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Bio-ethanol Production from Wheat Straw Using Different Pre-treatment Approaches

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by

Mohammed Oday Abdulsattar

(B. Eng, M. Eng, University of Technology, Iraq)

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Dedication

This thesis is dedicated to my father and mother, for their endless love, support and encouragement. I am truly thankful for having you in my life. This work is also dedicated to my sisters, Ban, Hanan and Jwan, who stand by me when things look tough and to all the people in my life who touch my heart.

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Papers

Abdulsattar, M.O., Abdulsattar, J.O., Greenway, G.M., Welham, K.J., Zein, S.H., Optimization of pH as a Strategy to Improve Enzymatic Saccharification of Wheat Straw for Enhancing Bioethanol Production, biomass and bioenergy. Under review.

Abdulsattar, M.O., Greenway, G.M., Zein, S.H., Optimization of the microwave pre-treatment on the wheat straw to increase the overall sugar recovery. Trends in biotechnology. In preparation

Abstract

The high level of pollution associated with greenhouse gas emissions and the rapid increase in energy demand across the globe raised the needs for clean, easily available, cheap and renewable energy sources to replace traditional fossil fuels. Among the different forms of renewable energy, bio-ethanol has been of great interest in recent decades since it has the ability to replace conventional transport fuel. A wide range of raw materials including food crops, molasses and lignocellulosic biomass have been utilised for bio-ethanol production. Among the variety of lignocellulosic biomass, agricultural waste such as Wheat Straw (WS) presents itself as a good candidate for bio-ethanol production on an industrial scale. In the current study, WS was pre-treated with several approaches including grinding, Steam Explosion (SE), Liquid Hot Water (LHW), microwave, Atmospheric Disk Refining (ADR) and pressurized disk refinering (PDR) with the aim to improve the total reduced sugar yield from Enzymatic Hydrolysis (EH). In grinding pre-treatment, WS was ground with a ceramic disk to various particle sizes (> 2000 µm to < 250 µm). The highest total reduced sugar yield after EH of 58.0% (wt/wt) was obtained from the sample with a particle size of < 250 µm. SE and LHW pre-treatment were carried out at the same severity of 4.65 and 3.35 by using distilled water or H_2SO_4 (3%, wt/wt), respectively. PDR pre-treatment experiments were conducted at a pressure ranging 4, 6, 8 and 10 bar. Moreover, the ADR pre-treatment was performed at the atmospheric pressure. Microwave pre-treatment time (min), temperature (°C), power (W) and distilled water volume (mL)effects on sugar recovery were investigated by the means of the Design of Experiments (DoE) software. Furthermore, microwave pre-treatment conditions were optimized. The maximum total reduced sugar yield of 92.1% was obtained from the

WS pre-treated by the PDR at 10 bar. Meanwhile, the highest total reduced sugar yield obtained from the ADR was 74.6%.

The hemicellulose removal in the liquid fraction for the SE, LHW and microwave was reported. The overall sugar recovery (including the total reduced sugar yield after EH and the extracted sugars in the liquid fraction (if applicable)) yield was calculated and reported. The overall sugar recovery yield for the SE and LHW pre-treatment with H_2O and H_2SO_4 (3%, wt/wt) was 72.4%, 82.7% and 69.5%, 85.6%, respectively. The highest overall sugar recovery yield of 93.4% was achieved by applying microwave pre-treatment method on the WS at 200 °C, 120 min, 900 W and with H_2O volume 30 mL. The microwave pre-treatment conditions were optimized to reduce the pre-treatment time. The optimum microwave pre-treatment time was found to be 200 °C, 42.8 min, 900 W and 30 mL at which the overall sugar recovery yield was 88.4%.

Moreover, the effects of the pH value during the EH process was evaluated to find the optimum pH value in which the total reduced sugar yield reach its maximum potential. The ground WS to the particle size of < 250 µm was used to investigate the pH effect on the EH process. Different enzymes cocktails including Celluclast 1.5L supplements with Novozymes 188, Cellic CTec2 and endo-1, 4-β-Xylanase were used in the pH study (pH 3.0 to 7.0). The highest concentration of the total reduced sugar liberated during EH was obtained by carrying out the EH at pH 5.8 - 6.0 for all the different enzymes used in the current study. At the optimum pH value (5.8 and 6.0), the total reduced sugar concentration after the EH for Celluclast 1.5L with Novozymes 188, endo-1, 4-β-Xylanase and Cellic CTec2 were found to be 7.0, 7.4 and 10.8 g L^{-1}, respectively.

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List of Abbreviations

DNS	3,5-dinitro-2-hydroxybenzoic acid
HMF	5-hydroxymethylfurfural
ASL	Acid soluble lignin
AIR	Acid insurable residue
AFEX	Ammonia fibre explosion
ADR	Atmospheric disk refiner
CBU	Cellobiose units
CCD	Central composite design
CBP	Consolidated bioprocessing
DoE	Design of experiments
DM	Dry matter
EH	Enzymatic hydrolysing
FPU	Filter paper unit
GC	Gas chromatography
GC-MS	Gas chromatography mass spectrophotometer
HDS	Hemicellulose derived sugars
HPLC	High performance liquid chromatography
IU	International unit
LHW	Liquid hot water
ENEA	National agency for new technologies, energy and sustainable economic development
NREL	National renewable energy laboratory
MSTFA	n-Methyl-n-(trimethylsilyl) trifluoroacetamide
PDR	Pressurized disk refiner
RSM	Response surface methodology
SHF	Separate enzymatic hydrolysis and fermentation
SSF	Simultaneous saccharification and fermentation
SE	Steam explosion
TMP	Thermo-mechanical pulping
WIS	Water-insoluble solids
WDM	Wet disk milling
WS	Wheat straw

List of Symbols

Symbols	Description	Unit
Y	Absorbance	---
ε	Absorptivity at specific wavelength	---
X	Concentration	mg L^{-1}
W	Mass	g
Power	Microwave power	W
MW	Molecular weight	g mol^{-1}
rpm	Revolutions per minute	rpm
Ro	Severity factor	---
T	Temperature	℃
t	Time	min
UV abs	UV-absorbance	---
V	Volume	L

Chapter One: Introduction

1.1 Background

Global request for energy is growing continuously over the last century corresponding to the fast world population expanding and the raising in industrial cities around the world, especially in the developing countries. Conventional fossil fuels such as coal, natural gas and crude oil are still the main resources for energy increasing demands.

Using fossil fuels is associated with carbon dioxide (CO_2) emissions and increasing greenhouse gases concentration in the earth's atmosphere. Moreover, the unstable oil market and energy supplying security motivated many countries to initiate extensive research in order to find substitutional non-petroleum based sources of energy (Ballesteros et al., 2006, Talebnia et al., 2010, Tran et al., 2019). EU has set a target that 25% of the total fuel used for transportation will be from biofuel production by 2030. As for the US Department of Energy, the target is to produce 60 billion gallons per year by 2030 (Himmel et al., 2007).

Several renewable energy sources such as biomass, solar cell, hydropower and wind turbines are being used worldwide. Among them, biofuels such as bio-ethanol or biodiesel derived from biomass materials present itself as the only convenient renewable energy resource that has the ability to replace transportation fuels (Hamelinck et al., 2005, Patni et al., 2013).

Among the different biofuels forms, bio-ethanol presents itself as the best candidate to be used as a transportation fuel and replace fossil fuels gradually. Bio-ethanol can be used in two ways in vehicles, either as an additive with the gasoline or as a sole fuel in vehicles (Licht, 2006, Goldemberg et al., 2019).

For example, in America, the bio-ethanol production from corn has been used as an additive to the gasoline (with 10% bio-ethanol by volume) since the 80's (Wang et al.,

1999). Recently, a bio-ethanol blend E85 (85% bio-ethanol and 15% gasoline by volume) has been used in vehicles and shows a significant reduction in greenhouse gas emission (Goldemberg et al., 2019). During the period between 2007 – 2017, world fuel bio-ethanol production increase from 13 to 27 million gallons (Association, 2017).

In the earliest attempt of bio-ethanol production, food crops (known as the first generation) such as sugar, starch and oily-crops were used as raw materials. Despite the high production of the bio-ethanol, using of the first generation biomass create a debate of fuel against food. Using the food crops for bio-ethanol production might threaten the sustainability of food supply world-wide, especially with the human population rapidly increasing (Taherzadeh and Karimi, 2007, Talebnia et al., 2010).

Lignocellulosic waste material (known as the second generation) acquired from agricultural waste, wood and grass presents itself as an interesting alternative for bio-ethanol production since it does not compete with food crops, high availability and relativity low price (Lin and Tanaka, 2006, Gomez et al., 2008).

Lignocellulose has been identified as the most abundant source of renewable biomass feedstock for biofuel production (Lin and Tanaka, 2006, Zhou et al., 2011). Lignocellulose consists of approximately 75% polysaccharide sugars (cellulose and hemicelluloses) which can be fermented to bio-ethanol (Bayer et al., 2007).

It has been estimated that more than 40 million tonnes of lignocellulose biomass including wood, straws and corn stover are produced annually (Sanderson, 2011).

Bio-ethanol production from lignocellulose biomass consists of 4 main processes (Mosier et al., 2005b):

1-Utilization or pre-treatment of the raw material.

Pre-treatment is an essential process which will determine the bio-ethanol yield and cost-effectiveness of the bio-ethanol production possess (Galbe and Zacchi, 2007). The objectives of pre-treatment are to remove lignin and de-crystallization of the lignocellulose biomass structure, prevent microorganism inhibitors forming and reduced the operating cost (Mosier et al., 2005b).

2- Enzymatic hydrolysis (EH) of cellulose and hemicellulose.

Hydrolysis process can be performed by using concentrated acid or with means of enzymes (Drapcho et al., 2008). With EH, the pre-treated biomass is usually exposed to cellulase and xylanase enzymes to breakdown cellulose and hemicellulose polymers to mainly glucose and xylose, respectively. The solid remaining after the EH is mostly lignin and un-hydrolysed cellulose as well as hemicellulose. In industrial large scale, the solid residue is normally burned and used to power the bio-ethanol production line plant (Zabed et al., 2016, Zabed et al., 2017). Recently, different aspects were explored to utilize the solid residue in order to extract valuables chemicals or convert it more useful materials (Rahikainen et al., 2011, Chen and Fu, 2016, Tomás-Pejó et al., 2017).

3- Fermenting the monosaccharides sugar with means of microorganism fermentation.

In the fermentation process, deferent microorganism might be used, among them, *Saccharomyces Cerevisiae* is the most common to be used for fermentation.

4- Distillation to purify bio-ethanol.

Based on the difference in water and ethanol boiling point. Ethanol distilation is carried out at 78 °C.

Different lignocellulose residues such as wheat, rice and barley straw as well as wood (soft and hard) can be used for bio-ethanol production. Among them, wheat straw (WS) presents itself as the second largest feedstock in the world after rice straw and

the largest in Europe (Kim and Dale, 2004, Balat et al., 2008). WS consists of 33 – 40% cellulose, 20 – 25% hemicellulose and 15 – 20% lignin (Prasad et al., 2007). According to statistics, WS worldwide production is approximately 690 kilotons in 2009 and around 730 million tons in 2014 (Zheng et al., 2018). As a result, WS serves as a main appropriate lignocellulosic feedstock for bioenergy in the 21st century and therefore, it was selected in his research. Figure 1-1 shows the bio-ethanol production from WS flow process.

After pre-treating the WS with different methods, the pre-treated WS was subjected to EH with means of different enzymes. The aqueous solution after EH was filtered and the liquid fraction was fermented with *S. Cerevisiae*

In this chapter, different types of pre-treatment, EH, fermentation and statistical method for optimization will be described with the examples of the related subject.

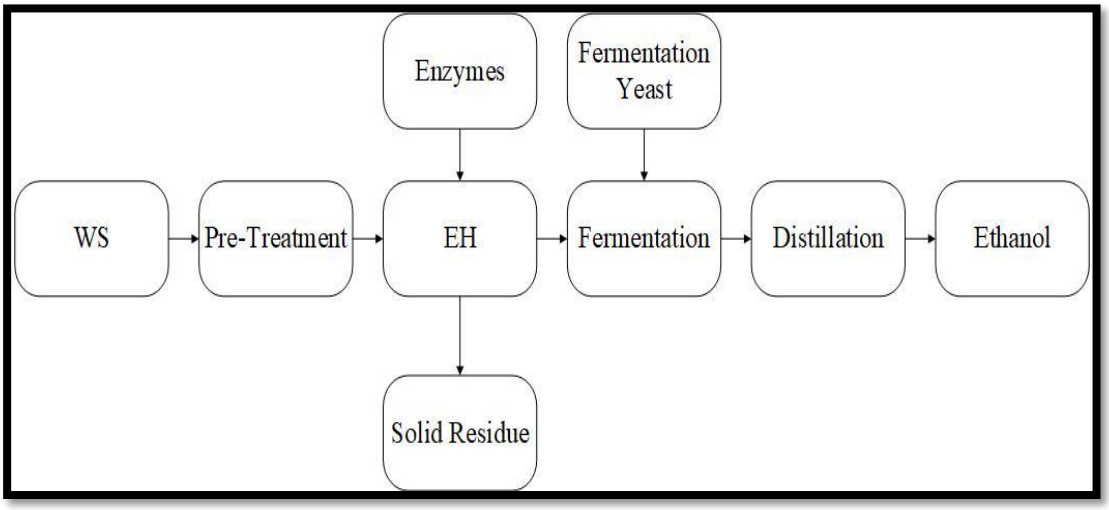


Figure 1-1. Bio-ethanol process flow diagram.

