted from biomass feedstock are key intermediates for further conversion to biofuel and other biorefinery products. The main process that used to break down hemicellulose and cellulose then convert to sugars is hydrolysis. Lignocellulosic material is highly recalcitrant to hydrolyze due to its rigid structure, particular by lignin which acts as physical barrier against hydrolytic enzymes penetration to the biomass microstructure. Therefore, an effective pretreatment technology is required in order to release the carbohydrates from lignin association and disrupt the cellulose structure so enzymes can penetrate the cell wall and depolymerize cellulose and hemicelluloses which will lead to the increasing of fermentable sugars from the enzymatic hydrolysis step [9].

Table 1.2 Composition of representative lignocellulosic feedstocks [10]

Lignocellulosic materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwood stems	40-55	24-40	18-25
Softwood stems	45-50	25-35	25-35
Corn cobs	45	35	15
Grasses	25-40	35-50	10-30
Paper	85-99	0	0-15
Wheat straw	30	50	15
Sorted refuse	60	20	20
Leaves	15-20	80-85	0
Cotton seed hairs	80-95	5-20	0
Newspaper	40-55	25-40	18-30
Waste papers from chemical pulps	60-70	10-20	5-10
Solid cattle manure	1.6-4.7	1.4-3.3	2.7-5.7
Switchgrass	45	31.4	12.0

Pretreatment technology is a prerequisite to facilitate the release of sugars from lignocellulosic biomass prior to enzymatic hydrolysis and fermentation. Different pretreatment methods are used to improve the availability of cellulose for enzymatic hydrolysis. These include biological pretreatments, physical pretreatments, chemical pretreatments, and physical-chemical pretreatments. These pretreatment methods are applied on different lignocellulosic biomass with different advantages and limitations. The best pretreatment for an individual biomass needs to be considered in terms of type of the lignocellulosic biomass, cost, safety, waste disposal concern, and desired products [11].

Pretreatment methods that focus on lignin removal and hemicellulose solubilization become more interesting because enzymatic digestibility is strongly related to lignin content, and that lignin removal greatly enhances enzymatic hydrolysis. It is also known that alkaline peroxide provides an effective approach delignification and hemicellulose hydrolysis [12] and is considered a promising approach for low-energy pretreatment of lignocelluloses. In this research, alkaline peroxide pretreatment for enzymatic saccharification of rice straw was developed based by comparing the use of conventional aqueous system with the aqueous-organic solvent systems as well as the use of different alkalines to increase the enzymatic saccharification of rice straw and enable efficient conversion to sugars.

1.2. Literature review

As described, the main objective of this research is to study the effect of alkaline hydrogen peroxide (AHP) pretreatment of rice straw using different alkaline and aqueous or aqueous/organic solvent systems based on enzymatic digestibility and sugar recovery. In this work, the optimal conditions of alkaline peroxide of rice straw was investigated. The previous researches related to this study are reviewed as follows.

1.2.1. Pretreatment

Utilization of lignocellulosic residues in a bioconversion process requires the pretreatment of raw materials which is an important step to promote the production of sugars by enzymatic saccharification [9]. Different pretreatment methods are used to improve the availability of cellulose for enzymatic hydrolysis by disruption of the recalcitrant material of the biomass. The pretreatment step results in an increase in the accessible surface area, cellulose decrystallization, partial cellulose depolymerization, hemicellulose and lignin solubilization, and modification of the lignin structure, which in overall lead to improving enzymatic digestibility of the biomass. The pretreatments are classified into physical, physical-chemical, chemical and biological processes. The applied methods usually use combination of different principles, such as mechanical together with thermal and chemical effects in order to achieve high sugar release efficiencies, low toxicants production, and low energy consumption [13].

Alkaline peroxide pretreatment

Alkaline peroxide pretreatment (AHP) is an environmentally friendly and effective process for pretreating lignocellulose materials. It utilizes lower temperature and pressure compared to other pretreatment technologies. The major effect of the alkaline peroxide pretreatment is the removal of lignin in cell wall substrates, thus improving the reactivity of remaining polysaccharides [14]. In order to improve efficiency of enzymatic hydrolysis, many factors (oxidizing agent concentration, pretreatment temperature, duration time, and solid loading) need to be optimized. The previous studies are presented below.

1.2.1.1 Optimization for fermentable sugar production

Sauro et al. (2013) evaluated the effects of peroxide concentration (2.5, 5, 7.5%, v/v) and reaction time for the conversion of rice hulls to simple sugars through enzymatic hydrolysis for the subsequent ethanol production. Peroxide concentration showed a positive effect on degradation of rice hulls structure providing a greater access of enzyme. The highest yield was attained from 7.5%, v/v hydrogen peroxide concentration, at pH 11.5, 90ºC for 1 h [15].

Saha et al. (2006) evaluated alkaline peroxide pretreatment and enzymatic saccharification for conversion of wheat straw cellulose and hemicellulose to fermentable sugars. The effects of alkaline H_2O_2 level (0-4.3%, v/v) on the pretreatment of wheat straw (8.6%, w/v) at pH 11.5 and 35ºC for 24 h were evaluated. Fig.1shows that sugar yield increased with increasing H_2O_2 up to 2.15 (v/v).

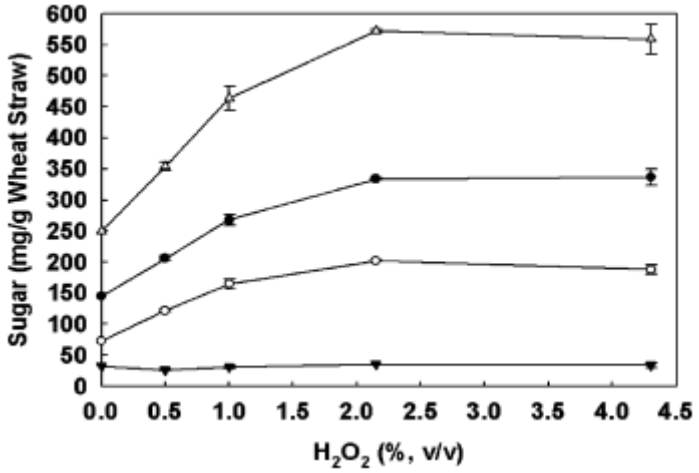


Fig. 1.2. Effect of hydrogen peroxide level (0-4.3%, v/v) for the pretreatment (pH 11.5, 35ºC, and 24 h) of wheat straw (8.6%, w/v) on its enzymatic saccharification (45ºC, pH 5.0, 120 h). Symbols used: ●, glucose; ○, xylose; ▼, arabinose; and △, total sugar.

The effect of duration of alkaline peroxide pretreatment on the enzymatic saccharification of wheat straw at 25 and 35ºC on its enzymatic saccharification (45ºC, pH 5.0, 120 h) were also investigated. The result is presented in Fig.1.3, and it is evident that the longer the pretreatment time led to the better yield of sugars by enzymatic saccharification [16].

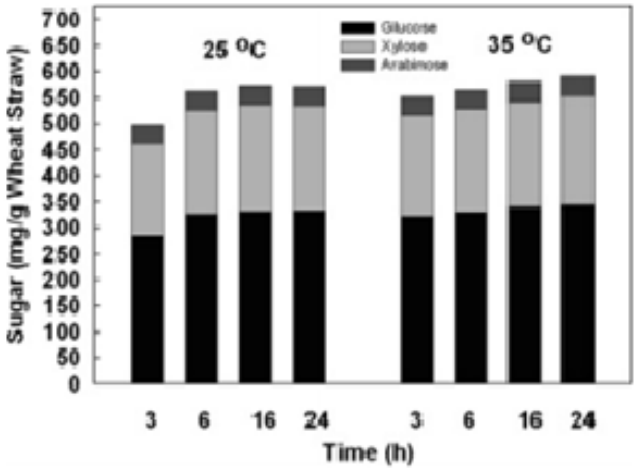


Fig. 1.3. Effect of duration of alkaline hydrogen peroxide pretreatment (2.15%, v/v; pH 11.5) of wheat straw (8.6%, w/v) at two temperature (25 and 35ºC) on its enzymatic saccharification (45ºC, pH 5.0, 120 h).

Saha et al. (2007) studied the parameters of alkaline peroxide pretreatment that affects the conversion of rice hull cellulose and hemicellulose to simple sugars. Initially, the effect of duration (6 and 24 h) of alkaline peroxide pretreatment (7.5%, v/v H_2O_2, pH 11.5) on the enzymatic saccharification of rice hull (15%, w/v) at 25 and 35ºC was studied. The resultant yield of total sugars in terms of mg per g rice hulls after enzymatic saccharification at 45ºC, pH 5.0 for 72 h were 149 ± 9 and 203 ± 12 for 6 and 24 h pretreatment, respectively at 25ºC. The total sugar yields after enzymatic saccharification under the same conditions were 211 ± 4 and 249 ± 1 mg/g hulls for 6 and 24 h pretreatment, respectively at 35ºC. It is evident that the effect of pretreatment time is more pronounced at 25ºC than at 35ºC. However, the yield of total sugars at 35ºC was much improved for both 6 and 24 h pretreatment in comparison to the same at 25ºC. Based on this result, it was decided to use 7.5% H_2O_2 (v/v) at 35ºC for 24 h for pretreating rice hulls (15%, w/v).

The effects of alkaline H_2O_2 levels (0-10%, v/v) on the pretreatment of rice hulls (15.0% w/v) at 35ºC for 24 h on enzymatic saccharification at 45ºC and pH 5.0 for 72 h is present in Fig. 1.4. It was found that the sugar yield was maximal at 7.5%, v/v H_2O_2. The individual sugar, as well as the total sugar yields were lower at both 3.75 and 10%, v/v H_2O_2 pretreatment [17].

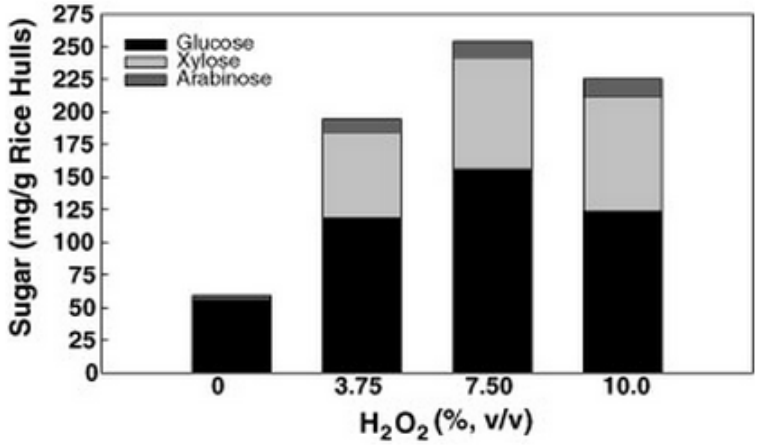


Fig. 1.4. Effect of hydrogen peroxide level (0-10.0, v/v) for pretreatment (pH 11.5, 35ºC, 24 h) of rice hulls (15%, w/v) on enzymatic saccharification (45ºC, pH 5.0, 72 h). A cocktail of three commercial enzymes was used.

Da Costa et al. (2013) studied parameters of alkaline peroxide pretreatment of cashew apple bagasse for ethanol production, which was evaluated on the conversion of the resultant cellulose into glucose. The effects of the concentration of hydrogen peroxide at pH 11.5, biomass loading and pretreatment duration performed at 35ºC and 250 rpm were evaluated after the subsequent enzymatic saccharification of the pretreated biomass using a commercial cellulase. The CAB used in this study contained 20.56 ± 2.19 % cellulose, 10.17 ± 0.89 % hemicellulose and 35.26 ± 0.90 % lignin. The pretreatment resulted in reduced lignin content in the residue solids. Increasing the H_2O_2 concentration (0-4.3%, v/v) resulted in the lowest (35%) mass yield of the pretreated solids, which indicates that 65% of bagasse mass was solubilized (Table 1.4). Lignin was the main solubilized component under this pretreatment condition. The mass yield of lignin in the pretreated solids was only 6.97 g; because the original composition of lignin in the raw CAB was 35.26 ± 0.9%, this result reveals that 80.2% of the lignin that was initially contained in the CAB was solubilized.

Table 1.3 Chemical composition in nature of bagasse and composition of bagasse that was pretreated using different hydrogen peroxide concentrations (0-4.3%, v/v) pH 11.5. The pretreatment was performed at 35ºC for 24 h using a biomass loading of 10%, w/v.

Components	Untreateded	0.0% H2O2 (v/v)			0.645% H2O2			2.15% H2O2 (v/v)			4.3% H2O2 (v/v)		
	Content (w/w)	Content (w/w)	Mass yield (g)	Losses	Content (w/w)	Mass yield (g)	Losses	Content (w/w)	Mass yield (g)	Losses	Content (w/w)	Mass yield (g)	Losses
Dry matter	100	100	97.61		100	82.49		100	53.56		100	35.14	
Cellulose	20.56± 2.19	20.55±0.77	20.06	2.44	26.81±1.77	22.12	0	28.99±1.17	20.61	0	32.94±0.44	11.57	43.70
Hemicellulose	10.17±0.89	9.29±1.62	9.07	10.84	15.78±4.13	13.02	0	15.49±0.97	11.04	0	11.22±0.61	3.94	61.23
Lignin	35.26±0.9	42.07±2.14	41.06	0	35.72±0.86	29.47	16.43	37.41±0.37	26.68	24.32	19.83±0.53	6.97	80.23
Extractives	7.79±0.60	8.34±0.64	8.14		4.43±0.06	3.65		6.37±0.06	4.54		8.73±0.43	3.07	
Ash	1.62±0.07	7.71±1.177	7.53		2.23±0.09	1.84		3.94±0.10	2.81		4.96±0.35	1.74	
Total	75.4±0.84	87.96±0.63	85.85		84.97±0.70	70.09		92.11±0.43	65.7		77.68±0.25	27.30	

The effect of alkaline peroxide pretreatment concentration (0-4.3%, v/v) on the enzymatic saccharification of CAB (10%, w/v) that was pretreated at 35ºC for 24 h was studied and shown in Fig 1.5. The pretreatment led to conversions higher than 50.7% after 72 h, whereas only 2.27% of the initial cellulose of the untreated CAB was hydrolyzed. An increase in the enzymatic digestibility was observed with increasing H_2O_2 concentration and increasing saccharification time [17].

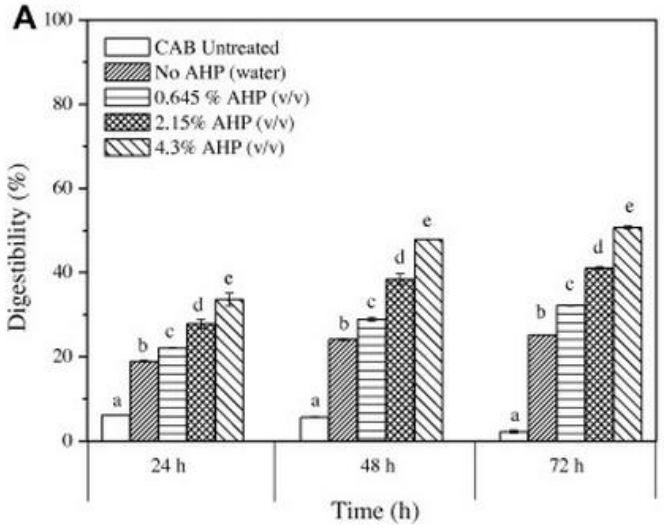


Fig. 1.5. Effect of the AHP concentration used for the pretreatment (pH 11.5, 35ºC, 24 h and 250 rpm) of CAB on cellulose digestibility on enzymatic hydrolysis (45ºC, 150 rpm and pH 4.8) of untreated CAB and pretreated CAB (10%, w/v) pretreated [17].

Ana Diaz et al. (2013) studied the effects of peroxide concentration and reaction time. Table 1.6 shows the results obtained when rice husks were pretreated for 2 h with alkaline solution (pH 11.5) at 90ºC at different peroxide concentrations (0, 2.5, 5.0 7.0%, v/v). It can be observed that the increasing of hydrogen peroxide concentration led to increasing solubilization of biomass components, which was highest (1.5821 g) at the highest H_2O_2 concentration. Thus, when peroxide concentration increased from 0 to 7.5% w/v, there was more solubilization of lignin and other compounds, decreasing cellulose crystallinity and improving its enzymatic digestibility [18].

Table 1.4 Effect of hydrogen peroxide concentration.

$[H_2O_2]$ (%v/v)	Solubilized compounds (g)	Hydrolysis Yield (%)
0	0.1350	3.54
2.5	1.0974 ± 0.0121	5.84 ± 1.47
5	1.2006 ± 0.0764	10.07 ± 3.40
7.5	1.5821 ± 0.0739	71.15 ± 5.57

The effect pretreatment time was also evaluated at 7.5%, v/v H_2O_2, which was defined as the optimal H_2O_2 concentration (Table 1.4). According to the data, the higher the pretreatment time, the higher the solubilized compound.

Table 1.5 Effect of the pretreatment time [16]

Time (h)	Solubilized compounds(g)	Hydrolysis Yield (%)
0.25	1.4897 ± 0.0380	69.01 ± 5.11
1	1.4320 ± 0.0378	81.60 ± 2.63
2	1.5821 ± 0.0739	71.15 ± 5.57
4	1.5442 ± 0.0191	76.80 ± 7.46

1.2.2. Hydrolysis

Enzymatic hydrolysis is the second step in the production of ethanol from lignocellulosic materials. It refers to the processes that convert the polysaccharides into monomeric sugars. The main hydrolysis product of cellulose is glucose, which can be obtained either enzymatically by cellulolytic enzymes or chemically by sulfuric or other acids. Hemicellulose, a branched polymer composed of (5-carbon) and hexose (6-carbon) sugars, can be hydrolyzed by hemicellulases or acids to release its component sugars. The fermentable sugars obtained from hydrolysis processes could be fermented into ethanol.

Saha and Cotta (2007) studied alkaline H_2O_2 pretreatment and enzymatic saccharification methods for conversion of rice hull cellulose and hemicelluloses to simple sugars.

The yield of sugars from diluted alkaline peroxide pretreated (7.50%, v/v H_2O_2; pH 11.5; 35ºC; 24 h) rice hulls (15.0%, w/v) after enzymatic saccharification (45ºC, pH 5.0, 72 h) by three commercial enzyme preparations (cellulase, β-glucosidase, and xylanase) using 0.12 ml of each enzyme preparation per g hulls was 428±12 mg/g (90% yield). The almost complete conversion (96%) of rice hulls to sugars was achieved by saccharifying the liquid and solid fractions separately after alkaline peroxide pretreatment [14].

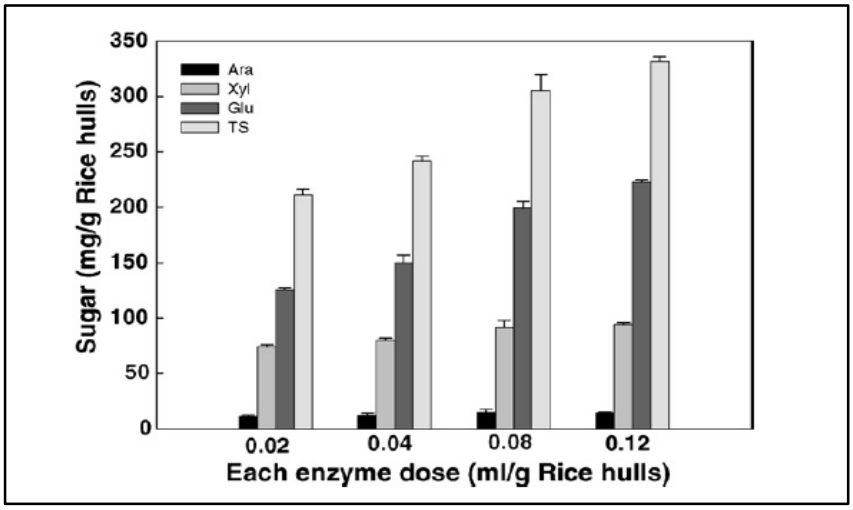


Fig. 1.6. Effect of each enzyme on the release of sugars at 72 h from AHP pretreated (7.5%, w/v; pH 11.5; 35^{o}C, 24 h) rice hulls (15.0%, w/v). A cocktail of three commercial enzyme preparations (cellulase, β-glucosidase and xylanase) was used [14].

1.3. Research Objective

The aims of this research are:

Alternative organic solvent

(1) To study the effect of co-solvent on solubility of lignin and hemicellulose fraction which would affect the pretreatment efficacy.

(2) The organic solvent was selected from various alcohols with different polarities and have been repeated for use in organosolv process.

Alternative alkaline

To study the use of organic alkaline with high volatility that could allow simple recovery.

1.4. Scope of Research Work

Alkaline-H_2O_2 pretreatment: Effects of reaction media i.e. solvent systems on efficiency of alkaline-H_2O_2 pretreatment were firstly evaluated in different aqueous-organic solvent systems using various alcohols under a fixed pre-defined reaction conditions. The reactions were screened based on enzymatic digestibility of the pretreated biomass under a

standard condition with *Accellerase® 1500* as sugar recovery yield from the native biomass. Released reducing sugars were analyzed using dinitrosalicylic acid (DNS) method [15] and the sugar profiles will be analyzed by HPLC.

Optimization of pretreatment parameters: Reaction parameters and conditions for pretreatment of rice straw including alkaline and solvent compositions, oxidizing agent concentration, temperature, time, and solid loading were optimized The experiments were designed and analyzed using Minitab software. The solid fraction was analyzed for compositions using NREL method and characterized by scanning electron microscope (SEM) and X-ray diffraction (XRD) analysis.

CHAPTER 2

THEORIES

2.1. Biofuel

As the world population increases, supporting economic growth expands, and energy consumption increases, most of the required energy is still acquired from fossil fuels but carbon dioxide (CO_2) emissions from widespread industrial consumption of fossil fuels (coal oil, and natural gas) is likely to continue to be a major contributor of greenhouse gases. Utilization of many types of renewable energy (wind, solar, etc.) is expanded. However they can be utilized to generate electricity, but not liquid transport fuels. Consequently, biomass has received much attention as a feedstock for biofuels (e.g. bioethanol and biodiesel).

2.2. Bioethanol

Bioethanol (ethyl alcohol) is a renewable energy source and the most-used liquid biofuel in the world. It can be produced from raw materials containing starch components and fermentable sugar, such as sugarcane and corn. It is also made from potato, rice, beetroot, and banana depending on the local availability. Ethanol can be directly employed as a fuel in vehicles or as gasoline oxygenate to increase the fuel oxygen content and allow for a better hydrocarbon oxidation that reduces the amount of aromatic compounds and carbon monoxide released into the atmosphere. For this reason, fuel grade ethanol is the market with the most rapid growth rate in America and Europe. However, the production of bioethanol from food crops such as grains (first generation biofuels) has resulted in an undesirable direct competition with food supply. A switch to a more abundant plant material which is lignocellulosic residue reduces pressure on the food crops. Large parts of these plant materials are made up of complex carbohydrates such as cellulose and hemicelluloses which can be converted to fermentable sugars [17].

2.3. Structure and chemistry of lignocellulosic biomass

Lignocellulosic biomass is made up of very complex biopolymers. It is primarily composed of cellulose, hemicellulose, and lignin in addition to a small amount of extractives, acids, and minerals [18]. Figure 2.1 shows the physical structure arrangement of lignocellulosic biomass. Figure 2.2 shows a general structure of lignocellulosic material.

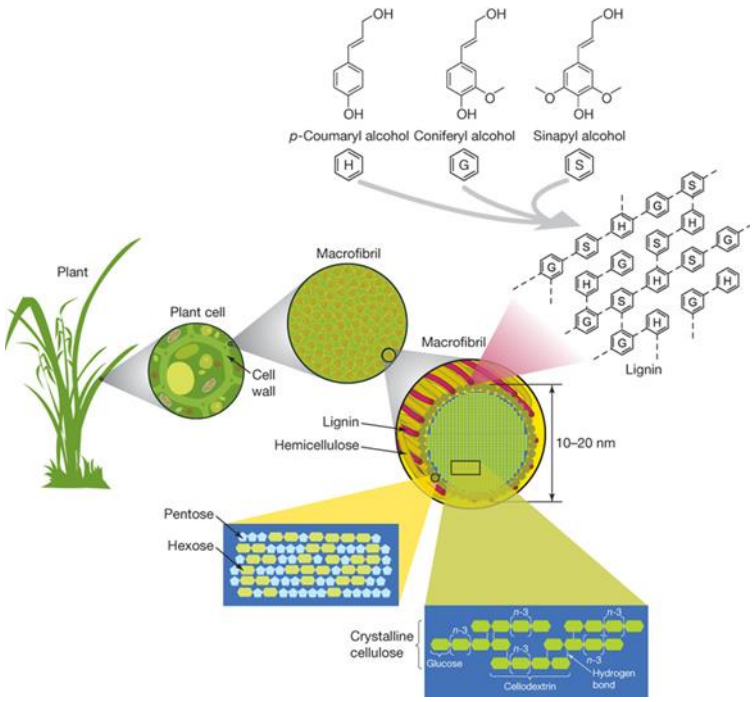


Fig. 2.1. Physical structure arrangement of lignocellulosic biomass [19].

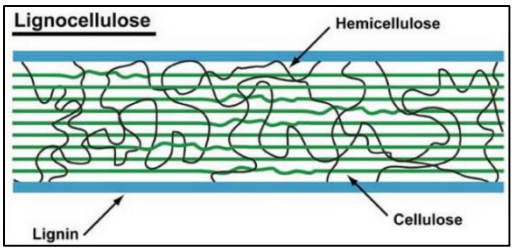


Fig. 2.2. Schematic structure of lignocellulosic material [20].

Cellulose is the most abundant component of lignocellulosic biomass, typically comprising 40-50% of the feedstock [21]. It is a polymer of glucose sugar molecules linked together via β-(1.4) bonds in long, straight parallel chains that are hydrogen-bonded to one another in a crystalline structure to form long fibers. It can be considered that the cellulose is a linear polymer made up of cellubiose monomers as shown in Figure 2.2.

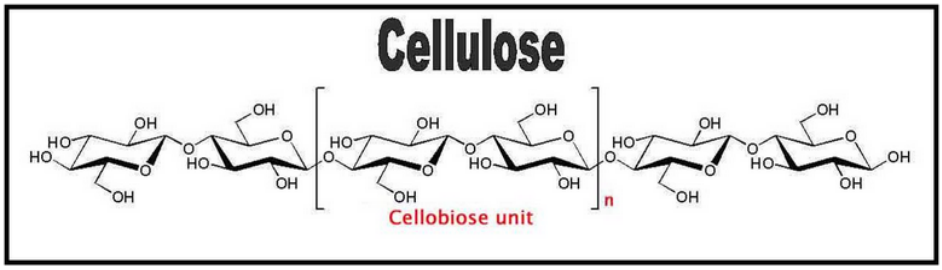


Fig. 2.3. Structure of single cellulose molecule [22].

Due to its linear nature and to the interactions by hydrogen bonds between the OH groups of the same chain or of the different chains, cellulose molecules are oriented by length leading to the formation of very stable crystalline structures [23]. These structures allow the bundles of cellulose chains to form rigid, difficult to break microfibers that contribute greatly to the recalcitrance of biomass. For this reason, the main function of cellulose in plants is structural, which explains its majority presence in the cell wall.

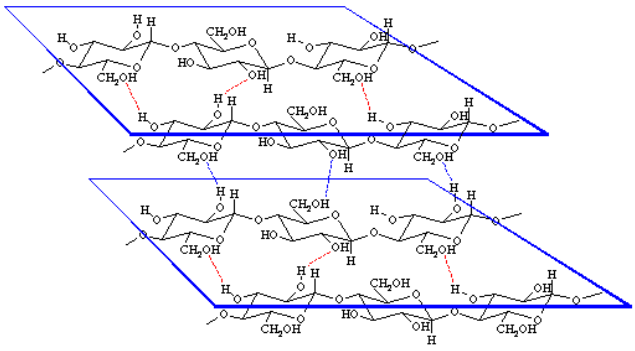


Fig. 2.4. Demonstration of the hydrogen bonding
that allows the parallel arrangement of the cellulose polymer chains [24].

Hemicellulose is the second-most abundant of lignocellulosic biomass, comprising 20-40%. Similar to cellulose, it is a carbohydrate polymer that have variable compositions and structure depending on the plant source [25]. Moreover, it represents a potential source of sugars for producing fuel and chemicals and it is highly digestible compared to cellulose. It is a branched amorphous polymer composed of xylose and arabinose (either 5-carbon sugars or pentoses), and galactose, glucose, and mannose (these latter sugars are hexoses) [26]. Other carbohydrate compounds like glucuronic acids are also present in hemicellulose structure. Furthermore, acetyl groups are esterified to some OH groups of its different sugars. Due to the predominance of xylose, hemicellulose can be considered as a xylan. For lignocellulosic materials derived from hardwood, the xylan backbone is composed of xylose units linked by β-(1, 4) bonds that branch through α-(1, 2) bonds with the methyl glucurunic acid [27]. In the case of xylan from softwood, the acetyl groups are less frequent, but there exist more branches due to the presence of α-(1, 3) between the xylose backbone and arabinose units. Considering its branched structure, hemicellulose does not form crystalline structures, but amorphous ones. Thus, this biopolymer is more soluble in water and has a higher susceptibility to the hydrolysis [28].

Lignin comprises 10 to 25% lignocellulosic biomass. This component is a very complex phenolic polymer composed of phenyl propane units linked by C-C and C-O-C bonds forming a three-dimensional amorphous structure [29]. The structural units of linin are the cinnamyl alcohols, which are differentiated by the various substitutions that aromatic ring present. The lignin has hydrophobic character and its main function is as inclusive material of a cell wall, i.e., as a sort of cement that fills the remaining gaps and sets, holding everything in place while excluding water from the polysaccharide environment [30].