1.2 Pre-treatment

1.2.1 Introduction

The first step in bio-ethanol production from lignocellulosic biomass materials is pre-treatment. The objectives of pre-treatment are to enhance the total sugars yield from

EH and sugar recovery by breakdown the biomass structure. Over the years, several approaches and methods were developed to pre-treat lignocellulosic materials (e.g. WS) (Kumar et al., 2019). The effectiveness of the pre-treatment is measured by the amount of cellulose produced with high digestibility, low inhibitors forming, low sugars lost and high sugar recovery. In general, pre-treatment can be categorized in to: biological, physical, chemical and physio-chemical pre-treatment. A combination of two or more pre-treatment methods might be used on the lignocellulosic materials such as size reduction with acid pre-treatment or size reduction with a steam explosion in order to further improve the sugar yield for EH. Moreover, combining two pre-treatment methods can also decrease inhibitors forming and reducing energy consumption through the process at the same time.

The main pre-treatment processes used for lignocellulosic materials, WS specifically, are reviewed in the following sections.

1.2.2 Biological Pre-treatment

Microorganisms such as white-rot fungi, soft-rot fungi and brown-rot fungi are adapted to pre-treat various lignocellulosic materials based on their selectivity of to degrade lignin and hemicellulose (Rouches et al., 2018, Tian et al., 2018). Biological pre-treatment is considered as an environmentally friendly, safe and consumes lower energy in comparison to other pre-treatment methods. Nevertheless, the hydrolysis reaction rate is very low and requires further improvement and optimization (Okano et al., 2005, Sánchez, 2009). White-rot fungi is the most exceedingly used fungi among other microorganisms. Different white-rot fungi such as cyathus stercoletus, eriporiopsis subvermispora, basidiomycetes, ceriporiopsis subvermispora, pleurotus ostreatus and ceriporia lacerate have been used to treat various lignocellulosic materials (Bari et al., 2015, Nayan et al., 2018, Bentil et al., 2018). In a study carried

by Hatakka (1983), 19 different types of white-rot fungi were used to pre-treat WS with the aim of improving the enzymatic saccharification of cellulose. The pre-treatment was carried at 28 °C for five weeks. The results show that when Pycnoporus cinnabarinus 115 was used, the reduced sugars yield reached 54.6% after enzymatic saccharification and the other fungi show a variation in the reduced sugars yield (Hatakka, 1983).

In a more recent study carried by López -Abelairas et al (2013), optimization of white-rot fungi pre-treatment on WS for production of bio-ethanol was conducted. Basidiomycetes P. eryngii (ATCC 90787) and I. lacteus (Fr. 238 617/93) were chosen to apply biological pre-treatment on the WS. Three different loadings were investigated 1.5, 3 and 24 mg of fungi to g of WS. The pre-treatment was carried at 28 °C in a shaker for 21 days. They compared the results obtained for the fungi pre-treatment with the results from mild alkaline pre-treatment. Under the optimum conditions, both cellulose and hemicellulose digestibility increased at the end of the pre-treatment (21 days) from 16% to 100% and from 12% to 87%, respectively. Glucose recovery yield and bio-ethanol yields were 84% and 74% after 14 days, respectively. It has been pointed out that the results are comparable with the steam explosion (López-Abelairas et al., 2013).

1.2.3 Physical Pre-treatment

Physical also known as mechanical pre-treatment involves size reduction of the lignocellulosic biomass materials in order to decrease the degree of polymerization and crystallinity which consequently advance sugar production through EH (Tian et al., 2018).

Various physical pre-treatment techniques such as (milling, chipping, or grinding) have been applied to treat the biomass. The selection of the pre-treatment methods

depends on the lignocellulosic material type and the desired particle size (Alvira et al., 2010, Sun et al., 2016b). To obtain fine particle sizes, firstly, chipping or/and grinding process is usually used to get particle size within 2 – 4 mm followed by milling to reach very fine particle size.

Diverse milling methods such as (vibro energy milling, colloid milling, hammer milling, two-roll milling, ball milling and air-jet milling) have been utilized to pre-treat the lignocellulosic biomass to promote EH (Taherzadeh and Karimi, 2008, Licari et al., 2016).

Milling lignocellulosic materials improve the EH and fermentation significantly. However, milling to very fine size increase the energy requirement. Moreover, the milling pre-treatment effectiveness depends on different variables such as moisture content and the final particle size. To improves the milling and grinding process, various approaches were suggested such as a combination between size reduction and chemical or thermal pre-treatment is important to reduce the physical pre-treatment energy consumption (Jin and Chen, 2006, Hendriks and Zeeman, 2009). Mani et al (2004) evaluated milling process effects using hammer milling fitted with three different screen sizes 0.8, 1.6 and 3.2 mm. Four types of biomass feedstock WS, barley straw, switch-grass and corn stover were investigated. The influence of particle size distribution, densities, bulk geometric mean diameter as well as moisture content were specified. It was reported that switch-grass consumed the highest specific energy consumption Whilst corn stover consumptions was the lowest. As for WS, the specific energy consumptions were 51.6 and 11.4 $kWht^{-1}$ for screen sizes of 0.8 and 3.2 mm, respectively (Mani et al., 2004).

In another study, Thomas et al. (2012) studied the grinding performance using two rows discs on WS, soybeans and maize in a multi-cracker system. The effects of disc

rotation speed, throughput, the gap between the two discs and the grinding disc types (ceramic versus steel discs) on the grinding performance were reported. The grinding performance was evaluated by monitoring specific energy consumption, particle size means and particle size distribution curves. It was reported that soybeans consumed the highest specific energy 2.8 $kWht^{-1}$ while WS consumed 1.8 $kWht^{-1}$ at the highest mean particle size (Thomas et al., 2012).

The effect of grinding processes of WS on enzymatic degradation was reported by Silva et al (2012). They used a pilot-scale grading system to obtained fine (800 – 50 μm) and ultra-fine (20 – 10 μm) particle size from WS. The WS powder were classified based on degree of crystallinity, particle-size distribution and sugars yield through EH for system effectiveness evaluation. The results showed an improvement in WS degradability by size reduction down to ∼100 μm particle size in which 36% and 40% total carbohydrate and glucose hydrolysis yields were obtained, respectively. A higher carbohydrate yield (46%) and glucose yields (72%) was recorded after using ball milling. The increasing yield might be a result of cellulose crystallinity reduction (from 22% to 13%) when ball milling was in used (Silva et al., 2012).

Mechanical disc refining has been used for decades in paper and pulp industry. Most recently, the possibility of using disc refining as a pre-treatment method for biomass has been explored. In general, the disc refining is carried at lower pressures and temperatures (Fang et al., 2011).

Size reduction pre-treatment is usually combined with other types of pre-treatment to improve EH. A study conducted by Pedersen and Meyer (2009) explored particle size followed by welt oxidation pre-treatment effects on WS surface structure and EH. The results demonstrate an improvement in glucose release after EH with size reduction. A further increase in glucose yield was recorded after introducing wet oxidation pre-

treatment as a second pre-treatment step. The smallest particles size 53 – 149 µm gave glucose up to 90% of the theoretical glucose yield when size reduction was combined with wet oxidation pre-treatment (Pedersen and Meyer, 2009).

Grinding and milling can also be done with the presence of H_2O in wet disk milling (WDM). Da Silva et al. (2010) compared between ball milling and WDM using straw and sugarcane as a lignocellulosic biomass raw materials. The ball milling was carried out at 400 rpm with total milling time 30 – 120 min. On the other hand, WDM was carried out by suspending 1 kg of the biomass in 15 L on H_2O overnight before adding another 5 L of H_2O prior to milling. The WDM was performed using a miller equipped with two nonporous ceramic disks. The gap between the ceramic disks was adjusted between 20 – 40 µm and rotation speed was 1800 rpm. It was reported that at the optimum conditions for ball milling and WDR, glucose and xylose yield for the straw were 72.1% and 36.7% and 56.8% and 44.9%, respectively.

In another study carried by Hideno et al (2009), the rice straw was pre-treated with WDM, hot-compressed water and ball milling. The maximum glucose and xylose yield after EH were 78.5% and 41.5%, 70.3% and 88.6%, 89.4% and 54.3% from the WDM, hot-compressed water and ball milling, respectively (Hideno et al., 2009). The WDM pre-treatment conditions were similar to (da Silva et al., 2010).

1.2.4 Chemical Pre-treatment

Several chemicals can be used to apply chemical pre-treatment on lignocellulosic materials such as alkalis, dilute and concentrated acids, ozone, ionic liquids, oxidation reagents and organic solvent (Li et al., 2009, Timung et al., 2015, Elgharbawy et al., 2016, Coca et al., 2016, Kim et al., 2016). The effects of chemical pre-treatment on the biomass are different according to the chemical being used. For example, acids (dilute and concentrated) are more effective in hemicellulose removal (hemicellulose

solubilisation). While alkaline, organsolv and oxidation reagents effects are more on lignin removal (de-lignification).

Acid pre-treatment (dilute and concentrated), alkaline and organosolv will be discussed in the following section as examples of chemical pre-treatment.

1.2.4.1 Acid

Inorganic acids such as sulphuric acid (H_2SO_4), hydrochloric acid (HCl), nitric acid (HNO_3) and phosphoric acid (H_3PO_4) are typically used for biomass acid pre-treatment. The acid pre-treatment can be classified into dilute and concentrated acid. The dilute acid pre-treatment is usually carried at either high temperature (around 180 °C) for short time or at a lower temperature (around 120 °C) with longer time (Alvira et al., 2010). The dilute acid pre-treatment is followed by EH for the solid fraction. As for the concentrated acid pre-treatment, it is carried at room temperature and does not require EH. Despite the advantage that EH is not required, the concentrated acid is less preference than dilute acid pre-treatment due to equipment corrosion, acid recovery, energy consumption and the high risk of inhibitors forming (Galbe and Zacchi, 2002, Alvira et al., 2010, Cardona et al., 2010, Beltramino et al., 2015). Dilute acid pre-treatment is a more preferable approach by industrial applications to pre-treat the biomass. As a result, it has been studied intensively on a wide range of lignocellulosic materials (Alvira et al., 2010).

The main effect of dilute acid pre-treatment is on increased hemicellulose solubility which increases the cellulose accessibility by enzymes. Acid pre-treatment has a low effect on lignin degradation (Sun et al., 2016b). Temperature, pre-treatment time and acid concentration are among the manipulated variable which controls the dilute acid pre-treatment.

In general, the liquid fraction after acid pre-treatment will mainly include sugars from hemicellulose hydrolysis (xylose, arabinose) as well as a small amount of glucose from cellulose. Acetic acid (CH_3COOH), furfural ($C_5H_4O_2$) and 5-hydroxymethylfurfural (HMF) might be also found and their concentrations are depending on the pre-treatment intensity (Saha et al., 2005, Gámez et al., 2006, Jönsson and Martín, 2016). Through time, many approaches and techniques were developed to improve the dilute acid pre-treatment by decreasing the inhibitors forming and increase the sugar yield. One of these approaches is by applying two steps pre-treatment. Firstly, the biomass is pre-treated with mild conditions and secondly, the solid residue is pre-treated with a hard condition. The advantage of this approach is to prevent or minimize sugars degradation (Galbe and Zacchi, 2002, Zhu et al., 2015b). The two-step pre-treatment also can be done oppositely by applying concentrated acid pre-treatment followed by a dilute acid condition, this technique was developed by National Renewable Energy Laboratory (NREL) in Golden, Colorado, US (Binod et al., 2010).

H_2SO_4 is the most favourite acid to perform dilute acid pre-treatment for lignocellulosic biomass materials. Baboukani et al (2012) investigated the optimum H_2SO_4 pre-treatment condition in order to improve sugar recovery and EH of WS. Three variables of the pre-treatment were evaluated: time (10 – 30 min), temperature (120 – 160 °C) and acid concentration (0.75 – 2.25%) by means of design of experiments software (DoE). A xylose yield up to 91% was reached by carrying out the pre-treatment at 140 °C with 1.5% acid concentration for 3.2 min. The maximum glucose yield (up to 50%) was accomplished with acid concentration 0.75%, temperature 160 °C and 30 min. The optimum sugar recovery conditions were 30 min, 1.53% acid concentration and pre-treatment temperature 147 °C (Baboukani et al., 2012).

Wu et al (2015) studied the optimization of H_2SO_4 pre-treatment of WS. The effect of temperatures range (120 °C, 130 °C and 140 °C) at a different H_2SO_4 concentration (2 wt%, 3 wt%, and 4 wt%) on the WS depolymerisation over time were investigated. The highest sugars yield (glucose 1.363 g L^{-1}, arabinose 1.203 g L^{-1} and xylose 8.934 g L^{-1}) were obtained when the WS was pre-treated at 130 °C for 75 min with 3 wt% of H_2SO_4. Moreover, at these the concentration of the microbial inhibitors were low (CH_3COOH 1.192 g L^{-1} and $C_5H_4O_2$ 0.526 g L^{-1}) (Wu et al., 2015).

Dihydrogen phosphate (H_2PO_4), sodium sulphite (Na_2SO_3), phosphoric acid (H_3PO_4) with hydrogen peroxide (H_2O_2), H_2SO_4 with metal, formic acid (CH_2O_2), maleic acid ($C_4H_4O_4$) and hydrochloric acid (HCl) are among the acids used for dilute acid pre-treatment.

In general, xylose recovery and glucose production vary between 80 – 100% and 58 – 80% depending on the pre-treatment temperature, time, acid concentration and the acids types (Kootstra et al., 2009, Hernández-Salas et al., 2009, Akimkulova et al., 2016, Jaisamut et al., 2016, Wang et al., 2016).

1.2.4.2 Alkali

The alkali pre-treatment improves the cellulose digestibility by reducing cellulose crystallinity with lower sugars degradation and inhibitors forming comparing to acid pre-treatment. The alkali pre-treatment affects the biomass structure in the means of hydrolysing the acetic ester and uronic acid which improves solubilisation of both lignin and hemicellulose (Talebnia et al., 2010, Kim et al., 2016, Loow et al., 2016, Xu et al., 2016). Removing lignin and hemicellulose will expose cellulose to enzymes during EH (Sun et al., 2016b).

The effectiveness of the alkali pre-treatment depends on the lignin percentage in the biomass. Low lignin content results in increase alkali pre-treatment efficiency.