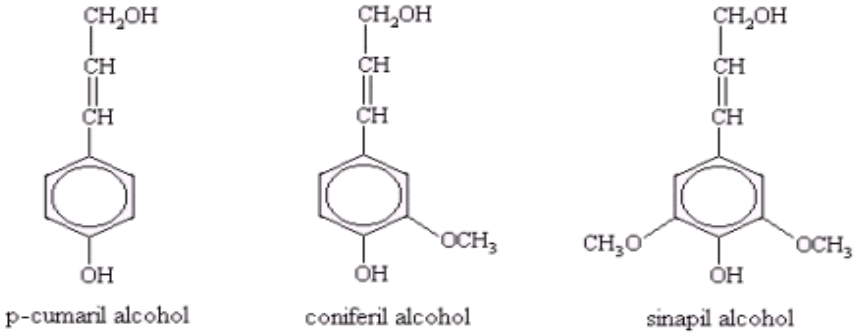


Fig. 2.5. p-coumaryl-, coniferyl- and sinapyl alcohol: dominant building blocks of the three dimensional polymer lignin [31].

The interaction and combination between the hemicellulose and lignin provide a covering shell to the cellulose making its degradation more difficult [32]. Precisely, the main aim of biomass pretreatment is to break the lignin seal and significantly reduce the proportion of crystalline cellulose in such a way that the enzymes hydrolyzing the cellulose can have greater access to this polysaccharide and convert it into fermentable sugars.

Rice straw is an underused agricultural waste in Thailand which is considered a potent lignocellulosic biomass for the production of bioethanol and organic chemicals. It content that can be readily hydrolyzed into fermentable sugars. In term of chemical composition, the straw predominantly contains cellulose (32-47%), hemicellulose (19-27%) and lignin (5-24%) with a high ash content mainly silica [33].

2.4. Pretreatment of lignocellulosic materials

Pretreatment of lignocellulosic biomass is a crucial step for the biochemical conversion of lignocellulosic biomass into biofuel. Its primary role is to disrupt the matrix of polymeric compounds that are physically and chemically bonded aims to render cellulose to accessible to the action of hydrolytic enzymes by altering the lignocellulosic cell wall [34]. Pretreatment involves the alteration of biomass so that enzymatic hydrolysis of lignocellulose and hemicellulose can be achieved more rapidly and with greater yield. Possible goals include the removal of lignin and disrupt of the crystalline structure of cellulose. The following criteria lead to an improvement in enzymatic hydrolysis of lignocellulosic material:

- Increasing of the surface area and porosity
- Modification of lignin structure
- Removal of lignin
- Partial depolymerization of hemicellulose
- Removal of the hemicellulose
- Reducing the crystallinity of cellulose

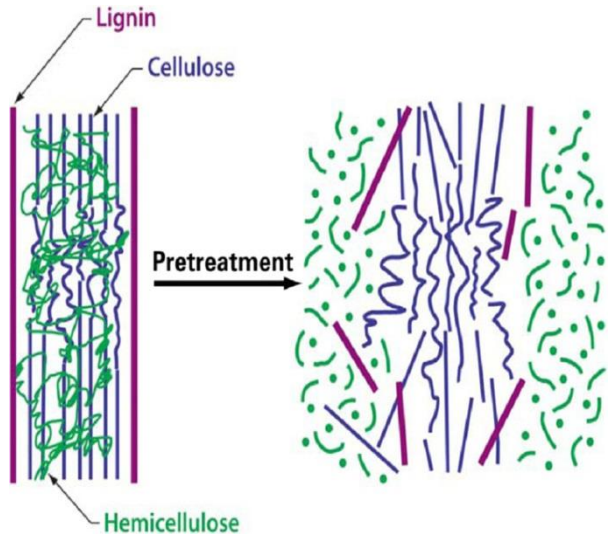


Fig. 2.6. Schematic of goals of pretreatment on lignocellulosic material **[**35].

In an ideal case the pretreatment employed leads to a limited formation of degradation products that inhibit enzymatic hydrolysis and fermentation, and is also cost effective. However, these are actually the most important challenges of current pretreatment technologies. In the following sections the most common pretreatment techniques of biomass are described [36].

2.4.1. Physical pretreatments

Coarse size reduction, chipping, shredding, grinding, milling are amongst the different mechanical size reduction methods that have been used to enhance the digestibility of lignocellulosic biomass. These treatments increase the available specific surface area, and reduce both the degree of polymerization (DP) and cellulose crystallinity [37]. The energy requirements of the physical pretreatment of agricultural materials depends on the final particle size and waste biomass properties.

Milling is physical pretreatment that cut the lignocellulosic biomass into smaller pieces. Reduction of particle size is often needed to make material handling easier and to increase the surface/volume ratio.

2.4.2. Chemical pretreatments

Chemical pretreatment utilizes different chemical agents such as ozone, acids, alkalis, peroxide, and organic solvents.

Acid pretreatment

The pretreatment can be done with dilute or strong acids. The main reaction that occurs during acid pretreatment is the hydrolysis of hemicellulose, especially xylan as glucomannan is relatively acid stable. Solubilized hemicelluloses (oligomers) can be subjected to hydrolytic reactions producing monomers, furfural, HMF and other (volatile) products in acidic environments. During acid pretreatment solubilized lignin is quickly condensate and precipitate in acidic environments. The solubilization of hemicellulose and precipitation of solubilized lignin are more pronounced during strong acid pretreatment compared to dilute pretreatment [38].

Alkaline pretreatment

It is well known that the alkaline pretreatment provides an effective delignification and chemical swelling of the fibrous cellulose. At the same time, the alkaline pretreatment can also cause condensation of lignin and modification of the crystal structure, which can introduce unwanted effects for lignin removal and cellulose degradation led to improving the reactivity of the remaining polysaccharide and enhance amount of reducing sugar extracted from biomass by enzymatic saccharification. The dominant effects of alkaline pretreatment occur on cellulose, hemicellulose, and lignin; however, it also partially removes waxes, silica, and waterproof cutin that coat plant tissue [39]. Full biomass delignification is difficult because lignin is located deep within the cell wall. Lignin has additional challenges such as hydrophobicity, physical stiffness, strong poly-ring bonds (C-O-C and C-C), and its tendency to recondence [40]. Because of the covalent bonds between lignin and hemicellulose, alkaline delignification tends to solubilize hemicellulose. Additionally, alkaline conditions degrade lignin and remove acetyl groups from hemicelluloses [41]. Moreover, alkaline also degrades some cellulose; however, it is much more resistant to attack than hemicellulose. All reactions are enhanced in the presence of an oxidative agent.

Sodium hydroxide

Among alkaline pretreatments, sodium hydroxide is perhaps the most widely used base. Alkaline delignification of wood with NaOH has long been used in industrial pulping and bleaching [42]. By using NaOH, salts are formed which can be incorporated in the biomass and need to be removed or recycled. Process conditions are relatively mild but reaction times can be long. These mild conditions prevent condensation of lignin, resulting in high lignin solubility, especially for biomass with low lignin content such as softwood and grasses [43]. Due to the mild conditions, degradation of sugars to furfural, HMF and organic acids is limited. The addition of air or oxygen to the reaction mixture greatly improves the delignification, especially highly lignified materials.

Ammonia

Pretreatment of biomass with aqueous ammonia at elevated temperatures causes compositional changes and biomass swelling. Delignification is achieved by cleaving bonds between lignin and hemicellulose, C-C, C=O, and C-O bonds of lignin macromolecules. This means that in ammonia pretreatments, the guaiacyl type of lignin is more readily hydrolysable than the syringyl type [44]. Ammonia causes significant changes in the biomass other than delignification. Pretreatment with ammonia is successful in several modes. Some of these are ammonia recycle percolation (ARP), soaking in aqueous ammonia (SAA), and AFEX.

Organosolv

Organosolv processes use an organic solvent or mixtures of organic solvents with water for the removal of lignin before enzymatic hydrolysis. Common solvents for this process include ethanol, methanol, acetone, and ethylene glycol. Temperatures used for the process can be as high as 200^{o}C, but lower temperatures can be sufficient depending on type of lignocellulosic biomass and the use of catalyst. Possible catalysts include inorganic or organic acids [45].

The solvent itself can be an inhibitor for the enzymatic hydrolysis and fermentation step. Therefore, the solvent must be partly removed prior to fermentation. Removal and recovery of the solvent is required for reducing its cost and environmental impact as well.

Oxidative delignification

To enhance delignification, an oxidative agent (e.g., oxygen or hydrogen peroxide) may be applied at the beginning, or during pretreatment. Delignification of lignocellulose can also be achieved by treatment with oxidizing agent such as hydrogen peroxide, ozone, oxygen or air. The effectiveness in delignification can be attributed to the high reactivity of oxidizing chemicals with aromatic rings [46]. Thus, the lignin polymer is converted into e.g. carboxylic acids. Since these acids formed will act as inhibitors in the fermentation step, they have to be neutralized or removed. In addition to an effect on lignin, oxidative treatment also affects the hemicellulose fraction of the lignocellulose complex. A substantial part of the hemicellulose might be degraded and can no longer be used for sugar production.

2.4.3. Physico-chemical pretreatment

Physico-chemical methods of pretreatment are remarkably more effective than physical ones.

Steam pretreatment/steam explosion

Steam explosion has been generally recognized as one of the most effective pretreatment technologies for breaking the crystalline structure of lignocellulose through chemical effect and mechanical forces attributed from sudden explosive decompression. During steam explosion pretreatment, hemicellulose is thought to be hydrolyzed by the acetic and other acids derived from acetyl groups at high temperatures [47]. On the other hand, lignin is redistributed and removed from the material. The removal of hemicelluloses is beneficial for exposing the cellulose surface and increasing enzyme accessibility to the cellulose microfibrils.

The steam explosion process offers several attractive features when compared to other pretreatment technologies including significantly lower environmental impact, less hazardous process chemicals, and greater potential for energy efficiency. It is remarkable that the conversional mechanical methods require 70% more energy than steam explosion to achieve the same size reduction.

Liquid hot water (LHW)

Liquid hot water is biomass pretreatment with water at high temperature and pressure. Other terms are hydrothermolysis, hydrothermal pretreatment, aqueous fractionation, solvolysis or aquasolv. It has the major advantage that the solubilized hemicellulose and lignin products are present in lower concentrations, when compared to steam pretreatment, due to higher water input. Due to these lower concentrations the risk on degradation products like furfural and the condensation and precipitation of lignin compounds is reduced [48]. Solvolysis by hot compressed water contacts water with biomass up to 15 min at temperature 200-300^{o}C.

Ammonia fiber explosion (AFEX)

Ammonia fiber explosion is an alkaline thermal pretreatment in which the lignocelluloses are exposed to liquid ammonia at high temperature and pressure for a period of time followed by a rapid pressure release. Herbaceous and agricultural residues are well suited for AFEX pretreatment. This method does not produce inhibitors for the downstream processes and small particle size is not required for efficacy [49].

This pretreatment has drawbacks of being less efficient for biomass containing higher lignin content (e.g. softwood newspaper) as well as solubilization of very small fraction of solid material, particularly hemicellulose.

2.4.4. Biological pretreatment

Biological pretreatment is based on the use of microorganisms such as brown, white, and soft-rot fungi for selective degradation of lignin and hemicellulose among which white-rot fungi seems to be the most effective microorganism. Biological pretreatment is safe, environmental friendly due to less energy requirement and mild condition conditions compared to other pretreatment methods. However, most of these processes are too slow, which limit their application at an industrial level foe ethanol production process [50].

Implementation of optimal pretreatment step will result in improved total yield of monomeric sugars in the hydrolysis step and the production of target fermentation products. Physical, chemical, thermal, and biological approaches have been studied for efficient pretreatment of lignocellulosic biomass. Each pretreatment technology has different advantages and disadvantages in terms of efficiency and economics, and is suitable for different biomass. They also possess different effects on the cellulose, hemicelluloses and lignin, the three main components of lignocellulosic biomass [51]. The effects of different pretreatments on physical/chemical composition of lignocellulosic biomass structure is

shown in Table 2.1 and the advantages, disadvantages and limitations of the different pretreatment methods are summarized in Table 2.2.

Table 2.1 Effects of the different pretreatments on the physical/chemical composition of lignocellulosic biomass structure [52].

	Increase accessable surface area	Decrystallization cellulose	Solubilisation hemicellulose	Solubilisation lignin	Fotmation furfural/ HMF	Alteration lignin structure
Mechanical	+	+				
ST/SE	+		+	-	+	+
LHW (batch)	+	ND	+	-	-	-
LHW (flowthrough)	+	ND	+	+/-	-	-
Acid	+		+	-	+	+
Alkali	+		-	+/-	-	+
Oxidative	+	ND		+/-	-	+
Thermal + acid	+	ND	+	+/-	+	+
Thermal + alkali (lime)	+	ND	-	+/-	-	+
Thermal + oxidative	+	ND	-	+/-	-	+
Thermal + alakali + oxidative	+	ND	-	+/-	-	+
Ammonia (AFEX)	+	+	-	+	-	+
CO_2 explosion	+		+			

+ = major effect; - = minor effect; ND = not determined

Table 2.2 Summary of various processes used for the pretreatment of lignocellulosic biomass [53].

Pretreatment process	**Advantages**	**Limitations and disadvantages**
Physical pretreatment	Reduce cellulose crystallinity	Power consumption usually higher than inherent biomass energy
Steam explosion	Causes hemicelluloses degradation and lignin transformation; cost effective	Destruction of a portion of the xylan fraction; incomplete disruption of the lignin-carbohydrate matrix; generation of compounds inhibitory to microorganisms
AFEX	Increases accessible surface area, removes lignin and hemicelluloses to an extent; does not produce inhibitors for downstream processes	Not efficient for biomass with high lignin content
Acid pretreatment	Hydrolyzes hemicelluloses to xylose and other sugars; alters lignin structure	High cost; equipment corrosion; formation of toxic substances
Alkali pretreatment	Removes hemicelluloses and lignin; increase accessible surface area	Long residence time required; irrecoverable salts formed and incorporated into biomass

Pretreatment process	Advantages	Limitations and disadvantages
Organosolv	Hydrolyzes lignin and hemicelluloses	Solvents need to be drained from the reactor, evaporated, condensed, and recycled; high cost
Biological	Degrades lignin and hemicelluloses; low energy requirements	Rate of hydrolysis is very slow

2.5. Enzymatic hydrolysis

Enzymatic hydrolysis is the process that using enzyme to break down starch or lignocellulose component into its constituent sugars at mild condition. Generally, hydrolysis can be performed by two pathways including the chemical hydrolysis and the enzymatic hydrolysis. Currently, enzymatic hydrolysis is the promising process for converting cellulosic compound to sugar for further conversion process due to its environmental friendliness. Commonly, enzymatic hydrolysis requires an enzyme for hydrolyzing the reactant. Theoretically, enzymes are proteins that catalyze the rates of chemical reactions. In enzymatic reactions, the molecules at the beginning of the process, called substrates, are converted into different molecules called products. Almost all chemical reactions in a biological cell need enzymes in order to occur at rates sufficient for life. Since enzymes are selected for their substrates and speed up only a few reactions from among many possibilities, the set of enzymes made in a cell determines which metabolic pathways occur in that cell. Like all catalysts, enzymes work by lowering the activation energy (Ea) for a reaction, thus dramatically increasing the rate of the reaction [54].