Therefore, it is more suitable for agricultural residues such as wheat, cereal, barley and rice straw than wood (Sanchez and Cardona, 2008, Kumar et al., 2009). The alkali pre-treatment is usually carried at temperature and time vary from a few seconds to days and at a relatively high temperature to room temperature, respectively. Long pre-treatment time and a high cost of alkali comparing to acid pre-treatment might be a drawback for this process (Talebnia et al., 2010).

Different alkaline such as calcium hydroxide ($Ca(OH)_2$), sodium hydroxide (NaOH), potassium hydroxide (KOH), ammonia solution (NH_4OH) and sodium carbonate (Na_2CO_3) are used for alkali pre-treatment. Among them, NaOH and $Ca(OH)_2$ has been used extensively (Talebnia et al., 2010, Sun et al., 2016b). The main effects of NaOH on the lignocellulosic materials are: reducing polymerization and degree of crystallization while causes swelling and increases the internal surface area of the cellulose, as well as disturbing the lignin structure (Taherzadeh and Karimi, 2008, SriBala et al., 2016). $Ca(OH)_2$ which is known as lime has been widely used to pre-treat diverse lignocellulosic materials. Pre-treatment with lime cause removing of lignin and acetyl groups from hemicellulose which subsequently improves EH process by increasing the cellulose accessibility and decreases the non-productive adsorption spots for the enzymes (Mosier et al., 2005b, Kim and Holtzapple, 2006, Gu et al., 2015). Lime has the preference to be used for lignocellulose materials pre-treatment over other alkaline due to the low cost, environmentally friendly and easy recovery process by reaction with CO_2 (Mosier et al., 2005b, Alvira et al., 2010).

Chang et al (1998) used WS and bagasse as a crop residue for lime pre-treatment. Four different variables were studied: lime loading, temperature, H_2O loading and pre-treatment time. Moreover, lime recovery with CO_2 was also performed. The results show that the optimum lime loading is 0.1 wt/wt and the water loading does not have

a significant effect on the digestibility. As for time and temperature, the results indicated that the sugar yield after EH from the biomass pre-treatment at high temperature (85 – 135 °C) for short period (60 – 180 min) is similar to the biomass pre-treated at lower temperature (50 – 65 °C) for longer time (1 – 8 days). As for the lime recovery, 86% was achieved.

McIntosh and Vancov (2011) examined dilute alkaline pre-treatment conditions on enzymatic saccharification of WS. The milled WS was pre-treated with NaOH at different concentrations 0.75%, 1.0% and 2.0% (wt/v). The solid loading ratio was fixed at 10% (wt/v). The reactions times were 30, 60 and 90 min. The pre-treatment was carried at 60 °C with a water bath and 121 °C in an autoclave (pressure = 15 psi). The results demonstrated excellent de-lignification, an increase in the cellulose content in the solid fraction and a high sugar yield after EH. The optimum conditions were found to be 30 min and 90 min with 2% NaOH for the autoclave and the water bath, respectively (McIntosh and Vancov, 2011).

In another study carried out by Jaisamut et al (2013), the optimization of alkali pre-treatment process on the WS was explored. In their study, the effects of four factors were investigated: temperature (80 – 160 °C), residence time (10 – 110 min), concentration of lime (0 – 0.1% g/g) and NaOH concentration (0 – 0.2% g/g) via DoE. The objective was increase lignin removal, glucose yield after EH and total sugars recovery (from cellulose and hemicellulose). The optimum pre-treatment conditions were found to be at 80 °C for 39 min, lime 0.06 g g^{-1} and NaOH 0.18 g g^{-1}. At these conditions, the lignin removal was 62.2 %, glucose yield from EH was 93.1% and the total sugars recovery from cellulose and hemicellulose was 80.3% (Jaisamut et al., 2013).

1.2.4.3 Organosolv

Organic solvent or as known as (organosolv procedure) is a pre-treatment method in which organic or aqueous solvent mixtures are used to pre-treat lignocellulosic materials. The major effects of organosolv pre-treatment are on lignin extraction (delignification) and hemicellulose removal which enhances the cellulose accessibility by enzymes and almost 100% glucose yield can be reached (Papatheofanous et al., 1995, Zhao et al., 2009, Zhao et al., 2017, Zhang et al., 2016b, Zhou et al., 2018).

Different types of organic solvent can be used for organosolv pre-treatment such as methanol, organic acid, acetone, ethylene glycol, ethanol and tetrahydrofurfuryl alcohol. Among them, ethanol is the most convenient solvent to be used due to ease recovery, low toxicity and low cost (Sun et al., 2016b).

The effects of the organic solvents on hemicellulose bonds and lignocellulosic linkages are limited. Therefore, acids such as H_2SO_4 and HCl are introduced to the organosolv process to enhance the lignin and hemicellulose removal (Huijgen et al., 2011). Applying the organosolv pre-treatment can be done at a high temperature around 180 °C or higher without the needs of acid or at a lower temperature in which the acid present is fundamental (Sun and Cheng, 2002).

Solvent recovery, high lignin recovery and low environmental effects are among the features which make organosolv pre-treatment suitable to apply on a range of lignocellulosic materials. Organosolv process faces few difficulties such as high organic solvents prices, handling the acids and apply an advance control system to deal with organic solvents volatility at the industrial production level (Pan et al., 2006, Sun et al., 2016b).

Solvents recovery by distillation and recycling the solvents is an essential step to reduce the operation cost. Furthermore, the presence of the solvents during EH could

inhibit enzyme activity and affects the fermentation microorganisms (Sun and Cheng, 2002, Zhao et al., 2009).

Wildschut et al (2013) evaluated the organosolv pre-treatment using ethanol-based on the WS to remove lignin and produce solid residue enriched with cellulose. Different parameters including the pre-treatment time, temperature, WS particle size, solvent concentration and the acid dosage were evaluated. Among these parameters, it was found that acid dose, pre-treatment temperature and ethanol concentration had a major effect on the process. It was reported that the highest glucose yield after EH was 86% at the optimum pre-treatment conditions (temperature 210 °C, 50% wt/wt aqueous ethanol) without using acid. Similar glucose yield was achieved by introducing H_2SO_4 (30 mM) with lower temperature (190 °C) and aqueous ethanol and (60%, wt/wt) (Wildschut et al., 2013).

1.2.5 Physio-Chemical Pre-treatment

Physio-chemical pre-treatments are usually performed at a high temperature and pressure. Physio-chemical processes target the hemicellulose fraction in the lignocellulose materials due to its high sensitivity to temperature (150 °C and higher) comparing to cellulose and lignin. Various types of physio-chemical pre-treatments such as SE, sulphur dioxide (SO_2)-steam explosion, CO_2 explosion, ammonia fibre explosion (AFEX), liquid hot water (LHW), wet oxidation, ionic liquid and microwave can be used to pre-treat lignocellulose materials (e.g. WS). Physio-chemical pre-treatments increase the hemicellulose solubilisation and they can be further improved by introducing acid to remove lignin and reduced cellulose crystallization (da Costa Lopes et al., 2013, Morone et al., 2017, Smuga-Kogut et al., 2017, Lorenzo-Hernando et al., 2018, Tang et al., 2018, Yang et al., 2018). In the

current study, LHW, SE, ADR, PDR and microwave pre-treatments were used to pre-treat WS.

1.2.5.1 Steam Explosion (SE)

In 1925, SE was developed by Mason and had been used in hardboard manufacturing (Wayman, 1980, Schultz et al., 1983). In the late of the 1970's, Iotech Canada, applied SE on the production of feed for ruminants. Based on the initial outcome, Iotech Ltd expanded the use of SE and introduced it as a pre-treatment method for aspen wood (Foody, 1980, Schultz et al., 1983). SE is one of the widely employed physio-chemical pre-treatment methods for lignocellulosic biomass (de Albuquerque Wanderley et al., 2013, Alvira et al., 2016). In SE pre-treatment, the biomass such as waste agricultural and wood is fed into a cylinder vessel and subjected to pressurized saturated steam (up to 1000 psig) for a certain period of time followed by a rapid reduction in pressure by the sudden decompression (Schultz et al., 1983, Cara et al., 2006). As a result, the biomass structure will be disturbed causing reduction in the degree of polymerization for lignin by cleavage of the /3-aryl-ether bonds (Schultz et al., 1983). Moreover, SE can disrupt the crystallinity of the cellulose, remove or hydrolyses hemicellulose (Lee et al., 2010, Singh et al., 2015) and increase surface area which improves EH (Shamsudin et al., 2012, Wang and Chen, 2016). SE is considered as an environment-friendly pre-treatment technique with a significantly lower environmental impact due to the usage of saturated steam (Egüés et al., 2012, Singh et al., 2015).

Due to the high pressure and temperature associated with the SE, there is the a risk of sugars and lignin degradation which could forms CH_3COOH as an inhibitory to the EH and fermenting microorganisms in subsequent (Jurado et al., 2009, Sharma et al., 2015, Alvira et al., 2016, Jönsson and Martín, 2016). As a result of the CH_3COOH forming, there is a risk for further degradation of the sugars due to acidic conditions.

A proposed solution is by applying post alkaline pre-treatment (Maekawa, 1996) or after the SE (Zhu et al., 2015a, Keshav et al., 2016, Wang and Chen, 2016) or applying two steps SE with removing the liquid fraction enriched with acids before applying the second SE (Chen et al., 2011a, Zhang et al., 2012, Wojtusik et al., 2018).

The main variables for SE pre-treatment are temperature, holding time and the biomass particle size (Alvira et al., 2016, Auxenfans et al., 2017). The effect of time (t) and temperature (T) is represented by the severity factor (log Ro) where, log Ro = log (t*e (T-100/14.75)) (Overend and Chornet, 1987a). The selection of the severity value mostly depends on the biomass feedstock type (Alvira et al., 2016). Similar hemicellulose hydrolysis and removal can be achieved by applying low temperature and long holding time (around 190 °C and 10 min) or with higher temperature and shorter holding time (around 270 °C and 1 min) (Duff and Murray, 1996, Martino et al., 2017). When applying milder severity conditions, cellulose loss can be neglected and the energy cost will be reduced but a higher dosage of enzymes is required (Garrote et al., 1999, Jacquet et al., 2015, Alvira et al., 2016). On the other hand, high severity causes significant removal of hemicellulose and therefore the biomass digestibility is improved. However, the risk of sugars degradation and inhibitors forming will be higher (Sipponen et al., 2014, Alvira et al., 2016, Niemi et al., 2017).

If large particle size is used during the SE pre-treatment then there is a risk of overcooking the outside of the biomass without pre-treating the interior of the sample due to low heat transfer rate which results in heterogeneous heating (Brownell et al., 1986, Ferreira et al., 2014). On the other hand, grinding or chipping the biomass materials with the aim of size reduction before the SE requires a significant amount of energy (Ballesteros et al., 2002, Liu and Chen, 2017).

Cara et al (2006) applied SE and alkaline-H_2O_2 de-lignification of olive tree wood to enhance EH. In the first pre-treatment step, the chipped wood was steam exploded at a range of temperatures (190, 210, 230 or 240 °C) with holding time of 5 min. After cooling the exploded samples in a cyclone to 40 °C, the samples were filtered and the solid fraction de-lignified in 1% (wt/v) H_2O_2 solution with 4% (wt/v) solid concentration for 45 min at 80 °C. Towards the end of the alkaline-H_2O_2 pre-treatment, the samples were filtered and the solid fraction was subjected to EH. At the optimum conditions with pre-treatment temperature at 190 °C, the lignin removal was up to 80% and the overall sugar recovery (in the liquid fraction and after EH) was 52.6% (Cara et al., 2006).

Chen et al (2008) studied the simultaneous saccharification and fermentation (SSF) of WS using SE pre-treatment coupled with alkaline-H_2O_2. The WS was firstly soaked with water overnight. SE was carried at a temperature of 198 °C, pressure 1.5 MPA for 10 min. The conditions were based on their previous research (Hongzhang and Liying, 2007). The solid fraction (slurry) was dried before applying the second stage of the pre-treatment. The dried solid was suspended in a solution of H_2O_2 at (4.0%, v/v) and (1%, wt/v) of NaOH and with a solid loading (10%, wt/v). The mixture was incubated at 25 °C for 120 h and with a shaking speed of 150 rpm. For compassion, Chen et al (2008) performed EH on the un-treated WS, WS pre-treated with SE only and WS pre-treated with SE with alkaline-H_2O_2. The results showed that the WS pre-treated with only SE produced a glucose concentration of 67.8 g L^{-1}, while the WS pre-treated with SE and alkaline-H_2O_2 gave a glucose concentration of 110.9 g L^{-1}. The bio-ethanol concentration and yield from the combined pre-treatment were 51.5 g L^{-1} and 81.1%, respectively (Chen et al., 2008).

Another established approach for SE is by impregnating the biomass in acid solution prior to SE process. Martinez et al (1990) studied the acid SE effect on the EH process using O. nervosum and C. cardunculus woods as lignocellulosic biomass. In their study, the wood biomass was chipped to a size of 4 mesh and pre-treated with SE in three levels. The first level is by applying SE on the dry biomass, the second level is applying SE of wet biomass (5 L of water to 1 kg of dry biomass) and the third level is by applying SE on the biomass pre-treated with acid. In the acid-SE pre-treatment, the biomass was submerged in 1% H_2SO_4 for 24 h at 25 °C with a solid/ liquid ratio of 1:4. SE was carried at a temperature ranged from 170 to 230 °C with a holding time of 1, 2, 4 and 8 min. The results showed that the SE at 230 °C for 1 – 2 min and 210 °C for 2 – 4 min on O. nervosum and C. cardunculus increased the saccharification efficiency to 90%. On the other hand, un-treated O. nervosum and C. cardunculus produced only 23% and 20%, respectively. Pre-treating the biomass with acid before SE was found to have a negative effect on the saccharification and the EH (Martinez et al., 1990).