Enzymatic hydrolysis of cellulose, the main component in lignocellulosic biomass is carried out by cellulase enzymes, which are highly specific. The hydrolysis product is mainly glucose which can be further converted into bioethanol and other chemicals. The utility cost of enzymatic hydrolysis is low compared to acid or alkali hydrolysis because enzyme hydrolysis is usually conducted at mild conditions (pH 4.8 and temperature 45-50^{o}C) and does not have corrosion problems. The process is also more environmental friendly and has been recognized as a potent method for glucose production from biomass.

Cellulases are usually a mixture of several enzymes. At least, three major groups of cellulases are involved in the hydrolysis process: (1) endoglucanase (EG, endo-1,4-D-glucanohydrolase, or EC 3.2.1.4), which attacks regions of low crystallinity in the cellulose fiber, creating free chain-ends; (exo-glucanase or cellobiohydrolase (CBH, 1,4-β-D-glucan cellobiohydrolase, or EC 3.2.1.91) which degrades the molecule further by removing cellobiose units from the free-chain ends; (3) β-glucosidase (EC 3.2.1.21) which hydrolyse cellobiose to produce glucose (Howard et al, 2003). In addition to the three major groups of cellulase enzymes, there are also a number of accessory enzymes that attack hemicellulose, such as glucuromidase, acetylesterase, xylanase, β-xylosidase, galactomannanase and glucomannanase [55]. During the enzymatic hydrolysis step, cellulose and hemicellulose are degraded into sugars which can be further fermented or chemically catalyzed to target products.

2.6. Ethanol fermentation

Ethanol can be produced from lignocellulosic materials, such as agricultural residues, wood, paper and yard waste in municipal solid waste and dedicated energy crops, which constitute the most abundant renewable organic component in the biosphere. The fermentation step is central in the overall fuel ethanol production process since it represents the actual transformation of the conditioned and pretreated raw materials into the main product, ethyl alcohol, using bioagent such as yeast or other ethanol-producing microorganism. Ethanolic fermentation is one of the most studied biological processes. Nevertheless, the need of increasing the efficiency of ethanol production including the usage of alternative feedstock has led to the development of new fermentation method with better technoeconomic and environmental indicators [56].

Traditionally, the most used microorganism for ethanolic fermentation is the yeast *Sacharomyces cerevisiae*. This is valid for practically every one of the main types of feedstocks employed for ethanol production: sucrose-based media, starchy material, and even lignocellulosic materials. However, there is a wider variety of process microorganisms employed [57].

CHAPTER 3
METHODOLOGY

Overall, this project focused on the studies of lignocellolosic biomass pretreatment by the alkaline peroxide pretreatment with different aqueous organic solvent processes (i.e. ethanol, isopropanol, and tert-butyl alcohol), alkaline catalyst (i.e. sodium hydroxide, ammonium hydroxide, and triethylamine). The effects of important operating parameters (i.e. solvent, temperature, resident time, concentration of hydrogen peroxide, solid loading) were studied and optimized.

3.1. Raw material preparation

Rice straw (*Oryza sativa*) was obtained from a paddy field in Pathumthani province. It was milled by a cutting mill and sieved through a 0.5 mm size screen. The physically processed rice straw was dried in a hot air oven at 70^{o}C for overnight and stored in plastic bag at room temperature before use.

3.2. Alkaline peroxide pretreatment of rice straw

First, varying the optimal conditions of alkaline peroxide pretreatment was studied using water as a solvent by varying the parameters that can affect pretreatment; temperature (25, 35, 45^{o}C), residence time (6, 18, 24 h), hydrogen peroxide concentration (2.5, 5, 7.5%, v/v), and biomass loading (5, 7.5, 10%, w/v). Experiments were analyzed through a (29 runs) factorial design in which the variables were evaluated at three levels. Operating conditions used in the different trials are shown in Table 3.1. The optimized condition was then applied for subsequent studies on studying effects of solvents and alkalines.

Table 3.1 Study of rice straw pretreatment using water as the medium

Run	Variables			
	H_2O_2 concentration (%v/v)	Temp (^oC)	Time (h)	Solid loading (%)
1	1	0	1	0
2	0	0	-1	-1
3	-1	0	-1	0
4	1	-1	0	0
5	0	1	-1	0
6	0	0	0	0
7	0	-1	-1	0
8	-1	0	0	-1
9	0	0	1	1
10	1	0	-1	0
11	0	-1	1	0
12	0	0	0	0
13	0	0	1	-1
14	1	1	0	0
15	-1	0	0	1
16	0	0	0	0
17	0	0	0	0
18	0	0	-1	1
19	0	1	0	1
20	0	1	1	0
21	1	0	0	-1
22	0	1	0	-1
23	1	0	0	1
24	-1	-1	0	0
25	-1	0	1	0
26	-1	1	0	0
27	0	-1	0	1
28	0	-1	0	-1
29	0	0	0	0

Dried rice straw of different weight is (5, 7.5, 10%, w/v) were added into 10 mL of (2.5, 5, 7.5%, v/v) hydrogen peroxide solution that already adjusted to pH 11.5 with NaOH. Alkaline peroxide pretreatments were performed in incubator at various pretreatment temperature (25, 35, 45^{o}C) for different residence time (6, 18, and 24 h). No further adjustments in pH were made during the course of the treatment. The insoluble residue was

collected by filtration and washed with distilled water several time until the pH of the filtrate was neutral before drying at 70^{o}C for overnight. The sample was stored at room temperature for enzymatic hydrolysis and composition analysis.

3.3. Enzymatic saccharification

Hydrolysis tests for each sample were performed to determine the improvement in enzymatic saccharification under the different pretreatment conditions applied. The solid rice straws were hydrolyzed in a 1 ml reaction contained 5% (w/v) pretreated biomass in 50 mM sodium citrate buffer pH 4.8 with 20 FPU/g and 5% sodium azide. The sample was incubated at 50^{o}C and shaking at 30 rpm for 72 hours. The samples were taken at time intervals and analyzed release reducing sugars using the DNS method [58]. The sugar profiles were analyzed by high performance liquid chromatography.

3.4. Optimization of pretreatment parameters

Reaction parameters and conditions for the pretreatment of rice straw e.g. oxidizing agent concentration, temperature, time, and solid loading were optimized using a systematic experimental design approach. The experiments were designed and analyzed using Minitab software (Company). The solid fraction was analyzed for compositions using NREL method [and characterized by scanning electron microscope (SEM) and X-ray diffraction (XRD) analysis.

3.4.1. Reducing sugar analysis

Chemical composition analysis

Both treated and untreated rice straw were analyzed by the standard NREL method [59].

Total reducing sugar

Concentration of releasing sugar after enzymatic hydrolysis was analyzed by DNS (Dinitrosalicylic acid) method [55] at wavelengths of 540 nm.

3.4.2. Scanning electron microscope analysis

The native and pretreated biomass microstructures were analyzed by a scanning electron microscope (SEM) using a JSM-5410F scanning electron microscope (JEOL, Tokyo, Japan). The samples were dried and coated with gold for analysis. An electron beam energy of 5 kV was used for analysis.

3.4.3. X-ray diffraction analysis

Crystallinity of the native and separated solid fractions was determined by X-ray diffraction (XRD) using an X'Pert PRO diffractometer (PANalytical, Almelo, The Netherlands). The samples were scanned in a range of 2θ=10°–30° with a step size of 0.02° at 500 kV, 30 mA and radiation at Cu Kα (λ=1.54 Å). Crystallinity was calculated according to the following equation [60].

$$\text{CrI (\%)} = \left(\frac{I_{002} - I_{amorphous}}{I_{002}} \right) \times 100$$

in which, I_{002} is the intensity for the crystalline portion of biomass (i.e., cellulose) at 2Θ = 22.4 and $I_{amorphous}$ is the peak for the amorphous portion (i.e., cellulose, hemicellulose, and lignin) at 2Θ = 18.0.

3.5. Statistical analysis

The experimental design and statistical analysis of the data were done by using SPSS for Windows version 11.5 (Softonic International S.A.; Barcelona, Spain). Regression analysis and analysis of variance (ANOVA) were used to evaluate the statistical significance of the model. The dataset was fitted as a second-order polynomial equation involving main effects and interaction effects for each variable. The fitting quality of the polynomial model equation was expressed by the coefficient of determination R^2. Differences in the means were considered significant when the p value for the null hypothesis was 0.05 or less.

CHAPTER 4
RESULTS AND DISCUSSION

4.1. Determination of releasing sugar of untreated rice straw

The components of rice straw before pretreatment were determined according to the standard methods of the National Renewable Energy Laboratory (NREL, USA). The chemical compositions of rice straw used as starting material in this study are shown in Table 4.1. The rice straw contained 35.5% cellulose, 13.8% hemicellulose and 25.0% lignin as the major content which made up the total carbohydrate content 49.3% of dry solid basis. It contained a high ash content of 22%. According to the composition analysis, rice straw contained a relatively lower content of cellulose and hemicellulose with slightly higher lignin compared to bagasse and corn stover. This nearly equals cellulose/hemicellulose/ lignin content and its high ash amount makes rice straw has unique characteristics for processing as compared to other lignocellulosic biomass. A complete compositional analysis was done for the dried rice straw samples.

Table 4.1 Chemical composition of rice straw used in this study.

	Composition of biomass (%)		
Component	**Rice straw [(a)]**	**Corn stover [(b)]**	**Bagasse [(b)]**
Cellulose	35.5	40.0	43.4
Hemicellulose	13.8	27.7	26.3
Lignin	25.0	23.1	24.4
Ash	22.0	6.5	1.9

(a) Current study (b) Reviewed paper [54]

As expected, the releasing sugar yield of 5% (w/v) dried untreated rice straw after enzymatic hydrolysis using commercial enzyme, *Accellerace® 1500*, mixed with 50 mM sodium citrate buffer (pH 4.8) and 5% sodium azide at the total volume 1 mL showed a low concentration of sugar because the lignin content in rice straw is the resistance against microbial attack that leads to difficulty to release the monomer (mostly sugars), commonly

referred to recalcitrance. Pretreatment is required to overcome biomass recalcitrance by disrupting the lignin seal and significantly reduce the proportion of crystalline cellulose in such a way that enzymes hydrolyzing the cellulose can have greater access to this polysaccharide and convert it into fermentable sugars.

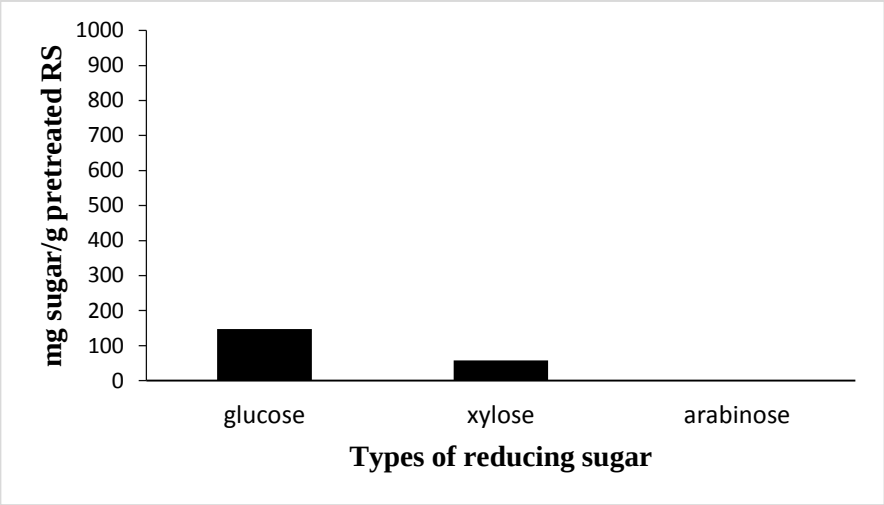


Fig. 4.1. Sugar recovery from enzymatic hydrolysis of untreated rice straw.

4.2. Optimization of conventional alkaline peroxide water-based pretreatment parameters

The initial study focused on the optimum conditions for the pretreatment of rice straw using alkaline peroxide water-based pretreatment. The dried rice straws were pretreated by alkaline (NaOH) peroxide pretreatment to prepare them for hydrolysis with *Accellerase®1500*. Effects of hydrogen peroxide concentration, pretreatment temperature, duration time, and solids loading were evaluated to determine the optimized conditions for improving enzymatic biomass digestibility. After pretreatment at different operating conditions were collected. The amount of total reducing sugars released from rice straw were analyzed by DNS method. A HPLC (high performance liquid chromatography) was used to analyze the components of the samples. Refractive index (RI) was used as a detector equipped with Bio- rad Aminex HPX-87H column (Bio-rad, Organic Acid Standard). 5mM H_2SO_4 was used as a mobile phase at flow rate 0.5 ml/min and operate at 65^oC.

Pretreatment of rice straw using hydrogen peroxide in the presence of alkaline catalysts led to increasing reducing sugar yield from enzymatic hydrolysis as compared to the native rice straw that is highly recalcitrant to enzymatic attack. The biomass loss physical and morphological integrity due to delignification reaction and disintegrated into small, highly dispersed fibers owing to the attack of hydroxyl (HO•) and superoxide radical anions (O_2-), by decomposition of the hydrogen peroxide, which can oxidize and degrade lignin. The oxidized lignin fragments was then dissolved in the liquid phase and resulted in increased digestibility of the solid fraction the effects of parameters in alkaline peroxide pretreatment (oxidant concentration, temperature, residence time, and solid loading) was evaluated by determining the amounts of released reducing sugars after enzymatic hydrolysis of the solid residues based on either pretreated or native rice straw (Table 4.2). The increase in reducing sugar yield from pretreated rice straw was markedly increased compared to reducing sugar from the untreated rice straw (166.37±6.65 mg per g native rice straw). The highest reducing sugar yield of 944.14 ±8.17 mg/g pretreated biomass equivalent to 687.95±5.71 mg/g native biomass was obtained using 7.5% biomass loading (w/v) treated with 7.5% H_2O_2 (v/v) at 35ºC for 18 h and hydrolyzed with Accellerase® 1500. This was equivalent to 82.35% recovery of available glucan and 16.48% recovery of xylan in the native rice straw. This pretreatment condition was applied as the initial optimal pretreatment conditions for subsequent study on the effects.

Table 4.2 Reducing sugars from enzymatic hydrolysis of the solid residues pretreated under different reaction condition.