The acid-SE can also be done by using gases such as SO_2 and CO_2. An example of using SO_2 with SE is the study conducted by Chacha et al (2011). In this study, the production of reduced sugars from pine (Pinus patula) wood residue pre- treated with SE was investigated. The wood chips were impregnated in different SO_2 concentrations (0.5, 1.5 and 3%) before applying SE. The SE was carried out in mild conditions (180 °C, 10 min, 10 bar and Log Ro 3.4) and in extensive conditions (225 °C, 5 min, 25 bar and Log Ro 4.4). The results showed that at low severity (Log Ro 3.4) with 1.5% SO_2, the total reduced sugar yield was 29%. On the other hand, by using high severity (Log Ro 4.4) with 3% SO_2, the yield increased and reached 91% (Chacha et al., 2011).

Acid SE can be used on the waste agriculture lignocellulose biomass such as rice, barley and WS. Chen et al (2011a) used H_2SO_4 with SE to pre-treated rice straw. The chopped rice straw was pre-treated with H_2SO_4 (1 – 15% wt/wt) at (125 – 165) °C for (2 – 5) min. After filtering the liquid fraction enriched with xylose, the solid fraction was steam exploded at 180 °C for 20 min with a pressure of 10 kg/cm^2. The results showed that using acid pre-treatment before SE increased the xylose recovery and enhanced the EH. At the optimum conditions, the cellulose recovery was approximately 90% (Chen et al., 2011a).

WS was also used as raw material for acid-SE pre-treatment. Linde et al (2008) studied the production of bio-ethanol using WS submerged H_2SO_4 and pre-treatment with SE. The WS was firstly immersed in a solution of 0.2% H_2SO_4 for 1 hr at room temperature with a loading ratio 20:1 (g liquid /g dry-matter (DM)). The aqueous solution was then filtered and the DM was (30%, wt/wt). The pre-treated solid fraction was then pre-treated again by applying SE a temperature ranged in 190, 200 and 210 °C and for 2, 5 and 10 min. The DM loading for the SE pre-treatment was 60 g in the small reactor and the optimum conditions were then applied in a bigger reactor with DM loading of 300 g. The highest glucose (from EH) and xylose (in the liquid fraction) recovery was achieved by using 0.2% H_2SO_4 followed by SE at 190 °C for 10 min. The glucose and xylose yields were 102% and 96% of the theoretical, respectively. The bio-ethanol yield at the optimum conditions was 67% of the theoretical glucose in the raw material (Linde et al., 2008).

1.2.5.2 Liquid Hot Water (LHW)

LHW is among the promising pre-treatment methods due to its low cost, environmental friendly and high efficiency (Carvalheiro et al., 2005, Zhuang et al., 2016). The main objectives of the LHW pre-treatment are to solubilize hemicellulose

which facilitates cellulose accessibility to enzymes and avoid forming inhibitors at the same time (Negro et al., 2003, Li et al., 2014). The main disadvantages of LHW are the risk of forming HMF, $C_5H_4O_2$ and phenolic compounds which are considered as inhibitors and toxic chemicals for the EH and the fermentation processes. Moreover, the relatively long period to heat up the reactor to the setting temperature specially for high LHW pre-treatments temperatures (Hendriks and Zeeman, 2009). Therefore, selecting the optimum LHW pre-treatment conditions are the key factors to prevent or reduce carbohydrate degradation and inhibitors formation (Pérez et al., 2007, Kim et al., 2014, Pedersen et al., 2011). Another suggestion to minimize inhibitors formation is by controlling the LHW pre-treatment pH and keeping it within the range of (4 – 7). Keeping the pre-treatment pH between (4 – 7) will reduce the biomass hydrolysis and therefore decrease the monosaccharides formation which will subsequently minimize the inhibitors forming (Weil et al., 1998, Mosier et al., 2005a, Pedersen et al., 2011, Kim et al., 2014).

Similar to the other hydrothermal pre-treatments, temperature and time are the main variables besides. In general, the LHW pre-treatment is conducted at a temperature ranged between 120 °C to 240 °C and with pre-treatment time of 0 min to 180 min (Nitsos et al., 2013, Michelin and Teixeira, 2016b).

The efficiency of the LHW pre-treatment is determined by the amount of hemicellulose dissolved in the liquid fraction and the amount of cellulose remaining in the solid fraction (water-insoluble solids (WIS)) (Pérez et al., 2008).

Pérez et al (2008) investigated the optimum LHW pre-treatment conditions with the aim of improving sugar recovery from WS using DoE software. The LHW pre-treatment process was conducted using chipped WS to a particle size of (0.5 – 2 cm) with loading ratio 1:10 (wt/v) solid/liquid, a temperature range of (170 – 220 °C) and

the pre-treatment duration of (0 – 40 min). DoE software was used with multiple responses (hemicellulose-derived sugars in the liquid fraction yield and EH sugars yield) followed by optimization. When combining the two responses, the optimum temperature was found to be 188 °C and the pre-treatment time of 40 min. at these optimum conditions, the hemicellulose-derived sugars (HDS) recovery and the sugars yield after were 43.6% and 79.8%, respectively. On the other hand, when each response was optimized separately a higher HDS recovery yield up to 71.2% and EH yield of 90.6% was achieved at 184 °C, 214 °C and for 24 min, 2.7 min, respectively (Pérez et al., 2008).

Michelin and Teixeira (2016) studied the LHW pre-treatment effect on different biomass (WS, brewers' spent grain, corn husk, Luffa sponge and corncob). The LHW pre-treatment was carried at 190 °C for 30 min corresponding to a severity (log Ro = 4.13). The solid-liquid loading ratio was 10% wt/v. The results demonstrated an increase in the glucose yield between un-treated and pre-treated samples except for the Luffa sponge. The glucose yield obtained from the WS increased by approximately 30% for the pre-treated WS compare to un-treated WS (Michelin and Teixeira, 2016b).

Sreenath et al (1999) studied the EH for un-treated fibre and LHW pre-treated fibre with and without dilute H_2SO_4 (0.07%). The LHW without H_2SO_4 was carried at 220 °C for 2 min. As for the LHW with the presence of H_2SO_4 (0.07%), the same duration was used with a temperature ranged between 175 – 225 °C. The results showed that the LHW with acid facilitated hemicellulose solubilisation and reduced fibre degradation in the solid fraction (Sreenath et al., 1999).

Another approach for LHW was suggested by Yu et al (2012) who proposed a two-step LHW on eucalyptus grandis to improve the sugar recovery in the liquid fraction and from EH of the solid fraction. The objective of applying two steps was to

accomplish complete saccharification for both cellulose and hemicellulose without using acid . The first LHW pre-treatment was carried at 180 – 200 °C for 0 – 60 min where the highest xylose yield of 86.4% was obtained at 180 °C for 20 min. The second LHW was conducted at a temperature range of 180- 240 °C with pre-treatment time 0 – 60 min. The optimum conditions were found to be 200 °C for 20 min. The total sugars recovered at the optimum conditions for both first and second LHW was 96.63% (Yu et al., 2010).

Lu et al (2012) applied LHW on reed as raw biomass for enzymatic saccharification and fermentation. The pre-treatment was carried at 170 °C, 180 °C, 190 °C, 200 °C and 210 °C for either 20 min or 40 min and by using different distilled water volume. EH was carried at 36 °C or 50 °C with pH 4.8 for 72 hrs on the Water Insoluble Solids (WIS) Whilst the fermentation was performed with Separate Hydrolysis and Fermentation (SHF) method. The optimum LHW pre-treatment conditions were found to be 180 °C, loading ratio 1:10 and with pre-treatment time 20 min. At these optimum conditions, the cellulose conversion was 82.59% after applying EH using 30 FPU g^{-1} DM and the bio-ethanol yield was 99.5% of the theoretical glucan yield (Lu et al., 2012).

Yang and Wyman (2004) described xylan and lignin removal in a flow and batch system from corn stover. A higher hemicellulose and lignin removal were obtained from the flow system compared to the batch system at the same severity. Moreover, adding H_2SO_4 to both systems increased the hemicellulose and lignin removal in the flow system whlie less lignin was removed in the batch system.

Several studies were done using the flow through system with the addition of an external acid. Some of these reports conclude that, adding acid during the flow through

system will have no significant effect or it is not the only factor affecting both lignin and hemicellulose solubility (Jacobsen and Wyman, 2002, Liu and Wyman, 2003)

1.2.5.3 Pressurized Disk Refining (PDR)

Pressurized disc refining is a relatively new approach for biomass pre-treatment. PDR is a combination of disc refining and SE. The biomass is usually submerged in a pressurized reactor vessel (compartment) with water or dilute acid. Once the required pressure and temperature is reached, the biomass is fed to the refining section. In the refining section, different gaps between the discs, discs types, numbers of the rotating discs and the holding time can be selected to achieve the maximum yield with lower energy consumption. Finally, the refined (pulp) biomass pressure will be reduced rapidly similarly to SE method (Pschorn et al., 2008, Schütt et al., 2012).

Fang et al (2011) investigated the application of continuous pressurised refining at pilot scale on WS. The WS was soaked with either H_2O or dilute H_2SO_4 (0.5%) before refining. The effects of refining residence time, steam pressure and temperature on the total reduced sugar yield was evaluated. The results showed that the WS pre-treated with H_2O only at high steam pressures (15 bar) and 198 °C for 6 min gave a total reduced sugar yield of 93.3% and an overall glucose yield of 85.8%. On the other hand, the WS pre-treating at the same conditions for shorter time 4 min gave a total reduced sugar yield of 88.7% and an overall glucose yield of 88.4%. Celluclast 1.5L supplemented with β-glucasidase enzyme at a ratio (2:1) were to perform the EH at pH 4.8. Although increasing retention time increases the total yield, the glucose yield was reduced due to degradation. Moreover, the optimum temperature and retention time for acid pre-treatment were 178 °C and 6 min, respectively (Fang et al., 2011).

similarly Gonzalez et al (2011) studied the effects of thermo-mechanical pulping on WS, sweet sorghum bagasse and corn stover. The biomass was pre-treated at two

temperatures 160 °C and 170 °C for 15 min with disk refining gap of 0.1524 mm. The results showed an increase in the sugar yield after EH from 25% to 40% by applying the pre-treatment at 160 °C and 170 °C, respectively. Further pre-treatment on the WS was applied by soaking the WS in CH_3COOH with longer steaming residence time (15 and 30 min) and using lower disk gap (0.0508 and 0.1524 mm). The data collected indicated that the sugar yield was improved when the WS was soaked in CH_3COOH and refined at a lower refiner gap (0.0508 mm). Nevertheless, CH_3COOH present causes major degradation in the hemicelluloses of the pulp (Gonzalez et al., 2011).

Schütt et al (2012) described the steam refining approach as an alternative for the conventional SE. Poplar (Populus balsamifera) wood chips were pre-treated with SE and steam refining at the same conditions for comparison. The temperature ranged between 205-215 °C and the residence time 10 – 20 min were investigated. The pre-treated biomass was subjected to EH using Celluclast 1.5L and Novozym 188 at 45 °C for 72 hr and pH 4.8. It was concluded that there is no fundamental difference between steam refining and steam explosion for the poplar wood chips in terms of sugars yields (Schütt et al., 2012).

1.2.5.4 Microwave

Microwave pre-treatment for softwoods and hardwoods was firstly used in the mid of the 1980's (Azuma et al., 1984, Ooshima et al., 1984). During the 1990's, there was no significant development on the microwave pre-treatment due to the maturity of the other types of pre-treatment such as chemical pre-treatments (Castro et al., 1993, Dominguez et al., 1997, Xu et al., 2011).

Over the last two decades, the microwave was rediscovered as an alternative method for the conventional heating (Hu and Wen, 2008, Xu et al., 2008, Binod et al., 2012). Microwave has the ability to apply direct electromagnetic field onto the object being

heated (Binod et al., 2012, Sun et al., 2016a, Swiergon et al., 2018). The high heating efficiency, short pre-treating time, reduction of energy requirements for the process, uniform heating of the samples and the ability to reach the target temperature in a short time are among the advantages which made the microwave pre-treatment attractive for biomass pre-treatment (Bajia et al., 2009, Xu, 2015, Mishra and Sharma, 2016, Loong and Idris, 2017, Ho et al., 2018).

Microwave radiation lies between radio frequencies and infrared in the electromagnetic spectrum. It coincides with the frequencies of 30 GHz to 300 MHz and wavelengths of (1 cm to 1 m) (Xu et al., 2008, Darekar et al., 2019). During the microwave irradiation, the sample molecules will rotate with a rotation frequency equal to the microwave frequency continually to cope with absorbed energy (Lewandowicz et al., 2000, Loong and Idris, 2017). In contrast with conventional conductive-convective heating, the reaction mixture absorbs the microwave energy and heats up from the interior of the mixture and spreads out to the reaction mixture boundaries (Nuechter et al., 2003, Xu et al., 2012, Ho et al., 2018).

There are several studies implied that the microwave irradiation might prompt a hot spot which could change the biomass structure (Hu and Wen, 2008). Furthermore, some studies suggested that the microwave irradiation could cause a degradation in the hemicellulose and lignin (Buranov and Mazza, 2010, Kuittinen et al., 2016).

Ooshima et al (1984) studied the microwave pre-treatment on rice straw and bagasse using water as a substrate solution. Different temperatures 170, 200 and 230 °C were used with a pre-treatment time vary between 0 to 15 min. The results showed an increase in the cellulose accessibility by 1.5 times for the rice straw pre-treated 170 °C for 5 min and by 3.2 times for the bagasse pre-treated at 200 °C for 5 min comparing to un-treated rice straw and bagasse, respectively (Ooshima et al., 1984).

Zhu et al (2006) studied the bio-ethanol production from WS pre-treated with microwave-assisted alkali. In their study, the WS was pre-treated with 1% NaOH aqueous solution using conventional and microwave-assisted alkali pre-treatment for 60 min and 25 min, respectively. The pre-treated WS was subjected to optimizing the simultaneous saccharification and fermentation (SSF) process. The results showed that the optimum bio-ethanol yield of 148.93 g kg^{-1} was achieved at pre-treatment time of 15 min, microwave power at 1000 W, NaOH concentration 10 kg m^{-3} and with loading ratio of 80 g kg^{-1} (wt/wt) (Xu et al., 2011).