Run	Variables				Weight recovery (%)	Reducing sugar (mg/g pretreated rice straw)	SD	Reducing sugar (mg/g native rice straw)	SD
	H_2O_2 concentration (%v/v)	Temp (^oC)	Time (h)	Solid loading (%w/v)					
1	7.5	35	24	7.5	70.94	969.45	5.81	687.70	4.12
2	5	35	6	5	56.03	931.00	2.82	521.61	1.58
3	2.5	35	6	7.5	46.96	704.22	6.52	330.69	3.06
4	7.5	25	18	7.5	60.06	963.81	1.04	578.90	0.63
5	5	45	6	7.5	51.43	910.14	3.36	468.07	1.73
6	5	35	18	7.5	49.98	903.77	9.61	451.74	4.80
7	5	25	6	7.5	53.01	897.12	7.87	475.58	4.17
8	2.5	35	18	5	53.20	782.06	3.47	416.06	1.85
9	5	35	24	10	50.65	880.39	7.78	445.93	3.94
10	7.5	35	6	7.5	72.52	968.20	5.28	702.10	3.83
11	5	25	24	7.5	50.02	899.41	6.82	449.85	3.41
12	5	35	18	7.5	51.41	907.31	8.49	466.43	4.36
13	5	35	24	5	48.95	942.13	5.48	461.14	2.68
14	7.5	45	18	7.5	66.44	978.62	8.66	650.21	5.75
15	2.5	35	18	10	65.96	631.56	10.18	416.57	6.72
16	5	35	18	7.5	48.51	904.45	2.39	438.78	1.16
17	5	35	18	7.5	49.68	908.98	4.77	451.60	2.37
18	5	35	6	10	52.01	878.37	4.27	456.82	2.22
19	5	45	18	10	49.33	892.69	3.99	440.33	1.97
20	5	45	24	7.5	49.32	919.26	3.19	453.36	1.57
21	7.5	35	18	5	69.90	984.14	8.17	687.95	5.71
22	5	45	18	5	51.14	952.49	3.06	487.10	1.56
23	7.5	35	18	10	52.01	955.70	3.59	497.02	1.87
24	2.5	25	18	7.5	57.74	683.79	2.93	394.81	1.69
25	2.5	35	24	7.5	58.29	722.98	5.81	421.46	3.39
26	2.5	45	18	7.5	54.05	763.16	5.41	412.52	2.92
27	5	25	18	10	50.21	872.30	2.70	437.98	1.36
28	5	25	18	5	48.87	928.62	4.15	453.81	2.03
29	5	35	18	7.5	48.68	905.03	10.55	440.57	5.14
untreated	-	-	-	-	100	166.37	6.65	166.37	6.65

The analysis of variance (ANOVA) was used to evaluate the quality of the model. It was designed to compare the variation due to the treatment factors with the variation due to random errors inherent in the measurements of the generated responses. The experimental dataset showed a high value of R^2 (99.4%) and R^2 adjusted (98.8%) which indicated a high dependence and correlation between the observed and the predicted values of response. In addition to this, the value of R^2 also indicates that 99.4% of result of the total variation can be explained by this model. The ANOVA results presented in Table 4.4 showed that hydrogen peroxide concentration, pretreatment temperature and biomass loading were considered to significantly affect ($p<0.05$) the reducing sugar from the enzymatic hydrolysis of rice straw (mg/g pretreated rice straw). Pretreatment time was not considered a significant factor under the experimental conditions. Interaction between some parameters e.g. temperature and solid loading was also found to affect the sugar yield obtained.

Table 4.3 Result of 1-way ANOVA

Model		Sum of squares	df	Mean square	F	Sig.
1	Regression	247368.32	14	17669.17	167.828	0.000[a]
	Residual	1473.94	14	105.28		
	Total	248842.26	28			

Table 4.4 Analysis of variance (ANOVA) for alkaline peroxide pretreatment in the water/NaOH system.

Factor	df	Sig.	Factor	df	Sig.
X_1	3	0.00	$X_1 \times X_4$	3	0.90
X_2	3	0.00	$X_2 \times X_2$	3	0.01
X_3	3	0.23	$X_2 \times X_3$	3	0.41
X_4	3	0.00	$X_2 \times X_4$	3	0.00
$X_1 \times X_1$	3	0.00	$X_3 \times X_3$	3	0.74
$X_1 \times X_2$	3	0.31	$X_3 \times X_4$	3	0.87
$X_1 \times X_3$	3	0.88	$X_4 \times X_4$	3	0.66

R^2 =0.994; X_1: H_2O_2 concentration, X_2: temperature, X_3: duration time, X_4: solid loading

The objective of the pretreatment and enzymatic saccharification steps is to release as much sugar as possible from raw materials in such a way that they can be fermented to ethanol. Table 4.5 HPLC showed the glucose and hemicellulose recovery in liquid fraction from enzymatic hydrolysis of pretreated rice straw under different operational conditions. The effect of each parameters on glucose recovery was determined when other parameters were fixed constant. After enzymatic hydrolysis, the increasing glucose recovery is detected in the experiment performed with increasing hydrogen peroxide concentration and solid loading. For those conditions that pretreated with 7.5, 5, 2.5%, v/v hydrogen peroxide, the amount of glucose recovery was in range between 72-82%, 56-78%, and 48-61%, respectively. For those conditions using 5, 7.5, 10%, w/v solid loading, the amount of glucose recovery was in range between 56-78%, 55-79%, and 56-72%, respectively. There was no substantial difference in glucose recovery using different pretreatment temperature and time.

Table 4.5 Glucose and xylose recovery from enzymatic hydrolysis of pretreated and untreated rice straw under conventional alkaline peroxide pretreatment using the water/NaOH system.

Run	Variables				Glucose recovery (%)	SD	Xylose recovery (%)	SD
	H_2O_2 concentration (%v/v)	Temp (^oC)	Time (h)	Solid loading (%w/v)				
1	7.5	35	24	7.5	77.54	0.19	16.82	0.24
2	5	35	6	5	78.61	0.41	14.82	0.43
3	2.5	35	6	7.5	47.93	1.11	19.16	1.00
4	7.5	25	18	7.5	79.33	0.15	16.08	0.13
5	5	45	6	7.5	70.36	1.22	25.13	1.35
6	5	35	18	7.5	66.57	0.38	22.99	0.72
7	5	25	6	7.5	68.45	3.33	23.11	0.71
8	2.5	35	18	5	57.17	0.47	27.75	0.28
9	5	35	24	10	63.63	0.54	25.75	1.11
10	7.5	35	6	7.5	76.79	0.30	17.33	0.30
11	5	25	24	7.5	66.05	0.22	20.27	5.71
12	5	35	18	7.5	68.73	0.66	27.26	0.74
13	5	35	24	5	72.62	0.73	21.92	1.25
14	7.5	45	18	7.5	79.27	0.24	15.68	0.06
15	2.5	35	18	10	60.22	3.83	32.72	1.65
16	5	35	18	7.5	63.43	1.76	25.31	0.11
17	5	35	18	7.5	65.31	3.97	22.41	1.25
18	5	35	6	10	62.51	0.64	24.50	0.77
19	5	45	18	10	62.77	1.80	26.88	0.95
20	5	45	24	7.5	67.75	0.97	24.03	0.03
21	7.5	35	18	5	82.35	0.11	16.48	0.02
22	5	45	18	5	75.10	1.27	22.99	0.32
23	7.5	35	18	10	72.93	0.05	23.66	0.35
24	2.5	25	18	7.5	55.96	0.34	30.99	1.78
25	2.5	35	24	7.5	60.94	0.48	31.11	1.46
26	2.5	45	18	7.5	57.01	0.24	31.50	0.95
27	5	25	18	10	56.07	0.94	25.50	0.07
28	5	25	18	5	68.50	0.59	22.00	0.56
29	5	35	18	7.5	64.92	0.64	21.25	0.89
untreated	-	-	-	-	22.54	1.03	17.22	1.26

Study of the effects of reaction parameters on alkaline hydrogen peroxide pretreatment

The effects of pretreatment parameters, which are hydrogen peroxide concentration (1.25, 2.5, 5, and 7.5%, v/v), pretreatment temperature (25, 35, 45, and 55ºC), reaction time (6, 12, 18, and 24 h), and solid loading (2.5, 5, 7.5, and 10%, w/v) were then evaluated by varying one factor at a time while other parameters were fixed under the initial optimal conditions. Increasing hydrogen peroxide concentration led to increasing sugar yield (Fig. 4.2). The highest reducing sugar of 825.45 mg/g pretreated rice straw equivalent to 404.47 mg/g native rice straw was achieved at 7.5%, v/v hydrogen peroxide concentration. The slightly increase in amount of the reducing sugar (mg/g pretreated biomass) were observed when increasing of pretreatment temperature and the pretreatment at 55ºC gave the maximal reducing sugar yield of 782.19 (mg/g pretreated rice straw) equivalent to 555.36 (mg/g native rice straw) when compared to that obtained at lower pretreatment temperature (Fig. 4.3). Pretreatment time led to slight increasing trend of reduced sugar yield. Majority of the reducing sugar was released during the first 6 h (Fig. 4.4). Majority of the reducing sugar was released during the first 6 h. The maximal reducing sugar yield of 549.97 mg/g pretreated biomass (360.77 mg/g native biomass) was obtained after 24 h pretreatment. Decreasing reducing sugar yield was found with increasing solid loading from 2.5-10% (w/v) with the highest sugar yield of 799.09 mg/g pretreated biomass (502.06 mg/g native rice straw) using the solid loading of 2.5% (Fig. 4.5).

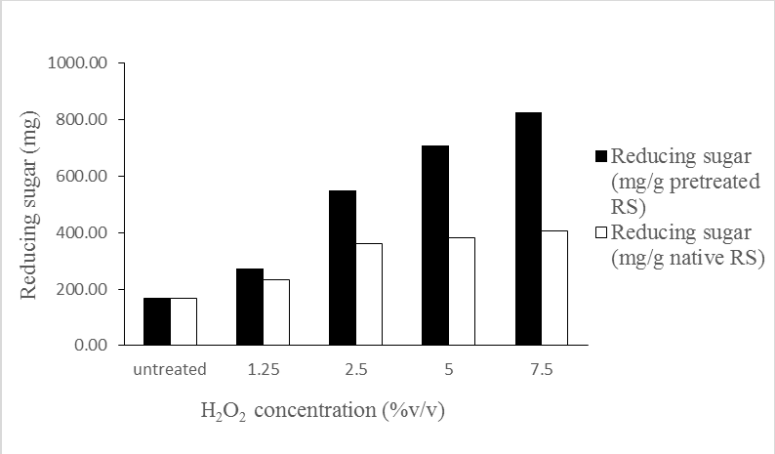


Fig. 4.2. Effects of hydrogen peroxide concentration of alkaline peroxide pretreatment on the total reducing sugar.

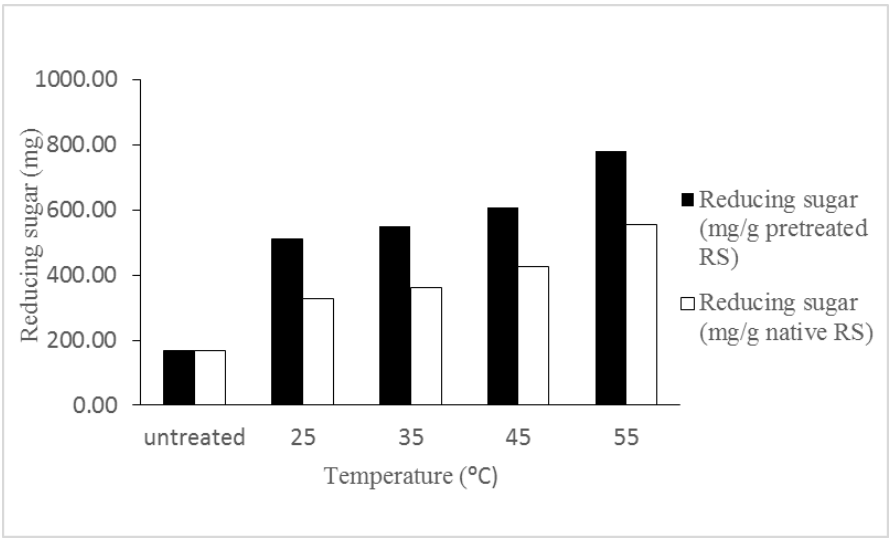


Fig. 4.3. Effects of reaction temperature of alkaline peroxide pretreatment on the total reducing sugar.

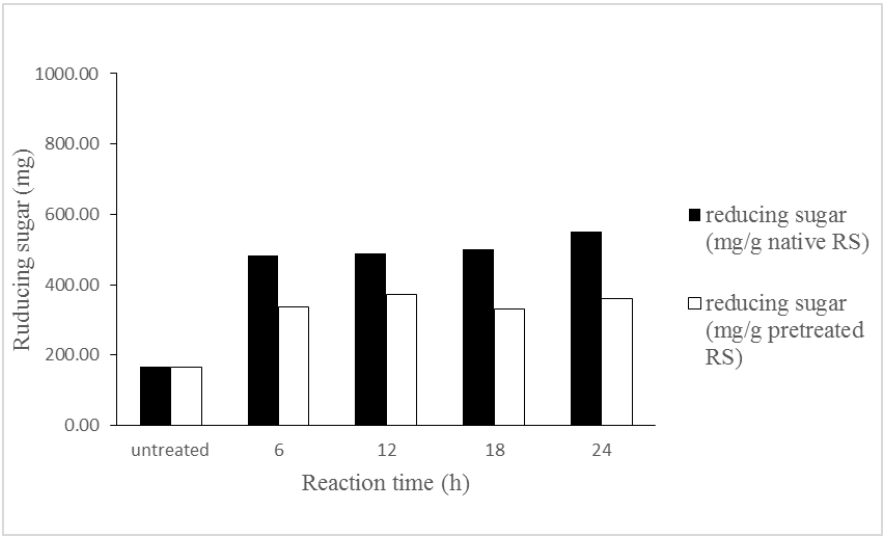


Fig. 4.4. Effects of reaction time of alkaline peroxide pretreatment on the total reducing sugar.

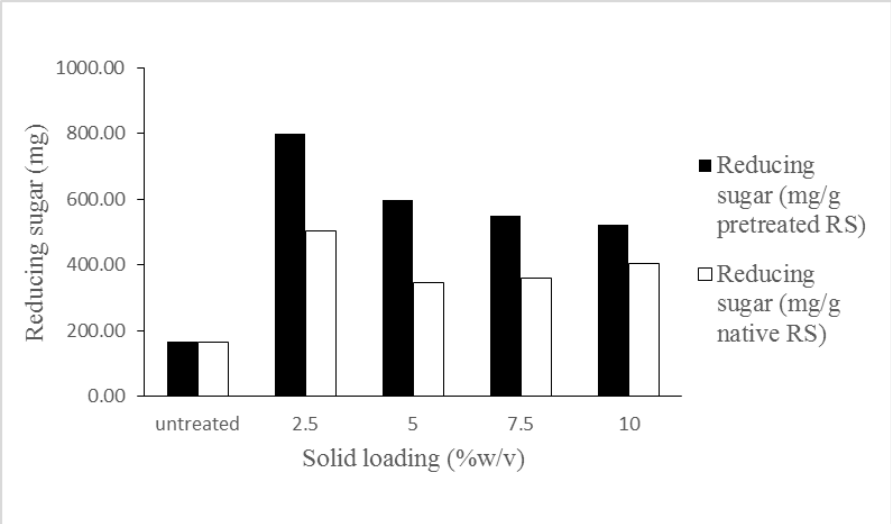


Fig. 4.5. Effects of solid loading of alkaline peroxide pretreatment on the total reducing sugar

4.3. Study on the effect of different types of alkaline and different types of organic solvents

The conditions for conventional AHP pretreatment was applied to study the effects of different alkaline catalysts and solvents on modification of the pretreatment process. The modified pretreatment reaction contained 7.5%, w/v solid loading of dried rice straw in 10 ml of the reaction mixture containing alkaline (ammonium hydroxide or triethylamine), 2.5% v/v hydrogen peroxide using an organic solvent (ethanol, isopropanol or tert-butyl alcohol as the co-solvent). The reactions were operated at 35ºC for 24 h. The solid and liquid fractions after pretreatment were separated by vacuum filtration. Then, the solid fraction was washed by deionized water until the pH equals to 7 and dried at 70ºC overnight. Table 4.6 showed that tert-butyl alcohol gave the highest reducing sugar among the three co-solvents in the presence of either ammonium hydroxide (459.54 mg/g pretreated rice straw) or triethylamine (199.36 mg/g pretreated rice straw).