Janker-Obermeier et al (2012) studied the microwave-assisted alkali pre-treatment of WS. Their main focus was the solubilisation of lignin and hemicellulose. The following variables were investigated: temperature 60 to 140 °C, NaOH concentration 2 to 5 (wt %) and time 10 to 60 min. For each run, the average power (W), energy input (J) and the average energy input per weigh of WS (J g^{-1}) were calculated and reported. According to their finding, a high xylan yields up to 73% (of theoretical xylans, wt/wt) could be achieved by using NaOH (4 – 5%). The lignin removal was found to be depending on energy input. The highest removal of lignin was found at low NaOH (about 2%, wt/wt), while xylan removal required higher NaOH loading (about 5%, wt/wt). It was concluded that a solubilisation of hemicellulose of more than 80% and lignin of 90% can be established without the risk of solubilizing high amounts or excessive degradation cellulose (Janker-Obermeier et al., 2012).

Panthapulakkal et al (2015) studied the xylan extraction from birch wood using microwave pre-treatment and the results were compared with the conventional heating. The microwave experiments were carried at 110 W with different time duration 0.5 to 18 min. As for the conventional experiments, the temperature was 90 °C with pre-treatment time duration of 5 to 240 min. The extraction for both

methods was done by using 4 wt% NaOH solution. The results showed that the maximum xylan yield was 60% for both methods. The microwave pre-treatment achieved the 60% xylan yield with 1:10 of the time in the conventional heating pre-treatment (Panthapulakkal et al., 2015).

Another application for the microwave pre-treatment is by using acids instead of alkaline. Gong et al (2010) used CH_3COOH and CH_3CH_2COOH as an organic acid to pre-treat the rice straw with the aim of improving EH. The influences of the pre-treatment time (5 – 12 min), power (100 – 700) W, acid concentration (2 – 25% wt), the additional of sodium bisulfite ($NaHSO_3$) and solid-liquid ratio (1:10 – 1:50) on lignin removal and the yield of reducing sugar after EH was investigated. The results indicate that the microwave power intensity has the highest influence on the pre-treatment followed by solid-liquid ratio, CH_3COOH concentration and the pre-treatment time, respectively. The optimum conditions were found to be 5 min irradiating time, 230 W microwave intensity, 25% acid concentration and 1:15 solid-liquid ratio. At these conditions, the lignin removal was 46.1% and 51.54% and total reduced sugar yields were 71.41 and 80.08% for CH_3COOH and CH_3CH_2COOH, respectively. It was also found that the presence of the $NaHSO_3$ catalyst improved the lignin removal but the concentration did not have a significant effect (Gong et al., 2010).

Chen et al (2011b) used dilute H_2SO_4 with microwave-assisted heating on the sugarcane bagasse with the aim of disrupting the lignocellulosic structure. Three different pre-treatment temperature (130, 160 and 190) °C were applied with two reaction times of 5 and 10 min. By monitoring the pre-treated sugarcane bagasse structure, it was found that at temperature 190 °C, the surface area of the particles

increased substantially and almost all the hemicellulose was removed. Furthermore, they concluded that the pre-treating time had no significant effect (Chen et al., 2011b) Zhu et al (2016) compared the sugar yield from sugarcane bagasse pre-treated with conventional and microwave assisted acid and alkali pre-treatment. The pre-treatment condition for both acid and alkali microwave assisted pre-treatment were: power 320 W, temperature 170 ± 5 °C, pre-treatment time (3 – 10) min and with 0.2 and 0.4 M concentration for H_2SO_4 and NaOH. On the other hand, the conventional pre-treatment was carried at 120 °C for 40 min with the same H_2SO_4 and NaOH concentration. The results showed that the highest lignin removal was achieved by using 0.4 M NaOH for 7 min. The maximum xylose yield in the liquid fraction with (86%) was accomplished by using (0.2M) of the H_2SO_4 for 7 min. Similarly to all studies on the microwave-assisted pre-treatment, it was found that the microwave assisted pre-treatment is faster compared to the conventional heating pre-treatment and achieved higher sugar yield (Zhu et al., 2016).

Another microwave-assisted approach was suggested by combining alkaline and acid pre-treatment for the biomass. Binod et al (2012) examined the microwave assisted pre-treatment using acid, alkali and a combination of alkali and acid pre-treatment on the sugarcane bagasse to enhance the enzymatic saccharification. Microwave-alkali and microwave-acid pre-treatment were conducted using 1% (wt/wt) NaOH and H_2SO_4, respectively. The biomass loading was 10%, pre-treatment time 1 – 30 min and with a microwave power range 100 – 850 W. In the microwave-alkali followed by acid pre-treatment, the sugarcane bagasse was firstly pre-treated with alkali then the sample was washed and air dried before applying the acid pre-treatment. The microwave-alkali pre-treatment gave a higher fermentable sugars yield of 0.665 g/g dry biomass at 600 W and 4 min pre-treatment time comparing to microwave-acid

which gave only 0.249 g g^{-1} dry at 100 W and 30 min. The combined pre-treatment method gave overall reducing sugar yield of 0.83 g g^{-1} dry biomass. Furthermore, it was found that at 450 W power with pre-treatment time 5 min and 1% NaOH, 90% of lignin was removed from the pre-treated sugarcane bagasse (Binod et al., 2012).

It is clear that the main advantage of microwave pre-treatment is reducing overall pre-treatment time and subsequently pre-treatment energy consumption.

1.3 Enzymatic Hydrolysis

The objective of enzymatic hydrolysis (EH) is to hydrolyse cellulose (and hemicellulose) to reduced sugars (hexose and pentose) which can be fermented to bio-ethanol. EH comprise cellulose and hemicellulose cleaving to monosaccharides sugars using enzymes (Binod et al., 2010, Bhaumik and Dhepe, 2015, Binod et al., 2019). The major component in the pre-treated biomass is usually cellulose and therefore cellulase enzymes are used to produce reduced sugars with glucose as the main monosaccharides sugar. On the other hand, hemicellulose hydrolysis produces several sugars including xylose and arabinose (Dutta and Chakraborty, 2018, Shokrkar et al., 2018, Philippidis, 2018). EH has been presented as the most effective method to liberate monosaccharides sugars (Talebnia et al., 2010). Applying EH will eliminate the corrosion risk associated with acid hydrolysis and decreases the process cost since EH is normally carried at mild temperature (40 – 50 °C) (Duff and Murray, 1996, Chen et al., 2018a, Yu et al., 2018).

The enzymes origin can be either bacteria such as Clostridium cellulovorans or fungi such as Trichoderma reesei and A. niger. Both bacteria and fungi enzymes have cellulases and the ability to hydrolyse biomass (Arai et al., 2006, Reilly et al., 2018, Sandhu et al., 2018, Kozaki and Miyake, 2019).

Commercial cellulase production concentrates on fungi due to the fact that most of the bacteria are anaerobic and therefore they have very slow growth rates. Moreover, some of the anaerobic bacteria such as Clostridium thermocellum and Bacteroides cellulosolvens despite having high specific activity, they have low production titres (Duff and Murray, 1996, Alvira et al., 2010, Herring et al., 2016, Cui et al., 2019).

On the other hand, fungi such as Trichoderma, Aspergillus, Penicillium and Schizophyllum have shown a high cellulases production. Among these fungi, Trichoderma is the most studied fungi for cellulase production (Talebnia et al., 2010, Herring et al., 2016).

Usually, the cellulases consiste of various enzymes mixture. The main three enzymes involved in cellulose hydrolysis to glucose in that mixture are:

1- Endoglucanase or endo-1, 4-Glucanohydrolase is responsible for cracking the low crystallinity regions of the cellulose polymer and generate free chain-ends.

2- Exo-glucanase or cellobiohydrolase is in charge of further degradation by extracting the cellobiose units from the free chain-ends

3- β-Glucosidase is accountable for hydrolysing the cellobiose to glucose.

Other enzymes might be present in the mixture and work as an auxiliary to hydrolysis hemicellulose such as xylanase and β-xylosidase (Coughlan and Ljungdahl, 1988, Wang et al., 2011, Nitta et al., 2012, Chen et al., 2018b). Figure 1-2 shows the enzymes function during EH of the cellulose to glucose.

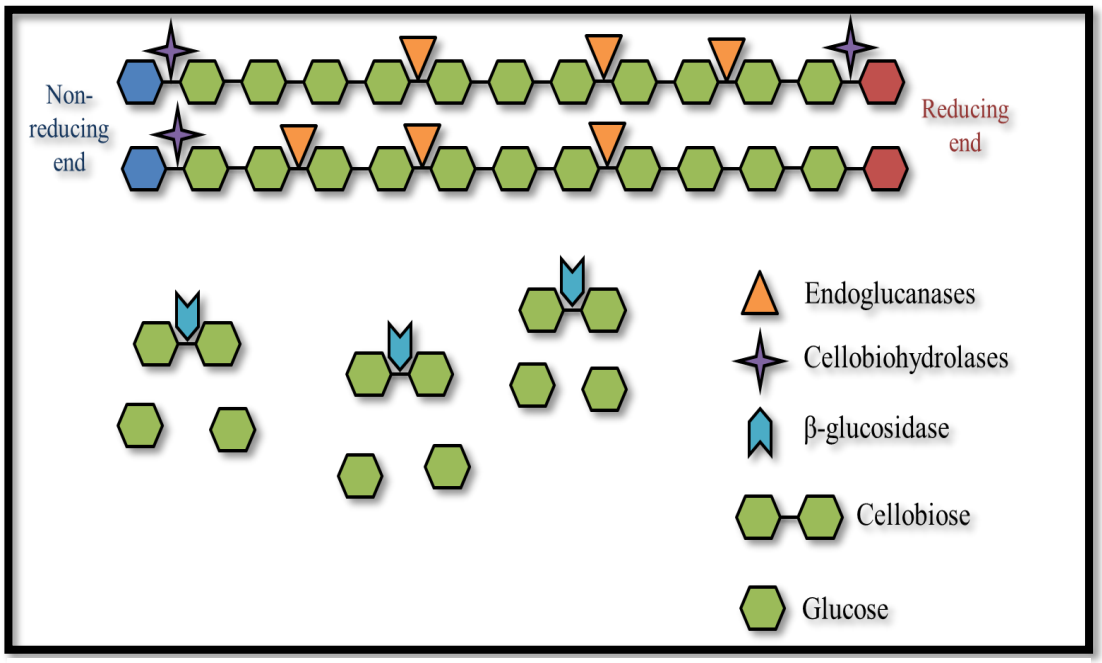


Figure 1-2. Cellulose enzymatic hydrolysis sequence to glucose monosaccharide sugar

When the biomass is hydrolysed by T. reesei cellulases enzymes, the addition of β-glucosidases will improve the saccharification. Furthermore, adding hemicelluloses enzymes will increase the cellulose conversion significantly (Beldman et al., 1984, Talebnia et al., 2010, Hu et al., 2015).

Several variables might influence the EH process and limited enzymes activities. Pre-treatment process selection and conditions, sugars degradation and inhibitors forming. Hydrolysis conditions (temperature and pH) selection effect enzymes performance. Enzymes mixture selection and enzymes activities are also important to accomplish the maximum reduced sugars yield. The presence of end-product (cellobiose) considered an inhibitor for many cellulases and can effects the EH negatively. This obstacle can be overcome by adding β-Glucosidase which hydrolysis cellobiose (Galbe and Zacchi, 2002, Teixeira et al., 2015, Gao et al., 2018, Wojtusik et al., 2019). Finally, the enzyme dosage (substrate concentration) must be taken into account. Increasing substrate concentration results in improving the EH yield, however high

substrate concentration might lower the EH rate and work as an inhibitor. Optimizing the EH conditions and enzymes dosage will not only increase the reduced sugars yield but at the same time decrease the operating cost (Penner and Liaw, 1994, Mithra and Padmaja, 2017, Reyes-Sosa et al., 2017). Enzymes mechanism can be classified to three main steps: cellulase enzymes adsorption on the cellulose surface, cellulose biodegradation to fermentable sugars and final cellulase desorption.

Enzymes activity and loading are expressed by different units. Cellulase enzymes activity is usually measured by filter paper unit (FPU) where FPU is defined as the amount of enzyme releasing 1 μmol of reducing sugar from filter paper per min. In general, 2.5 – 30 FPU g^{-1} (g of cellulose or glucan or biomass DM) is used for hydrolysis. β-glucosidase is measured by cellobiose units (CBU). The β-glucosidase loading is often measured as a ratio to the FPU. The common ratio for biomass hydrolysis is between 1:1 to 1:2 FPU/CBU (Zhang and Lynd, 2006, Gao et al., 2010, Samayam and Schall, 2010). The enzyme activity can also be measured by the international unit (IU), where IU is defined as the amount of enzymes that releases 1 μmol of reducing sugar per min from the substrate (Sun and Cheng, 2002, Van Dyk and Pletschke, 2012, Michelin and Teixeira, 2016a).

The activity of the cellulase enzyme diminishes during hydrolysis. It has been suggested that irreversible enzyme adsorption on cellulose is partially responsible for the deactivation (Zhang et al., 2016a, Zheng et al., 2016). Supplementing of surfactants through the hydrolysis can modify the cellulose surface property and minimized irreversible binding of cellulase on cellulose at the same time. As a result, the cellulose conversion to monomeric sugars can be enhanced (Cheong et al., 2007, Jeoh et al., 2017, Jiang et al., 2017, Lou et al., 2018). Different surfactants can be added to further improve EH such as non-ionic Tween 20, 80, 81, sophorolipid,

bacitracin and rhamnolipid. Among them, Tween 20 is the most effective supplementing detergent for enhancing of EH (Tabka et al., 2006, Araújo et al., 2018, Chen et al., 2018b).

Different commercial cellulase, β-glucosidase and xylanase enzymes are being used for EH. Celluclast 1.5 l with Novozym 188 and Cellic® CTec (1, 2 and 3) from Novozymes are the most used enzymes for cellulose hydrolysis. As for hemicellulose, the endo-1, 4-β-xylanase and Cellic® HTec (1, 2 and 3) from Novozymes is the common enzyme. In a study carried by Da Costa Lopes et al (2013), pre-treated WS was subjected to EH using Celluclast® 1.5 L and Novozym 188 enzyme solutions. The WS was suspended in 0.1 M sodium citrate buffer (pH 4.8) and 2% (wt/wt) of sodium azide solution was added to prevent the organism's growth. The hydrolysis was carried at 50 °C for 72 hr in an incubator-shaker with 150 rpm (da Costa Lopes et al., 2013). Mclntosh and Vancov (2011) investigated EH process on pre-treated WS using Cellulase (NS50013), β-glucosidase (NS50010) and xylanase (NS50030) from Novozymes (Bagsværd Denmark). Different enzymes loading were used. The highest totally reduced sugars were achieved by using (10 FPU cellulase, 10 CBU β-glucosidase and 1.5 FXU (farvet xylan unit) xylanase per gram of pre-treated solids. The EH was performed at 50 °C for 72 hrs and with shaking speed of 150 rpm. Pre-treated WS loading was 5% wt/v in a 0.05 M citrate buffer (pH 5.2) and 0.01 M of sodium azide was added to prohibit microbial contaminant growth. The results showed that besides the pre-treatment effects, enzymes loading and mixing have a high effect on the EH process. Adding all enzymes will not increase the total reduced sugar yield only but at the same times decreases cellulase enzyme loadings (McIntosh and Vancov, 2011).

Lan et al (2013) studied the effect of pH during EH on the reduced sugars yield. Lodgepole pine trees were pre-treated with different pre-treatment approaches and used to investigate the hydrolysis pH effect. Celluclast 1.5L supplemented with β-glucosidase (Novozyme 188) and Cellic CTec2 enzymes cocktails were used. The celluclast and Cellic CTec2 dosage was between 7.5 – 15 FPU g^{-1} of glucan Whilst β-glucosidase loading was 1.5 FPU/CBU per gram of glucan. The EH was carried out at different pH values and it was found that the optimum pH was between 5.2 – 6.2 (Lan et al., 2013).

Saha and Cotta (2007) described pH and temperature effects during EH of pre-treated WS. They investigated the pH value and the temperature ranged between (3.5 – 6.5) and (25 – 70 °C), respectively. Several enzymes were used including Celluclast 1.5 L and Novozyme 188 for cellulose hydrolysis and Viscostar 150 L for hemicellulose hydrolysis. It was reported that a pH of 5.0 and a temperature of 45 °C are the optimum condition for EH (Linde et al., 2008).

1.4 Fermentation

One of the key processes in the bio-ethanol production system in fermentation. The sugars released after EH is fermented with microorganism yeast. Fermentation can be carried out in two main approaches: separate EH and fermentation (SHF) in which hydrolysis is implemented followed by fermentation and (SSF) were both hydrolysis and fermentation are executed at the same time.

In SHF method, high reduced sugar yield can be reached by optimizing hydrolysis conditions. Similarly, fermentation can reach its highest potential by optimizing fermentation conditions. On the other hand, applying SSF methods can increase bio-ethanol yield by minimizing hydrolysis inhibition and at the same time reduces the operating cost since both hydrolysis and fermentation is carried simultaneously. The

major disadvantage of SSF is that the optimum temperature of EH is different from most fermenting microorganisms temperature (da Silva et al., 2010, Kaur et al., 2018, Kadhum et al., 2019, Sudiyani et al., 2019). Most research on bio-ethanol production from biomass states that fermentation is carried out in an anaerobic system at moderate temperature (28 – 35 °C) Whilst the optimum temperature for EH is around 50 °C (Haynes et al., 2018, Jansen et al., 2018). Several attempts were made to overcome this obstacle by using thermo-tolerant microorganisms like *Candida Lusitaniae* and *Kluyveromyces Marxianus* or a mixed culture such as *Rettanomyces Clausenii* and *Saccharomyces Cerevisiae* (Pandey et al., 2019, Suzuki et al., 2019).

Beside SHF and SSF, there is another method named consolidated bioprocessing (CBP). In this process, cellulase pre-treatment, cellulose hydrolysis and fermentation are performed in one-step. Different genetic modified microorganisms can be used to apply CBP method on biomass such as *Clostridium Thermocellum*, *Cellulolytic Thermophilic* bacterial cells belonging to the genus *Caldicellulosiruptor*, *Saccharolytic* and/or *Xylanolytic Thermophilic* bacterial cells belonging to the genus *Thermoanaerobacter* and *Clostridium Cellulolyticum* (Hasunuma and Kondo, 2012, Curvers and Svetlitchnyi, 2018, Kumar et al., 2018, Braga et al., 2019).

Hexoses (C6 from the cellulose) and pentoses (C5 from the hemicellulose) are the dominating sugar monosaccharides released from the lignocellulosic biomass after EH are hexoses (C6 from the cellulose) and pentoses (C5 from the hemicellulose). The prsences of hexoses and pentoses raise a problem in the fermentation process especially at industrial scales due to the lack of microorganism that has the ability to ferment both these sugars with high yield and rate (McIntosh et al., 2016, Arshad et al., 2017). Different approches were suggusted to overcome this dropback. One of these approaches is to ferment C6 and C5 by adding two microorganisms to establish

a co-fermentation. The main limitation of this method is that the C6 and C5 microorganism environmental conditions are different. Most of the studies investigating the co-fermentation (co-cultures) stated that, despite the high glucose (C6) fermentation rate, xylose (C5) fermentation rate was usually slow as results of oxygen conflict requirements between the two microorganisms. Another fermentation approach suggested to improve sugars fermentation is by using a modified microorganism strain to gain the ability to ferment both glucose and xylose to bio-ethanol (Chen et al., 2018c, Liu et al., 2018, Papapetridis et al., 2018, Van de Velde et al., 2018, Patiño et al., 2019, Wang et al., 2019). A range of different microorganisms are used for cellulose and hemicellulose fermentation. *Saccharomyces Cerevisiae*, *Kluyveromyces Marxianus* and *Zymomonas Mobilis* are most used microorganisms to ferment cellulose (C6) sugars. Among the different yeasts, *S. Cerevisiae* shows the highest bio-ethanol yield up to 90- 97% of the theoretical glucose concentration and with high fermentation rate (Talebnia and Taherzadeh, 2006, Jørgensen, 2009, Tomás-Pejó et al., 2009, Moreno et al., 2017, Puligundla et al., 2019).

1.5 Response Surface Method

Response surface methodology (RSM) is a mathematical and statistical technique used to solve multivariable equations by creating sufficient relationship between observed results and experimental factors (Bezerra et al., 2008, Sadhukhan et al., 2016). The RSM was developed during the 1950's by Box and co-operators (Gilmour, 2006). RSM applies linear or square polynomial functions to characterize the studied system and explore experimental conditions with the aim of optimization (Ribeiro et al., 2010). RSM designing and optimizing depends on six different stages: independent variables selection based on their significant impacts on the system, experimental design selection, analysing the model mathematic–statistical by means of polynomial

function fitting, examination of the model fitting, verifying the model validation and the possibility to implement factors towered optimization by identifying the optimum values under study (Bezerra et al., 2008, Montgomery, 2008).

In the RSM, the first step includes using a simple design to fit the first-order model. In the simple design module, a first-order linear model will be sufficient. In most cases, a second-order polynomial model will be used based on the manipulated variables numbers and the interaction between them (Gilmour, 2006).

The central composite design (CCD) with axial points and centre point techniques is used to describe the second-order polynomial module. The CCD with centre point or as called face-centred cubes has the ability to define both linear and quadratic models (Box et al., 1978, Edmondson, 1994). The CCD method can evaluate three-level (or higher) factors with a minimum number of experiments while providing comparable results. To evaluate k factors: CCD required a factorial design including nfact = 2k points, minimum xi = −1 and maximum xi = +1 points for i = 1, 2, …, k, a start on an axial points formed by nax = 2*k and a total runs number equal to n_c at the centre point. The CCD can be also carried with a rotatable method by enabling the alpha, where $\alpha = (2k)^{1/4}$. By using the alpha, one higher and one lower values than the given range will be assumed by the DoE (Bezerra et al., 2008, Chattoraj et al., 2013).

DoE software can be used to apply the CCD method to find the mathematical design model and analyse the model. After the selection of the factorials and response number, DoE will suggest a number of experiments with a face centre or rotatable method. DoE will analysis the experiments data statistically and estimate the effects and interaction between them in order to generate the process equation to fit the data and estimate the response surface (Baboukani et al., 2012). The analysis will evaluate the intensity of the effects and determine the domain variables in the process.

CCD method is used extensively to optimize the biomass pre-treatment condition in order to improve the sugars yield. In most cases, the total reduced sugar yield after EH is selected to be the response to evaluate the pre-treatment conditions. Hemicellulose recovery, lignin extraction, inhibitors concentration and enzymes load are also selected individual or combined with sugar yield in a multiple responses study (Baboukani et al., 2012, Kim and Han, 2012, Yemiş and Mazza, 2012, Jaisamut et al., 2013, Zhang et al., 2013).

1.6 Research Objectives

The aim of the current study is to investigate the bio-ethanol from WS using different pre-treatment methods. This objective will be established through the following steps:

i. Study the effect of grinding, LHW, SE, ADR, PDR and microwave pre-treatment methods on the WS. The effectiveness of the pre-treatment method will be determined based on the total reduced sugar yield after the EH process.

ii. Optimise the microwave pre-treatment conditions using DoE. This will be accomplished by assessing the microwave pre-treatment time (5 – 120 min), temperature (50 – 200 °C), power (200 – 900 W) and liquid volume (20 – 40 mL) effects on the overall sugar recovery yield.

iii. Investigate the pH value effect on the total reduced sugar concentration during EH. This goal will be established by performing EH using Celluclast 1.5L supplement with Novozymes 188, Cellic CTec2 and endo-1, 4-β-Xylanase enzymes in sodium citrate buffer solution with pH value ranged (3 – 7).

1.7 Scope of Study

The global warming caused by the CO_2 emissions and the energy increasing demand due to rapid expansion in population has become one of the greatest problems to humanity worldwide. The scope of the current study is to adopt diverse pre-treatment

methods and evaluate their effect on the sugar yield and subsequently the ethanol yield from WS. The WS was pre-treated by a novel two disk ceramic grinder (WALDNER). The WS was grounded to various particle size ranged from > 2000 µm to <250 µm. The grounded WS was subjected to the EH and fermentation process to obtain the bio-ethanol as the final product.

LHW and SE pre-treatment were carried in 500 mL (PARR) reactor and 10 L batch plant, respectively. LHW and SE experiments were performed in the National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA) facility in Italy as a part of a collaboration project. The novelty of this project is by conducting LHW and SE without and with H_2SO_4 (3%, wt/wt) at the same severity for comparison on WS. The pre-treated WS was subjected to EH and fermentation to produce the bio-ethanol.

Disk refiner pre-treatment was achieved in ADR and PDR. In the ADR approaches, the WS was suspended in distilled water at 55°C for 60 min. The wet WS was refined at refiner plate gaps of 1 mm, 0.5 mm and 0 mm. The refined WS was passed through screen mesh (149 microns) and dewatered using a Vincent CP4 screw press.

As for the PDR pre-treatment, the WS was pre-treated by refining at high pressure followed by SE. The WS was pre-treated with Andritz Sprout-Bauer 12 inch (30.5cm) pressurized refiner. Varied pressures (4, 6, 8 and 10 bar) during the refining stage were investigated in a pilot scale continuous pressurised disc refining plant. Both of the ADR and PDR pre-treatment experiments were conducted at Bangor University as part of their contribution to the collaboration work. Sugar and ethanol yield from EH and fermentation process was performed in the University of Hull to evaluate the pre-treatment effectiveness.

As for the microwave pre-treatment, a novel approach was adopted by using distilled water to pre-treat the WS at different temperature, time, power and water volume. This investigation was performed to omit the using of the chemicals (such as alkali and acid) which are used usually in the microwave pre-treatment. The effect and interaction of microwave pre-treatment conditions were investigated using a central composite design (CCD). Furthermore, the microwave pre-treatment conditions were optimized by the means of DoE software.

The effect of pH during EH on the total reduced sugar yield was investigated. Different enzymes were applied to conduct the EH at pH ranged in (3 – 7).

Chapter Two: Materials and Methods

2.1 Introduction

In this chapter, the chemicals, equipment and methodology which were used in the current study will be detailed. The methodology is outlined in the order which the research was carried out. Starting with Section 2.2, all the materials that have been used in the current study were specified. The description of the equipment used in the current study for processing and characterisation is presented in Section 2.3. Section 2.4 illustrates the four steps of bio-ethanol production including pre-treatment, enzymatic hydrolysis, fermentation and distillation. Finally, Section 2.5 covers the analytical methods, which have been used to characterize the raw material, determine the sugars produce after enzymatic hydrolysis.

A simplified flow diagram for the work sequence carried out in the current study is given in Figure 2-1.