Table 4.6 Reducing sugar yields from alkaline peroxide pretreatment of rice straw using different alkaline catalysts (ammonium hydroxide and triethylamine) and organic solvents (alcohol).

Run	Alkaline	Organic solvent	Weight recover (%)	Reducing sugar (mg/g pretreated rice	SD	Reducing sugar (mg/g native rice straw	SD
1	NH_4OH	Ethanol	86.62	232.14	7.20	201.09	6.24
2	NH_4OH	Isopropanol	83.26	318.45	5.78	265.14	4.81
3	NH_4OH	Tert-butyl alcohol	80.43	459.54	4.67	369.59	3.76
4	TEA	Ethanol	89.12	179.41	2.92	159.89	2.61
5	TEA	Isopropanol	91.67	186.65	6.40	171.11	5.87
6	TEA	Tert-butyl alcohol	85.78	199.36	3.98	171.02	3.41

*The reaction (10 ml total volume) contained 7.5% (w/v) rice straw, 2.5% (v/v) H_2O_2, and 6-7 ml organic solvent while NH_4OH (2 ml) or TEA (3 ml) was added to adjust pH to 11.5.

Table 4.7 demonstrates that glucose was the major released sugar from enzymatic hydrolysis followed by xylose. The maximum glucose recovery 40.68 and 26.79% was obtained from NH_4OH/tert-butyl alcohol and TEA/tert-butyl alcohol, respectively. The alkaline peroxide pretreatment of rice straw generally resulted in enrichment of cellulose content as a result of partial solubilization of hemicellulose and removal of lignin. The results revealed that the use of various organic solvents and alkalines gave the higher reducing sugar and glucose recovery (20-40%) than the native rice straw especially the use of both alkaline in tert-butyl alcohol but it was still lower when compared to those of the conventional water-based/NaOH process.

Table 4.7 Glucose and xylose recoveries from alkaline peroxide pretreatment using different alkaline catalysts (ammonium hydroxide and triethylamine) and organic solvents (alcohol).

Run	Alkaline	Organic solvent	Glucose recovery (%)	SD	Xylose recovery (%)	SD
1	NH_4OH	Ethanol	28.87	0.20	13.01	1.57
2	NH_4OH	Isopropanol	29.53	0.23	17.16	2.30
3	NH_4OH	Tert-butyl alcohol	40.68	0.35	23.30	0.92
4	TEA	Ethanol	21.50	0.29	7.94	0.28
5	TEA	Isopropanol	25.71	3.38	8.17	0.53
6	TEA	Tert-butyl alcohol	26.79	1.09	8.57	0.67

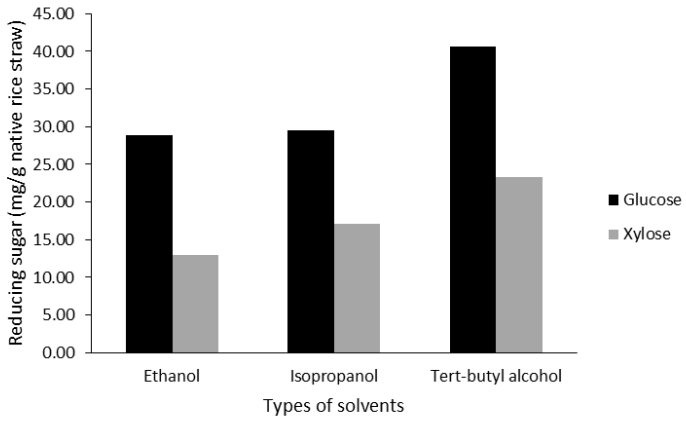


Fig. 4.6. Reducing sugar yields from enzymatic hydrolysis of rice straw pretreated by ammonium hydroxide in various types of co-solvents. The pretreatment reaction contained 7.5%, w/v solid loading treated with 2.5%, v/v hydrogen peroxide at 35ºC for 24 h.

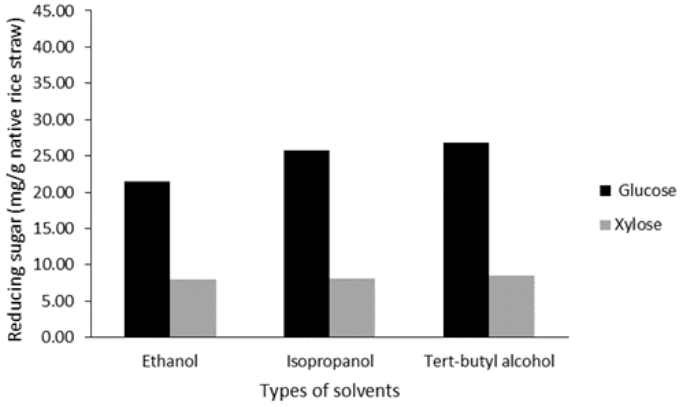


Fig. 4.7. Reducing sugar yields from enzymatic hydrolysis of rice straw pretreated by triethylamine in various types of co-solvents. The pretreatment reaction contained 7.5%, w/v solid loading treated with 2.5%, v/v hydrogen peroxide at 35ºC for 24 h.

In the next step, the effect of hydrogen peroxide concentration in the modified reaction contacting organic alkalines and co-solvents was studied. Increasing hydrogen peroxide concentration from 2.5 to 5 and 7.5%, v/v led to the increase in reducing sugar yield (Table 4.8). A maximum yield of releasing sugar of 532.30 mg/g pretreated rice straw was obtained from the pretreatment of rice straw using ammonium hydroxide as the alkaline catalyst in the reaction mixture containing tert-butyl alcohol as a co-solvent. The alkaline

peroxide pretreatment using TEA/tert-butyl alcohol gave lower reducing sugar when compared to NH_4OH/tert-butyl alcohol in every concentrations of hydrogen peroxide.

Table 4.8 Effect of hydrogen peroxide concentration on reducing sugar yields by using different alkalines (ammonium hydroxide and triethylamine) using tert- butyl alcohol as a co-solvent.

Run	H_2O_2 concentration (%v/v)	Alkaline	Organic solvent	Weight recover (%)	Reducing sugar (mg/g pretreated rice straw	SD	Reducing sugar (mg/g native rice straw	SD
1	2.5	NH4OH	Tert-butyl alcohol	80.43	459.54	4.67	369.59	3.76
2	2.5	TEA	Tert-butyl alcohol	85.78	199.36	3.98	171.02	3.41
3	5	NH_4OH	Tert-butyl alcohol	82.12	471.48	6.90	387.17	5.67
4	5	TEA	Tert-butyl alcohol	88.85	202.30	2.86	179.74	2.54
5	7.5	NH_4OH	Tert-butyl alcohol	78.56	532.30	19.39	418.16	15.23
6	7.5	TEA	Tert-butyl alcohol	86.73	258.61	12.06	224.29	10.46

*The reaction (10 ml total volume) contained 7.5% (w/v) rice straw, 2.5-7.5% (v/v) H_2O_2, and 2-7 ml organic solvent while NH_4OH (2-4 ml) or TEA (3-5ml) was added to adjust pH to 11.5.

Table 4.9 demonstrates that the increasing of hydrogen peroxide concentration resulted in the increasing of glucose recovery in both ammonium hydroxide and triethylamine system by using tert-butyl alcohol as a co-solvent. The maximum glucose recovery of 49.81 and 31.37% was obtained from 7.5%, v/v hydrogen peroxide concentration in NH_4OH/tert-butyl alcohol and TEA/tert-butyl alcohol, respectively.

Table 4.9 Glucose and xylose recoveries from alkaline peroxide pretreatment at different hydrogen peroxide concentration using different alkaline catalysts (ammonium hydroxide and triethylamine) and organic solvents (alcohol).

Run	H_2O_2 concentration (%v/v)	Alkaline	Organic solvent	Glucose recovery (%)	SD	Xylose recovery (%)	SD
1	2.5	NH4OH	Tert-butyl alcohol	40.68	0.35	23.30	0.92
2	2.5	TEA	Tert-butyl alcohol	26.79	1.09	8.57	0.67
3	5	NH_4OH	Tert-butyl alcohol	49.60	0.33	9.81	1.06
4	5	TEA	Tert-butyl alcohol	27.70	1.11	5.35	0.46
5	7.5	NH_4OH	Tert-butyl alcohol	49.81	2.19	25.58	2.21
6	7.5	TEA	Tert-butyl alcohol	31.37	1.90	7.70	0.47

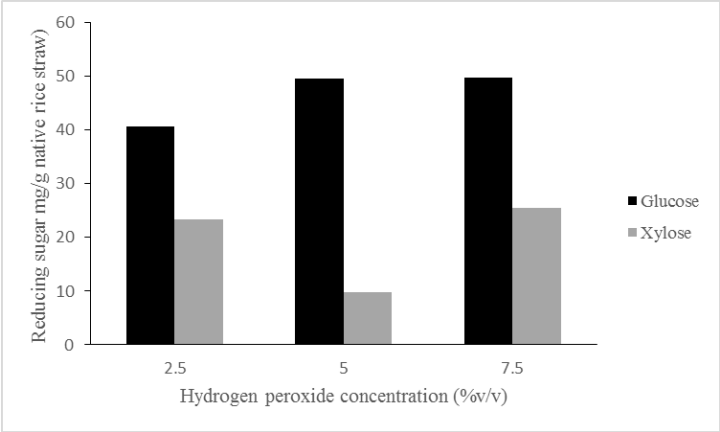


Fig. 4.8. Reducing sugar yields from enzymatic hydrolysis of rice straw pretreated by ammonium hydroxide in different hydrogen peroxide concentrations. The pretreatment reaction contained 7.5% (w/v) solid loading treated with different hydrogen peroxide concentrations (2.5-7.5%, v/v) at 35^{o}C for 24 h.

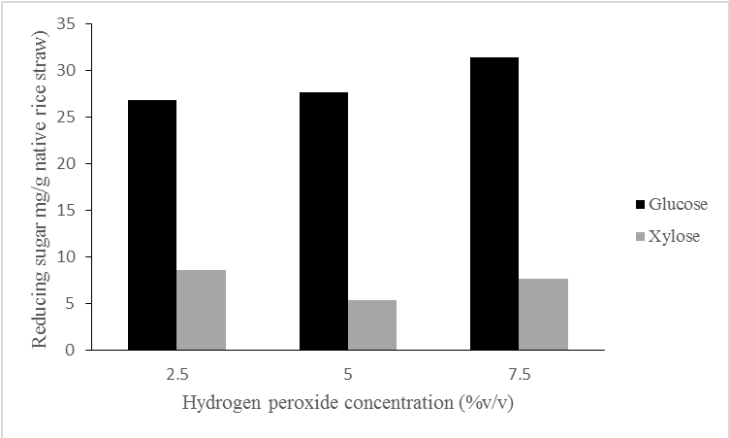


Fig. 4.9. Reducing sugar yields from enzymatic hydrolysis of rice straw pretreated by triethylamine in different hydrogen peroxide concentrations. The pretreatment reaction contained 7.5%, (w/v) solid loading treated with different hydrogen peroxide concentrations (2.5-7.5%, v/v) at 35^{o}C for 24 h.

From the above results, it was revealed that alkaline peroxide pretreatment of rice straw using the tert-butanol/NH_4OH system resulted in progressively high sugar releasing compared to tert-butanol/TEA. In the experimental data (Fig.4.4), it showed that the lower the solid loading, the higher the reducing sugar production. The effect of solid loading on tert-butyl alcohol/NH_4OH pretreatment of rice straw was studied. The biomass loading was reduced from 7.5 to 5 and 2.5 %, w/v, and operating temperature was also reduced from 35 to 25ºC to prevent evaporation of ammonium hydroxide since the boiling point of ammonia is 36ºC, under fixed conditions for other parameters (7.5%, v/v hydrogen peroxide concentration, 24 h operating time). As expected, the lowest solid loading (2.5%, w/v) demonstrated the highest reducing sugar of 569.63 mg/g pretreated rice straw (Table 4.10).

Table 4.10 Effect of hydrogen peroxide concentration on sugar recovery by using different ammonium hydroxide in tert- butyl alcohol.

Run	Solid loading (% w/v)	Alkaline	Organic solvent	Weight recover (%)	Reducing sugar (mg/g pretreated rice straw	SD	Reducing sugar (mg/g native rice straw	SD
1	2.5	NH_4OH	Tert-butyl alcohol	79.97	569.63	6.59	413.17	25.17
2	5	NH_4OH	Tert-butyl alcohol	80.75	545.01	4.80	440.37	3.88
3	7.5	NH_4OH	Tert-butyl alcohol	81.56	535.11	5.63	436.65	4.60

*The reaction (10 ml total volume) contained 2.5-7.5% (w/v) rice straw, 7.5% (v/v) H_2O_2, and ml organic solvent while NH_4OH (2-4 ml) was added to adjust pH to 11.5.

In Table 4.11, it was found that the increase of solid loading reduced glucose recovery. The maximum glucose recovery 62.76% from the native biomass was obtained from 2.5% (w/v) solid loading.

Table 4.11 Glucose and xylose recovery from alkaline peroxide pretreatment using different alkaline catalysts (ammonium hydroxide and triethylamine) and organic solvents (alcohol).

Run	Solid loading (%w/v)	Alkaline	Organic solvent	Glucose recovery (%)	SD	Xylose recovery (%)	SD
1	2.5	NH_4OH	Tert-butyl alcohol	62.76	1.09	29.33	0.83
2	5	NH_4OH	Tert-butyl alcohol	53.28	3.89	24.60	2.04
3	7.5	NH_4OH	Tert-butyl alcohol	46.49	0.45	17.22	0.61

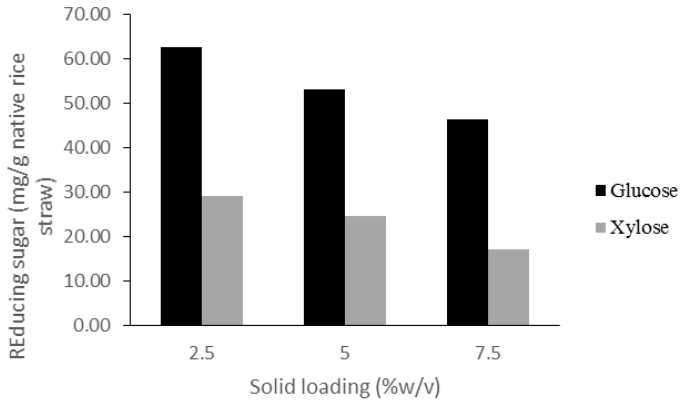


Fig. 4.10. Glucose and xylose recoveries from enzymatic hydrolysis of rice straw pretreated by tert-butyl alcohol/NH_4OH in different hydrogen peroxide concentrations. The pretreatment reaction contained 7.5%, w/v solid loading treated with different hydrogen peroxide concentrations at 35ºC for 24 h.