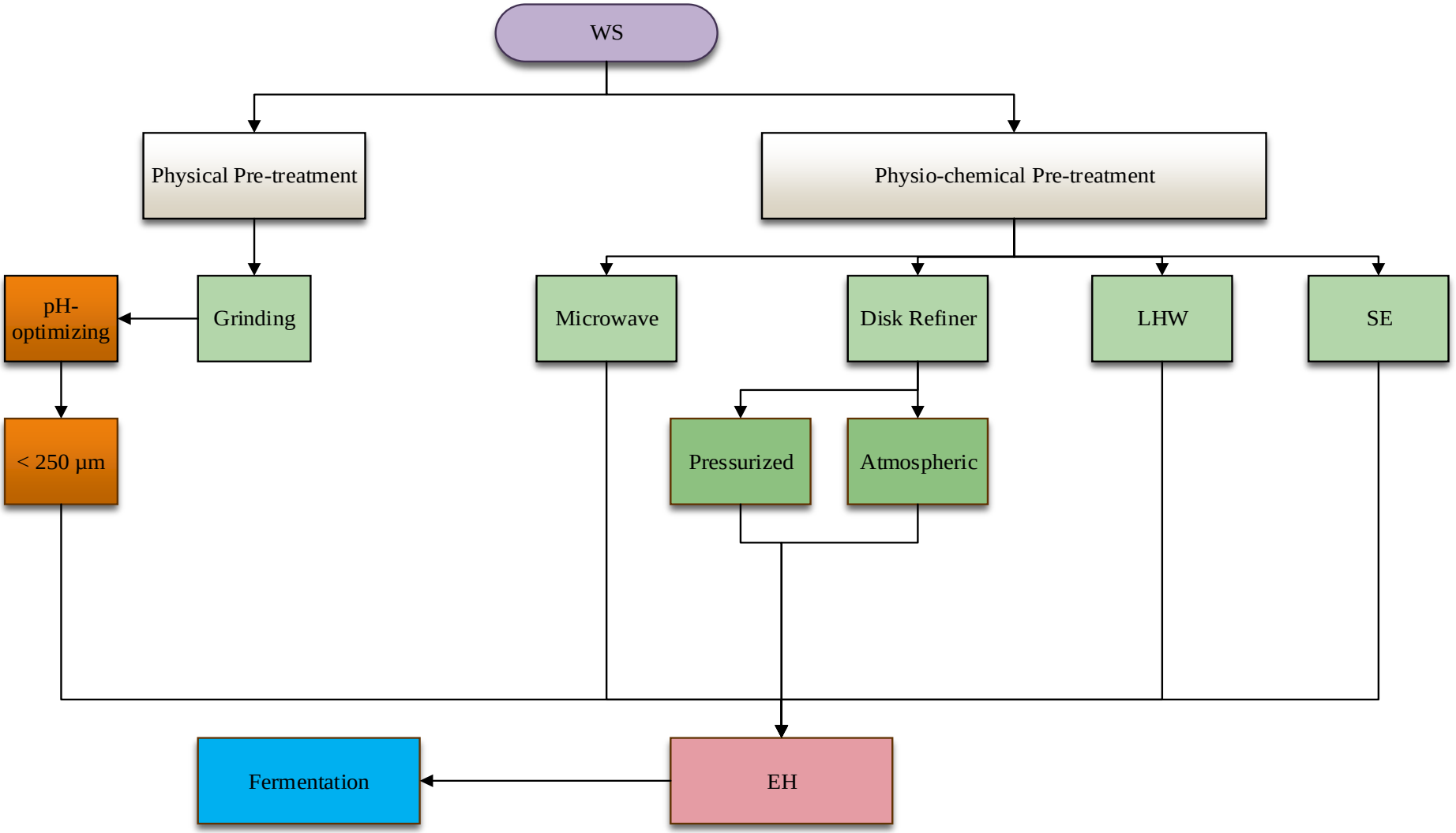


Figure 2-1. Work steps flow diagram.

2.2 Materials and Methods

A list of all the chemical reagents and the sources from which they were purchased as well as their properties and uses are shown in Table 2-1.

Table 2-1. List of the chemicals, their properties and usage

No.	Chemical	Supplier	Properties (purity)	Usage
1	Calcium Carbonate ($CaCO_3$)	ACROS Organics	99%	HPLC Analysis
2	Cellic CTec2	Novozymes	≥ 110 FPU/g	Hydrolysis Enzyme
3	Cellulase from Trichoderma reesei, Celluclast® 1.5L	Sigma-Aldrich	≥ 700 EGU/g	Hydrolysis Enzyme
4	D (+)-Arabinose	ACROS Organics	99%	GC-MS Analysis
5	D (+)- Cellobiose	Alfa Aesar	98%	GC-MS Analysis
6	D (+)-Galactose	ACROS Organics	99%	GC-MS Analysis
7	D (+)-Glucose	Fisher Scientific	analytical reagent grand	GC-MS Analysis
8	D (+)-Xylose	Alfa Aesar	98%	GC-MS Analysis
9	3, 5-Dinitrosalicylic acid ((O2N)2C6H2-2-(OH)CO2H)	ACROS Organics	98%	DNS-Reagent Preparation
10	endo-1,4-β-Xylanase from Trichoderma Longibrachiatum	Sigma-Aldrich	≥ 7.7 units/mg solid	Hydrolysis Enzyme
11	Ethanol (C_2H_5OH)	VWR Chemicals	99.94%	GC- Analysis
12	Glucosidase from Aspergillus Niger	Sigma-Aldrich	≥750 U/g	Hydrolysis Enzyme
13	Hydrochloric Acid (HCl)	Fisher Scientific	~ 37% S.G 1.18	pH Adjusting
14	L (+)- Mannose	Alfa Aesar	99%	GC-MS Analysis
15	Methoxyamine Hydrochloride (CH_3ONH_2)	Sigma-Aldrich	98%	GC-MS Analysis
16	N-Methyl-N-(trimethylsilyl) Trifluoroacetamide ($C_6H_{12}F_3NOSi$)	Fisher Scientific	97% ACROS Organics	GC-MS Analysis

17	Peptone	Oxoid LTD	pH 6.2	Yeast Growth Media
18	Potassium Sodium Tartrate Tetrahydrate ($KNaC_4H_4O_6 \cdot 4H_2O$)	ACROS Organics	99%	DNS-Reagent Preparation
19	Propanol (Propan-1-ol) (C_3H_8O)	Fisher Scientific	99%	GC- Analysis
20	Pyridine (C_5H_5N)	ACROS Organics	≥ 99%	GC-MS analysis
21	Saccharomyces Cerevisiae Yeast Type II	Sigma-Aldrich	--	Fermentation Yeast
22	Sodium Azide (NaN_3)	Fisher Scientific	99%	Sterilising Hydrolysis Media
23	Sodium Citrate Buffer Solution	Alfa Aesar	pH 5.0 , 0.5M	Hydrolysis Media
24	Sodium Hydroxide (NaOH)	Fisher Scientific	99% analytical reagent grand	DNS-Reagent Preparation and pH Adjusting
25	Sulphuric Acid (H_2SO_4)	Fisher Scientific	>95% S.G 1.83	Wheat Straw Analysis
26	Wheat Straw	Local farmer (Hull)	--	Process Row Material
27	Wheat Straw	Local farmer (Bangor)	--	ADR and PDR pre-treatment
28	Wheat Straw	Local farmer (Hull)	--	Process Row Material
29	Yeast Extract	Sigma-Aldrich	pH 7 (0.5% solution)	Yeast Growth Media

2.3 Equipment

2.3.1 WALDNER Grinder LADY - Grain Mill

WALDNER grinder LADY was purchased from WALDNER-biotech, GmbH, Germany. Figure 2-2 shows the schematic diagram of the WALDNER grinder.

The grinder contains two ceramic stone disks with a diameter of 90 mm, the top ceramic stone is attached to the adjustable lid. By twisting the lid, the gap between the two mills ceramic stone can be selected to obtain the desired WS particle sizes. The top lid of the grinder is a funnel shape to facilitate sample loading from the top. The

hopper capacity is 1 g, the hopper surrounding is equipped with two wipers to ensure all the grounded sample has been discharged from the hopper.

The mill housing is made from stone pine wood, it is equipped with a motor of 250 V, 50 Hz and 400 W to achieve an output for fine milling of 120 g min^{-1}.

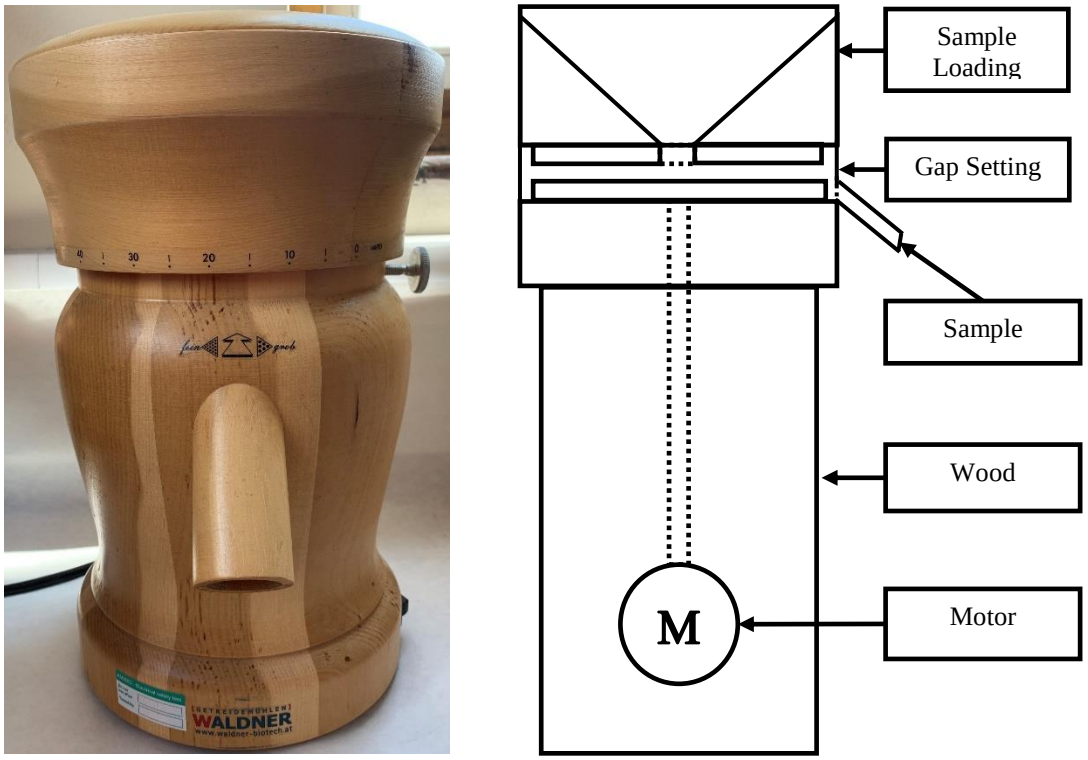


Figure 2-2. WALDNER Lady Grinder schematic diagram

2.3.2 Orbital Incubator Shaker

An orbital incubator shaker, Stuart SI500 from, Cole-Parmer Scientific Experts Ltd was used to perform the EH and fermentation processes. The shaker is equipped with an orbital shaking diameter 16 mm and a motion adjustable between 30 to 300 rpm. The incubator has a digital timer which ranged from 1 sec to 9 days. The temperature can be set from ambient temperature up to 60 °C ± 0.5°C.

2.3.3 Sieve

The sieving was carried out using a Retsch AS 200 Sieve from Retsch.com. The Retsch AS 200 Sieve is equipped with 5-different sieving plates (2000, 1000, 710,

500, 250) µm and a pan. The maximum loading for the Retsch AS 200 sieve is 3 kg with a time setting (1 – 99) min.

2.3.4 Microwave

A Milestone (Ethos EX, 1200 W) microwave reactor system, equipped with six 200 mL cylindrical PTFE rotary vessels, was used WS pre-treatment. Figure 2-3 shows the Ethos EX microwave and the PTFE rotary vessels reactor system. The microwave is equipped with an industrial magnetron that delivers up to 900 W of power, for rapid heating even of high-throughput rotors with a maximum working temperature of 200 °C. A heavy-duty airflow system is fixed with the ETHOS EX which is placed on top of the cavity to cools the external surfaces of the vessels rapidly.

The temperature was monitored and controlled continuously with an Automatic Temperature Control system ATC-FO Fiber-Optic. The temperature during the microwave use was monitored and controlled by placing the Fiber-Optic temperature sensor inside the PTFE ceramic thermos-well of the reference vessel.

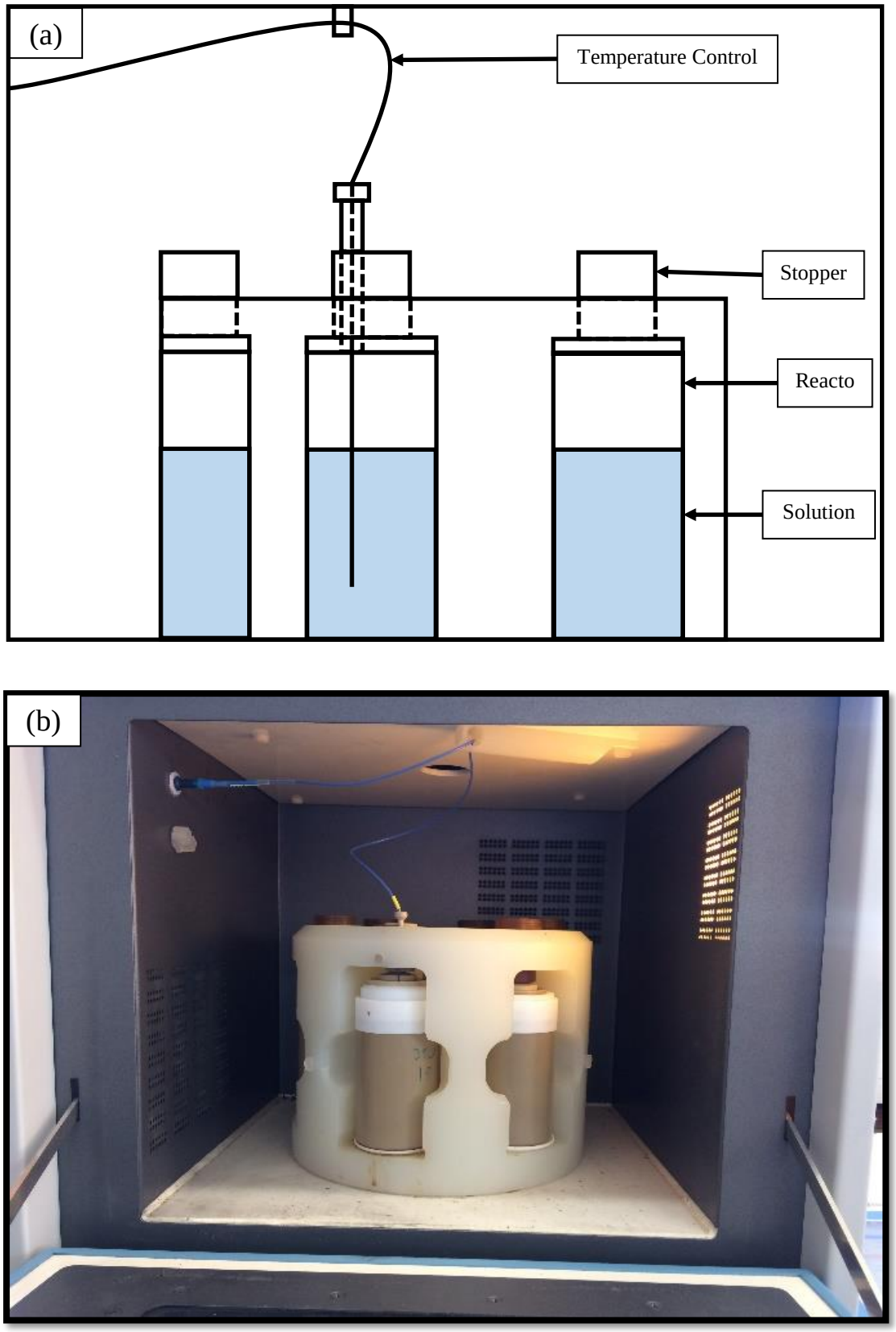


Figure 2-3. Ethos EX microwave and the PTFE rotary vessel reactors system: (a) Schematic and (b) picture.