Overall, the results showed that the use of organic co-solvents did not show any marked effects on improving the sugar yields obtained from enzymatic hydrolysis. In the next step, the effect of using ammonium hydroxide as an alternative alkaline catalyst in the conventional water-based reaction was studied. The results in Table 4.12 showed that high reducing sugar yield and glucose recovery were obtained from the water/NH_4OH reaction. The highest reducing sugar yield of 520 mg/g native biomass and 55% glucose recovery were obtained when pretreated 2.5%, w/v biomass loading with 5% (v/v) hydrogen peroxide concentration at 35ºC for 24 h.

Table 4.12 Sugar recovery of alkaline peroxide pretreatment using the water/NH_4OH system

Run	Variables				Alkaline	Solvent	Weight loss (%)	Reducing sugar/g pretreated	Reducing sugar/g native	Glucose recovery (%)
	H_2O_2 concentration (%v/v)	Temp (°C)	Time (h)	Solid loading (%w/v)						
1	1.25	25	24	2.5	NH4OH	water	6	253	238	28
2	1.25	25	24	5	NH4OH	water	10	258	232	27
3	1.25	25	24	7.5	NH4OH	water	13	236	207	24
4	2.5	25	24	2.5	NH4OH	water	12	515	452	47
5	2.5	25	24	5	NH4OH	water	15	459	391	35
6	2.5	25	24	7.5	NH4OH	water	16	389	328	32
7	5	25	24	2.5	NH4OH	water	11	656	498	53
8	5	25	24	5	NH4OH	water	17	573	477	51
9	5	25	24	7.5	NH4OH	water	19	557	450	46
10	1.25	35	24	2.5	NH4OH	water	18	417	343	40
11	1.25	35	24	5	NH4OH	water	18	404	330	32
12	1.25	35	24	7.5	NH4OH	water	17	361	299	30
13	2.5	35	24	2.5	NH4OH	water	20	626	500	53
14	2.5	35	24	5	NH4OH	water	19	547	441	48
15	2.5	35	24	7.5	NH4OH	water	22	537	420	46
16	5	35	24	2.5	NH4OH	water	19	769	520	55
17	5	35	24	5	NH4OH	water	24	629	477	52
18	5	35	24	7.5	NH4OH	water	23	636	450	50

*The reaction (10 ml total volume) contained 2.5-7.5% (w/v) rice straw and 1.25-5% (v/v) H_2O_2 while NH_4OH (0.5-3 ml) was added to adjust pH to 11.5.

4.4. Physical analysis of pretreatment effects by scanning electron microscope (SEM)

The microstructures of the native and pretreated rice straws obtained from the alkaline peroxide process were analyzed by scanning electron microscopy (SEM). The results displayed an intact surface of the lignocellulosic biomass covered by lignin, which acts as the protective shield to external chemical and microbial attacks. This inhibits enzyme accessibility to cellulose and hemicellulose components in the hydrolysis process (Fig.4.11).

The intact biomass structure thus limited the enzymatic hydrolysis of the native rice straw which resulted in low sugar yields obtained.

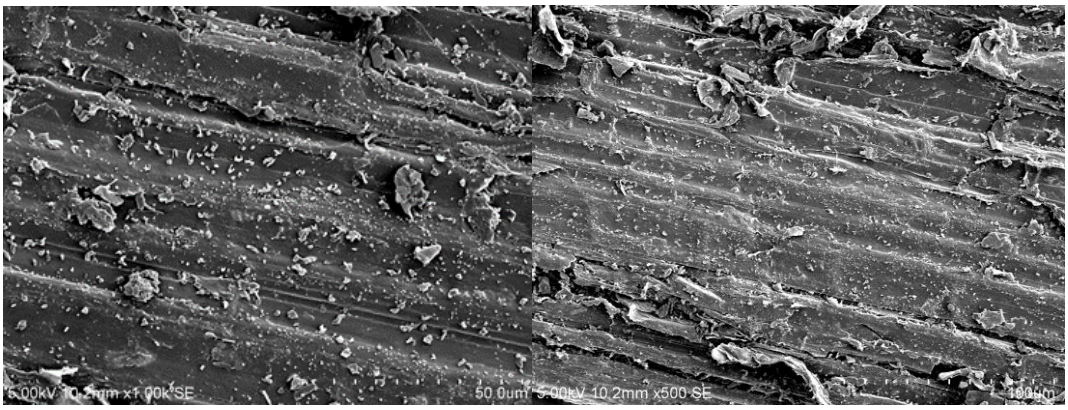


Fig. 4.11. Physical structure of native rice straw as analyzed by SEM magnify 500X.

4.4.1. Scanning electron microscope analysis of rice straw pretreated with conventional alkaline peroxide water-based/ NaOH pretreatment

The effects of alkaline peroxide pretreatment on rice straw under various conditions are shown in Fig 4.12. The results illustrated the structural changes on biomass morphology from the effects of oxidative agent under alkaline conditions. Peeling of the rice straw surface of observed with formation of papillae structure. This indicates the removal of lignin as well as hemicellulose from the biomass which led to the increasing external surface area and the porosity of the biomass. Thus, resulted in increasing enzymatic accessibility to the cellulose microfibrils and led to markedly increasing in sugar yield obtained from the pretreated biomass.

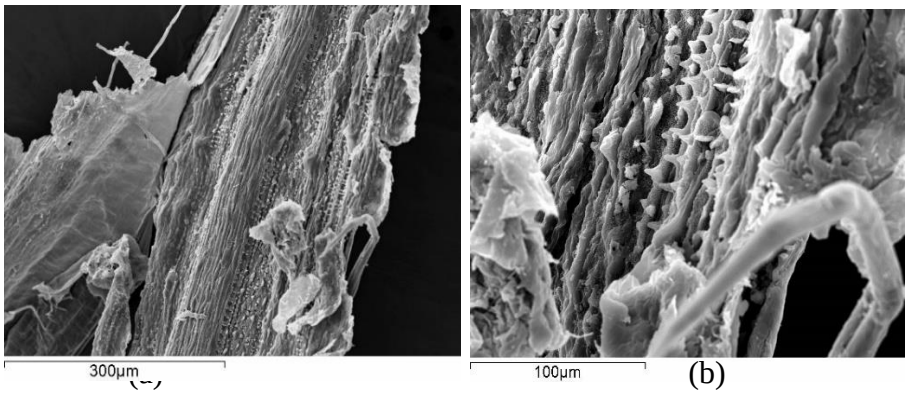
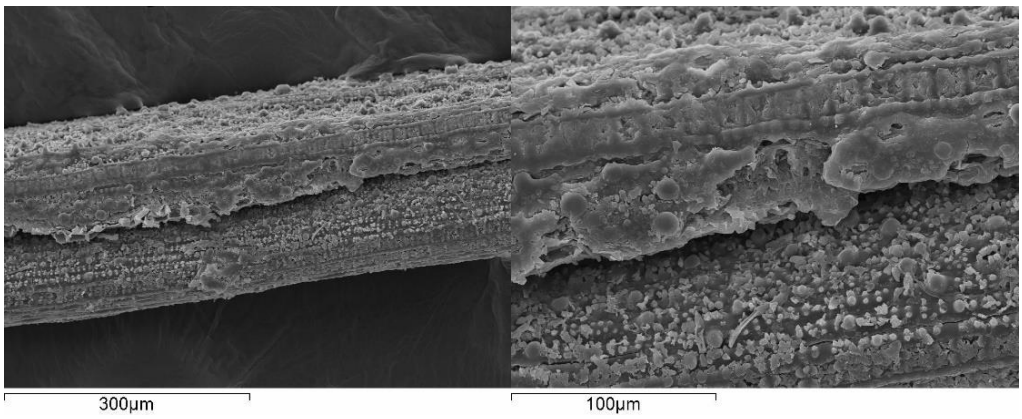


Fig. 4.12. Morphology changes in rice straw after alkaline peroxide pretreatment. 5%,w/v ground rice straw was treated with 7.5%, v/v H_2O_2 , pH 11.5 as adjusted by catalyst and 35^{o}C for 18 h (a) 200X, (b) 500X

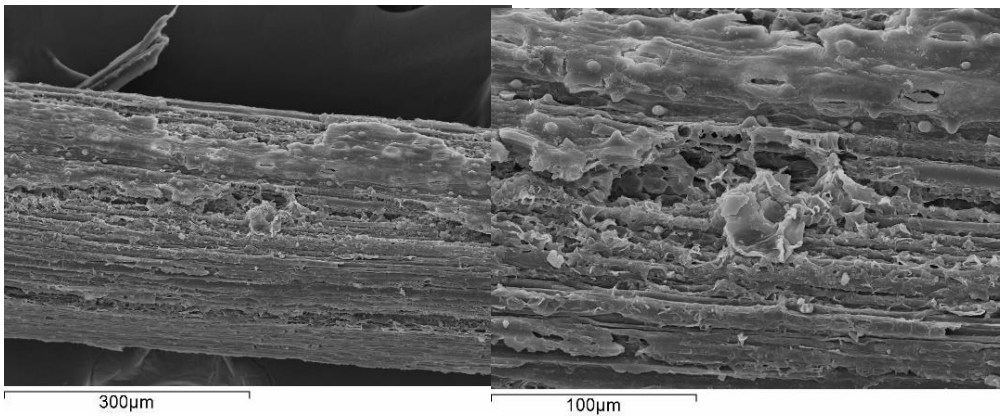
4.4.2. Scanning electron microscope analysis of rice straw pretreated with alkaline peroxide pretreatment using NH_4OH as a catalyst with different co-solvents.

The effects of modified alkaline peroxide pretreatment on rice straw in the reaction medium containing different co-solvents (ethanol, isopropanol, and tert-butyl alcohol) with NH_4OH as the catalyst are shown in Figs. 4.8 to 4.11. The SEM pictures indicates peeling of the surface of the biomass pretreated under all conditions due to removal of lignin but to a less extent compared with the observed using the conventional alkaline peroxide pretreatment using water and NaOH. The most distinct change in the modified reactions was observed with the reaction containing tert-butanol as the co-solvent which was in accordance high reducing sugar obtained from enzymatic hydrolysis.



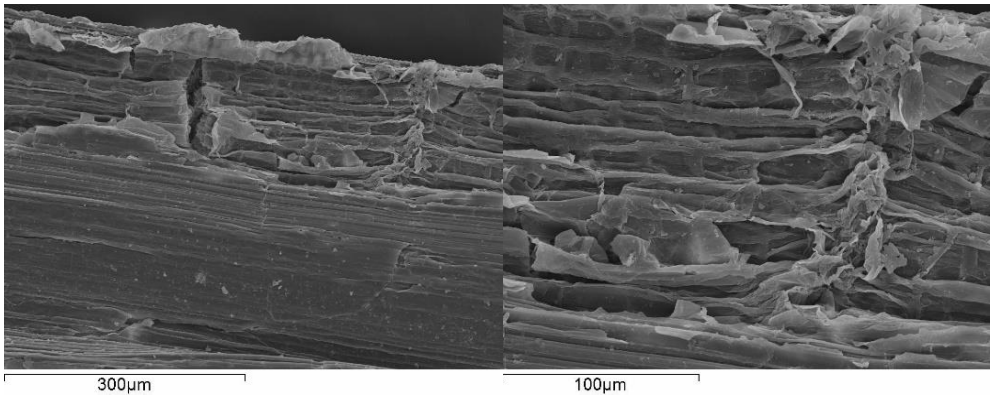
(a) (b)

Fig. 4.13. Physical structure of rice straw pretreated with modified alkaline hydrogen peroxide pretreatment using ethanol and NH_4OH. The reaction contained 7.5%, w/v rice straw loading pretreated with 2.5% (v/v) H_2O_2 using ethanol as a co-solvent with NH_4OH as the alkaline catalyst and incubated at 35^{o}C for 24h (a) 200X, (b) 500X



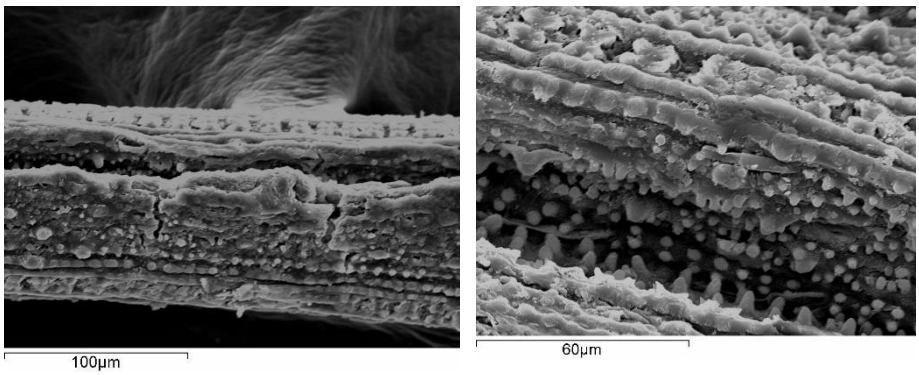
(a) (b)

Fig. 4.14. Physical structure of rice straw pretreated with modified alkaline hydrogen peroxide pretreatment using isopropanol and NH_4OH. The reaction contained 7.5%, w/v rice straw loading pretreated with 2.5% (v/v) H_2O_2 using isopropanol as a co-solvent with NH_4OH as the alkaline catalyst and incubated at 35^{o}C for 24h (a) 200X, (b) 500X



(a) (b)

Fig. 4.15. Physical structure of rice straw pretreated with modified alkaline hydrogen peroxide pretreatment using tert-butyl alcohol and NH_4OH. The reaction contained 7.5%, w/v rice straw loading pretreated with 2.5% (v/v) H_2O_2 using tert-butyl alcohol as a co-solvent with NH_4OH as the alkaline catalyst and incubated at 35^0C for 24h (a) 200X, (b) 500X



(a) (b)

Fig. 4.16. Physical structure of rice straw pretreated with modified alkaline hydrogen peroxide pretreatment using water and NH_4OH. The reaction contained 7.5%, w/v rice straw loading pretreated with 2.5% (v/v) H_2O_2 using water as a co-solvent with NH_4OH as the alkaline catalyst and incubated at 35^oC for 24h (a) 200X, (b) 500X

4.5. X-ray diffraction

Table 4.13 Crystallinity of rice straw pretreated with alkaline hydrogen peroxide process under different conditions.

Sample	Crystallinity Index (%)
Untreated rice straw	81.6
H_2O_2+NaOH+H_2O	52.1
H_2O_2+NH_4OH+Ethanol	76.2
H_2O_2+NH_4OH+Isopropanol	75.1
H_2O_2+NH_4OH+Tert-butyl alcohol	73.4
H_2O_2+NH_4OH+H_2O	67.3
H_2O_2+TEA+Ethanol	79.3
H_2O_2+TEA+Isopropanol	78.5
H_2O_2+TEA+Tert-butyl alcohol	77.4

The results showed that the native biomass is highly crystalline (CrI = 81.6) while pretreatment led to decreasing CrI under all experimental conditions (Table 4.7). The lowest CrI of 52.1 was found for rice straw pretreated with the conventional AHP (H_2O_2 + NaOH + H_2O) followed by that pretreated in water using NH_4OH as the alkaline catalyst. Substantial decrease in CrI was observed for the rice straw pretreated using NH_4OH with co-solvents while only slight reduction in CrI was found for those pretreated using TEA in the presence of co-solvents compared to the native sample. Crystallinity is an important feature affecting enzymatic saccharification of cellulose. The reduction in CrI could be due to the changes in cellulose crystal structures by disrupting inter- and intra- chain hydrogen bonding of cellulose fibrils.

This decrease in cellulose crystallinity indicates that the recovered product is highly amorphous, and therefore has an increase in cellulose surface accessibility, and would theoretically enable more efficient enzymatic hydrolysis. The decrease in CrI of the pretreated biomass was in contrast to several pretreatment methods e.g. hydrothermal pretreatment using dilute acid as the catalyst, liquid hot water and steam explosion in which increase in CrI was found due to their effects on removal of the amorphous hemicellulose

and/or lignin from the biomass with no modification of the crystallinity of the cellulose fraction.

CHAPTER 5
CONCLUSION

Pretreatment is a pre-requisite step in a sugar platform biorefinery to improve the enzymatic digestibility of the intact native biomass in order to obtain feasible sugar yields for further conversion to biofuels, biochemical, and other value-added products. Alkaline hydrogen peroxide pretreatment is considered an efficient and eco-friendly pretreatment process which has been previously reported for pretreatment of various agricultural residues. The conventional alkaline pretreatment process is operated in a low temperature range and clean as the process generated only water as the by-product from decomposition of H_2O_2. Under the alkaline conditions, H_2O_2 dissociates to hydroperoxy anion which reacts with reactive groups in lignin, which leads to solubilization of lignin by cleavage of lignin ring, aryl ether bond, or other linkages within lignin [34]. When compared to other pretreatment alkaline peroxide pretreatment tend to be gentler, operating conditions is less critical and appears to be most effective method in breaking ester bonds between lignin and polysaccharides. The modification of the alkaline hydrogen peroxide process for pretreatment of rice straw was explored in this study.

The effects of key reaction parameters (H_2O_2 concentration, solid loading, temperature, and time) on efficiency and selectivity of the alkaline pretreatment process were studied. The optimized reaction condition (5%, w/v solid, 7.5%, w/v H_2O_2 adjusted to pH 11.5 with NaOH and incubated at 35ºC for 18 h) led to the highest reducing sugar yield of 984.14 mg/g pretreated biomass, which is equivalent to 687.9 mg/g native biomass from enzymatic hydrolysis under the standard conditions with the commercial cellulase. Glucose recovery was equivalent to 82% from the native rice straw. Only a very low level of pentose was found under the optimized condition, suggesting high efficiency of the developed pretreatment process on increasing digestibility of the cellulose fraction and its high selectivity on removal of the hemicellulose and lignin from the native biomass. The glucose recovery yield in this study was comparable to those previously reported for alkaline hydrogen peroxide pretreatment on other lignocellulosic biomass e.g. bamboo [56].

The incorporation of an organic co-solvent in the reaction was examined in order to see its effects on increasing solubility of the extracted lignocellulosic components. Tert-butanol was found to be the best co-solvent according to the sugar yield obtained compared to other solvents in this study. However, the uses of different co-solvents (ethanol,

isopropanol) into the reaction led to lower sugar yield (20 - 60% glucose recovery) from enzymatic hydrolysis of the solid residues as compared to the conventional alkaline hydrogen peroxide pretreatment process. Several organic solvents e.g. alcohols have been explored as the sole solvents or co-solvents for organosolv pretreatment of various biomass at an elevated temperature range. The results thus showed no significant improvement effects of the co-solvent on efficiency and selectivity for the low-temperature alkaline hydrogen peroxide pretreatment reactions.

The use of organic alkaline reagents i.e. NH_4OH and TEA could provide an alternative to NaOH with the advantages on recycling by evaporation due to its high volatility at relatively low temperatures. The use of these organic alkalines led to decreasing reducing yield compared to the conventional conditions using NaOH. The lower pretreatment efficiency could be due to the lower alkalinity of the organic alkalines to NaOH which is a strong base. However, the results thus suggested the potential of using organic alkalines in the modified alkaline hydrogen peroxide pretreatment process.

Enzymatic digestibility of the pretreated substrate and the sugar recovery were in accordance with the observed changes to physical structures and crystallinity of the pretreated biomass as shown by SEM and XRD. The results thus suggested that the improvement in enzymatic digestibility of the rice straw was due to the disruption of the intact lignocellulose surface due to removal of hemicelluloses and lignin which led to increased enzyme accessibility to the cellulose microfibers and the reduction in cellulose crystallinity.

REFERENCES

[1]. Dilip, A., et al. Sustainable energy for developing countries, S.A.P.I.EN.S [Online], 2.1 | 2009, Online since 27 November 2009, connection on 16 January 2014. URL: http://sapiens.revues.org/823.

[2]. Alya, L., et al. 2012. Lignocellulosic biomass for bioethanol production: Current perspectives, potential issues and future prospects. Progress in Energy and Combustion Science. 38: 449-467.

[3]. Hongzhang, C., et al. 2010. Key technologies for bioethanol production from lignocelluloses. Biotechnology Advances. 28: 556-562.

[4]. Nathan, M., et al. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresource Technology. 96: 673-686.

[5]. Anuja, N., et al. Biomass Production from Biomass. http://www.ika.rwth aachen.de/r2h/index.php/Hydrogen_Production_from_Biomass

[6]. Seksan, P., et al. Overview of Biomass Utilization in Thailand: http://www.aist-riss.jp/old/lca/ci/activity/project/biomass/report/041028_paper/thailand_paper.pdf

[7]. Demirbas, A., et al. 2001. Biomass resource facilities and biomass conversion processing for fuel and chemicals Energy Converts Manage 43:1357-1378.

[8]. Yuya, Y., et al. 2010. Alkaline peroxide pretreatment for efficient enzymatic saccharification of bamboo. Carbohydrate Polymers. 79: 914-920.

[9]. Vishnu, M., et al. 2012. Trends in bioconversion of lignocelluloses: Biofuels, platform chemicals & biorefinery concept. 38: 522-550.

[10]. Kamm, B., et al. 2004. Principles of biorefineries. Appl Microbiol Biotechnol. 64: 137-145.

[11]. Gierer, J. et al. 1985. Chemistry of delignification. Part 1: General concept and reactions during pulping. *Wood science and Technology*, 19, 289-312

[12]. Farid, T., et al. 2010. Production of bioethanol from wheat straw: An overview on pretreatment, hydrolysis and fermentation. Bioresource Technology. 101: 4744-4753.

[13]. Freire, C., et al. 2006. Effect of oxygen, ozone and hydrogen peroxide bleaching stages on the contents and composition of extractives of Eucalyptus globulus kraft pulps. *Bioresource Technology*, 97 (3), 420-428.

[14]. Taherzadeh, M. 2008. Pretreatment of lignocellulosic wastes to improve ethanol ethanol and biogas production: A review. *International Journal of Molecular Sciences*, 9 (9), 1621-1651

[15]. Miller, G.L., 1995. Using dinitrosalicylic acid for determination of reducing sugar. Anal. Chem. 31, 426–428.

[16]. Golova, O., et al. 1973. *Degradation of cellulose by alkaline oxidation*. Russian Chemical Reviews, 42(4), 327-338.

[17]. Lewin, M., et al. 1971. *Investigation of biomass degradation mechanism in pretreatment of switchgrass by aqueous ammonia and sodium hydroxide*. Bioresource Technology, 101 (21), 8185-8191

[18]. Badal, C., et al. 2007. *Enzymatic saccharification and fermentation of alkaline peroxide pretreated rice hulls to ethanol*. Enzyme and microbial technology. 41: 528-532.

[19]. Jessica, A., et al. 2013. *Alkaline hydrogen peroxide pretreatment of cashew apple bagasse for ethanol production*: Study of parameters. Bioresource Technology. 139: 249-256.

[20]. Ana, D., et al. 2013. *Pretreatment of Rice Hulls with Alkaline Peroxide to Enhance Enzyme Hydrolysis for Ethanol Production*. Chemical Engineering Transaction. 32: 949-954.

[21]. C.A. Cardona. Process synthesis for fuel ethanol production. New York: CRC Press; 2010.

[22]. NSF MUSES: Cellulosic Ethanol: A Future Fuel from Forests. http://www.sfi.mtu.edu/ FutureFuelfromForest/LignocellulosicBiomass.htm

[23]. Plant Structure & Function. Cellulose and Lignin. http://sci.waikato.ac.nz/farm/content/plantstructure.html

[24]. Harmsen, P., et al. 2010. Literature Review of Physical and Chemical Pretreatment Processesfor Lignocellulosic Biomass. http://www.ecn.nl/docs/library/report/2010/e10013.pdf

[25]. Hamelick, P., et al. 2003. *Prospects for ethanol from lignocellulosic biomass: techno-economic performance as development processes*. Report NWS-E-2003-55, Utrecht University, the Netherlands.

[26]. Lee, J. 1997. *Biological conversion of lignocellulosic biomass to ethanol*. Journal of Biotechnology 56:1-24.

[27]. Biomass, biofuel, and biochemicals. 2008. More about lignocellulosic biomass pretreatments. http://biofueloutlook.blogspot.com/2008/11/more-about-lignocellulosic-biomass.html

[28]. Hendriks, F., et al. 2009. *Pretreatments to enhance the digestibility of lignocellulosic biomass*. Bioresources Technology. 100: 10-18.

[29]. Oh, K., et al. 2001. *Pretreatment of lignocellulosic biomass using combination of ammonia recycled percolation and dilute acid process*. Journal of Industrial and Engineering Chemistry, 8 (1), 64-70.

[30]. Saha, B., et al. 2006. *Ethanol production from alkaline peroxide pretreated enzymatically saccharified wheat straw*. Bitechnology Progresss, 22 (2), 449-453.

[31]. Zhao, Y., et al. 2008. *Enhanced enzymatic hydrolysis of spruce by alkaline pretreatment at low temperature,* Biotechnology and Bioengineering, 99(6), 1320-1328.

[32]. Lopez, R., et al. 2000. Alkaline surface modification of sugarcane bagasse. *Advanced Composite Material*, 9(2), 99-108

[33]. Heitz, M., et al. (1987) Physical-chemical characterization of lignocellulosic substrates pretreated via autohydrolysis: an application to tropical woods. *Biomass*, 13 (4), 255-273.

[34]. Zhao, X., et al. 2009. Organosolv pretreatment of lignocellulosic biomass for enzymatic hydrolysis. Applied Microbiology and Biotechnology, 82 (5), 815-827.

[35]. Cateto, C., et al. 2011. Enzymatic hydrolysis of organosolv Kanlow swithgrass and its impact on cellulose crystallinity and degree of polymerization. Energy and Environmental Science,4(4), 1516-1521.

[36]. Sun, F., et al. 2008. Organosolv pretreatment by crude glycerol from pleochemicals industry for enzymatic hydrolysis of wheat straw. Bioresource Technology, 99(13), 5474-5479.

[39]. Zhang, Y., et al. 2007. Fractionating recalcitrant lignocellulose at modest reaction conditions. Biotechnology and Bioengineering, 97 (2), 214-224.

[40]. White, R., et al. 1987. Effect of lignin content and extractives on the higher heating value of wood. Wood and Fiber Science, 19, 446-452.

[41]. Brodeur, G., et al. 2011. Chemical and physicochemical pretreatment of lignocellulosic biomass: a review. Enzyme Research, 17.

[42]. Malinen, R., et al. 1972. Studies on the reactions of carbohydrates during oxygen bleaching. Part 1. Oxidative alkaline degradation of cellubiose. Papperi ja Puu, 54, 451

[43]. Kim, T., et al. 2003. Pretreatment of corn stover by aqueous ammonia. Bioresource Technology, 90 (1), 39-47

[44]. Oh, K., et al. 2001. Pretreatment of lignocellulosic biomass using combination of ammonia recycled percolation and dilute acid process. Journal of industrial and engineering Chemistry, 8 (1), 64-70

[45]. Thompson, N.S., et al. 1977. Study of the carbohydrate peeling and stopping reactions under the conditions of oxygen –alkali pulping, Projest 3265, report three: a progress report to members of the Insitute of Paper Chemistry. Appleton, Wisconsin: the Institute.

[46]. Lee, J.W., et al. 2011. Efficiencies of acid catalysts in the hydrolysis of lignocellulosic biomass over a range of combine severity factors. Bioresour. Technol. 102, 5884–5890.

[47]. Lu, Y., Mosier, N.S., 2007. Biomimetic catalysis for hemicellulose hydrolysis in corn stover. Biotechnol. Prog. 23, 116-123.

[48]. Menon, V., et al. 2012. Trends in bioconversion of lignocellulose: Biofuels, platform chemicals & biorefinery concept. Prog. Energ. Combust. Sci. 38, 522-550.

[49]. Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M., Ladisch,M., 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour. Technol. 96; 673-686.

[50]. Saha, B.C., Iten, L.B., Cotta, M.A., Wu, Y.V., 2005. Dilute acid pretreatment, enzymatic sacchrification, and fermentation of rice hulls to ethanol. Biotechnol. Prog. 21, 816-822.

[51]. Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D. 2006. Determination of sugars, byproducts, and degradation products in liquid fraction process samples. Laboratory Analytical Procedure.

[52]. Jonsson, L.J., Alriksson, B., Nilvebrant, N.O., 2013. Bioconversion of lignocellulosic: inhibitors and detoxification. Biotechnol. Biofuels 6, doi: 10.1186/1754-6834-6-16.

[53]. Lee JY, Ryu HJ, Oh KK. Acid-catalyzed hydrothermal severity on the fractionation of agricultural residues for xylose-rich hydrolyzates. Bioresour Technol 2013; 132:84-90.

[53]. Gupta, R., Lee, Y.Y., 2010. Investigation of biomass degradation mechanism in pretreatment of switchgrass by aqueous ammonia and sodium hydroxide. Bioresour. Technol. 101(21), 8185-8191.

[54]. Adney, B., Baker, J., 1996. Measurement of cellulase activities, Chemical Analysis and Testing Task. Laboratory Analytical Procedure.

[55]. Miller, G.L., 1995. Using dinitrosalicylic acid for determination of reducing sugar. Anal. Chem. 31, 426–428.

[56]. Yamashita, Y., 2010.Alkaline peroxide pretreatment for efficient enzymatic accharification of bamboo. Carbihydrate Polyers, 74, 914-920