2.3.5 Carousel Reactor

A Carousel 6 plus reaction station from Radleys innovations for chemistry, was used for the distillation process. The reactor is fixed with a water-cooled aluminium reflux head and the cooling system has an insulated cooling reservoir to maintain the temperatures for long periods. The hotplate has even stirring to ensure equal distribution of the temperature with a maximum heating temperature at 180 °C. The Carousel reactor is equipped with 6 Azeotropic round bottom flasks.

2.3.6 UV-Spectrophotometer

During this study, the total reduced sugar concentration released through EH was measured using a Jenway 7310 spectrophotometer from cole-parmer scientific experts. The Jenway 7310 feature measurement modes for concentration, transmittance percentage, spectrum scanning and absorbance.

2.4 Methods

2.4.1 Pre-treatment

2.4.1.1 Grinding Pre-treatment

The WS (harvest winter 2014) was subjected to a grinding pre-treatment process. For each test, around 10 g of the WS was used. The WS was firstly chopped with a 2200 W Luvele blender miller for 5 min to achieve WS sample length around of 2000 µm before grinding. During the WS grinding pre-treatment, the gap between the two ceramic disks was set on different values to establish a different range of particle sizes. The gap was adjusted by twisting the top lid of the grinder in order to achieve different particle sizes. The grounded WS samples were then sieved to separate the different particle sizes.

To get the finest size (powder WS), the WS was firstly grounded with a 250 µm gap. Afterwards, the gap was reduced by twisting the top lid until the two ceramic stone

starts to touch each other. The WS sample was then grounded for the second time. The grounded WS sample was then sieved and the fine WS size was separated from the rest of the WS particle sizes. After grinding all the samples, the collected WS powder was mixed and ground once more to get a homogenised mixture of dry powder WS with a particle size less than 250 µm.

The amplitude for the Retsch AS 200 was set at 0.99 mm g^{-1}, the sieving was carried out for 3 min with 1 sec interval. The equipment was supplied with the following size sieve plates: 2000, 1000, 710, 500, 250 µm and a pan. The collected WS from the different sieve was used in enzymatic hydrolysis and further processing.

2.4.1.2 Bangor University Atmospheric Disk Refining

As a part of collaborative work between the University of Hull, Bangor University and Vivergo Fuels Ltd the WS was pre-treated with an atmospheric disk refiner (ADR) in Bangor University. The atmospheric disc refiner equipment used consisted of three components: a rotating disc plate connected to the motor, a stationary disc plate, and housing.

WS (3 kg) was initially pre-processed with hammer mill Miracle Mill (Type 3RR) and passed through a 2 mm screen. The WS recovery was about 2.9 kg. The hammer milled WS was then sieved using a Farleygreen Sieve-master (a three decks vibrating sieve) to identify the particle size distribution. The results for the particle size were 0.289 kg for size > 4mm, 0.909 kg for 2-4 mm and 1.722 kg for < 2mm.

The results showed that the majority of the milled WS had a particle size of less than 2 mm which rise a difficulty and limitation of larger scale refining trials. Fine particle size (3 mm and less) caused clogging for the refiner plates and slowing drainage of the refined fibre. This complication is one of the main drawback and limitation in the pulp and paper industry. Furthermore, in this approach, if the fine particle size was

removed then the material lost will be high. A Pierret N40 forage chopper with a 0.5 inch (12.7 mm) cut length was introduced as a replacement of the Miracle Mill in order to minimize the amount of 'fines' produced. The chopped / milled WS samples were suspended in a hot water at 55 °C for 60 min to produce a specific consistency. The consistency requirement for the atmospheric disc refiner was 2% (1 kg of fibre suspended in 50 L of hot water). This value was recommended by Bangor University to facilitate the sample loading to the disc refiner. The suspended WS was fed into the rotating plates via a screw feed with a rate of 1 kg per batch. The refining process cycles took between 2.0 – 2.5 min. The refined WS was then passed through a screen mesh (149 µm) and dewatered using a Vincent CP4 screw press. Figure 2-4 shows the ADR equipment at the Bio-Composites Centre in Bangor University and the refiner plate.

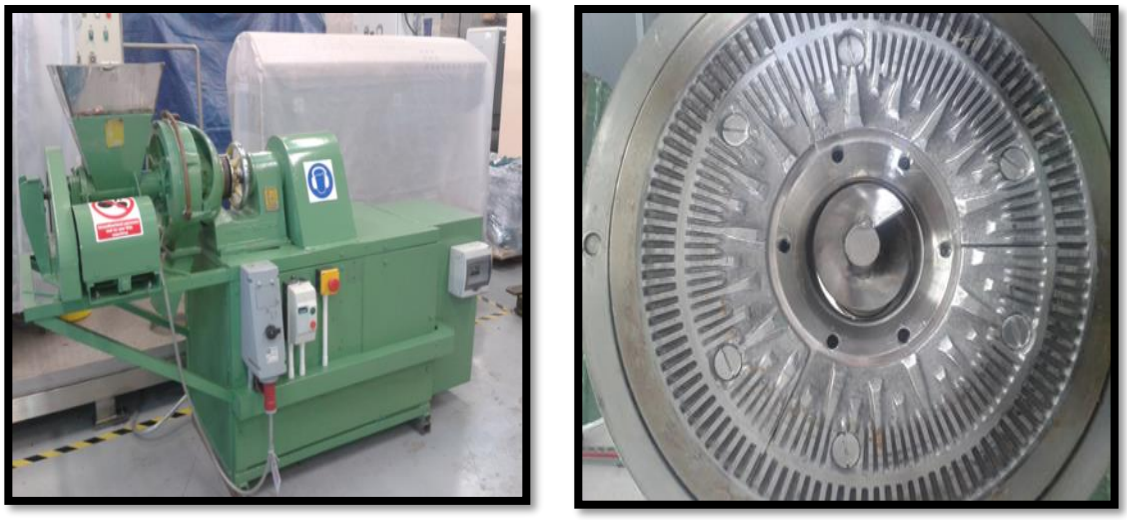


Figure 2-4. Atmospheric disc refining equipment and the refiner plate (Bangor University).

2.4.1.3 Bangor University Pressurized Disk Refining

The Andritz Sprout-Bauer 12 inch (30.5cm) pressurised disk refiner (PDR) was used for the WS pre-treatment at high pressure. The equipment is designed to process 50 kg of material per hour.

During the PDR pre-treatment, the WS was firstly pre-processed with the forage chopper with a 0.5 inch (12.7mm) cut length and then transferred to the PDR system. The WS was fed into the MSD (modular screw device) where a 'plug' was formed and the desired refining pressure was archived. The plug was passed through a heated cooker with 1 min residence time before entering a 60 L digester for another 1 min at the selected pressure.

The steam was fed into the refining zone. The refining zone contained two 12 inch diameter refining plates with one rotating disc. After refining, the refined WS was collected through a cyclone. Figure 2-5 shows the thermo-mechanical pre-treatment process diagram.

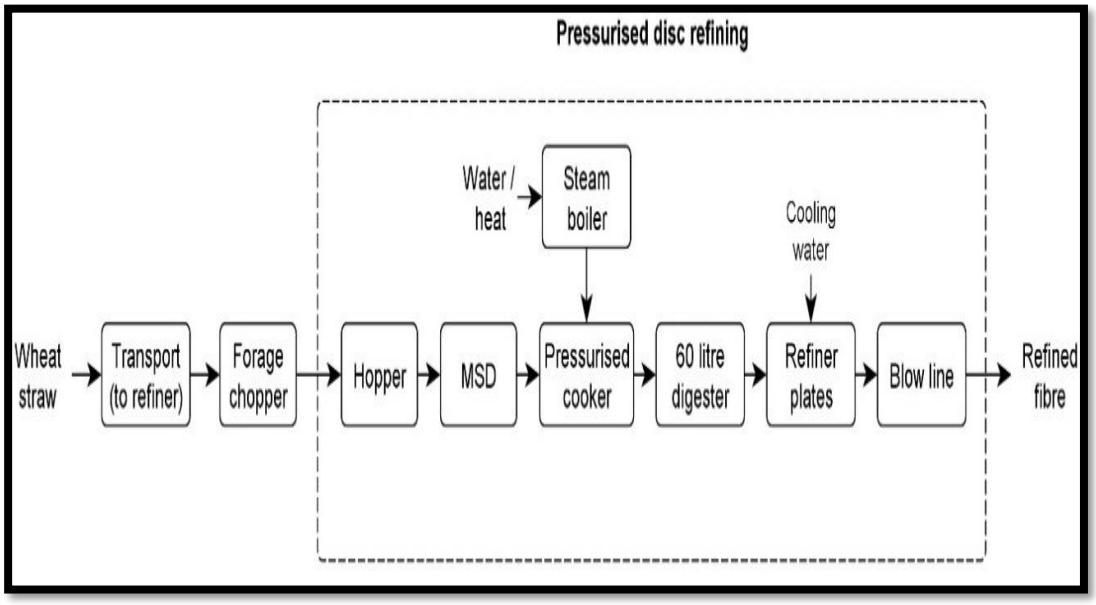


Figure 2-5. Pressurized disk refiner pre-treatment process diagram (Bangor University).

Four refining process trials were carried out:

1- Using a 'low intensity' refining plates (Andritz D2-516 plates) at a pressure of 4, 6, 8 and 10 bar, with a disk refining speed of 2500 rpm at a plate gap of 4 µm.

2- Using the same pre-treatment conditions with Andritz D2-503 plates, this plate has a bi-directional pattern and a bar angle of 0°, which gives more emphasis on grinding.

3- Using low intensity (blunter) plates, the same pressure range and plate gap was used but with a longer pre-treatment time (15 – 28 min).

4- The WS was pre-treated with D2-516 plates at the same pressure rang with even longer pre-treatment time 30-45 min (in order to obtain more detailed energy monitoring data).

The WS collected after all the trials were dried in a 100 m long flash drier with fan assistance. Figure 2-6 shows the various stages for the pressurized refining process.

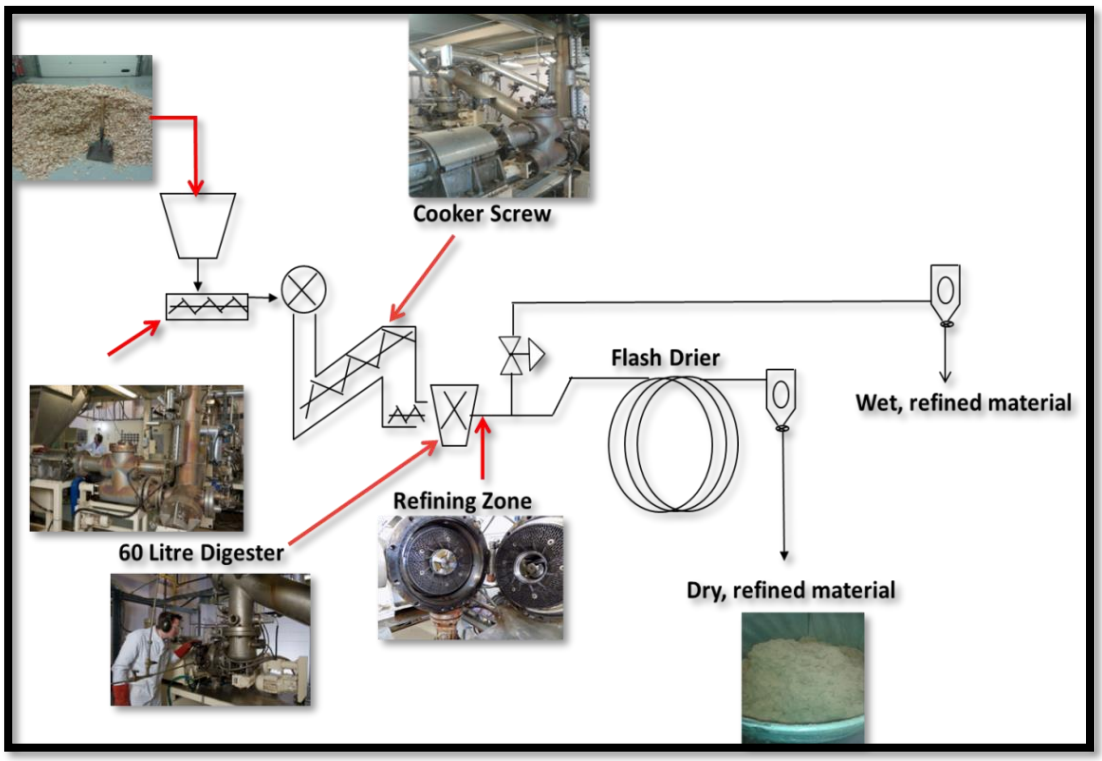


Figure 2-6. Continuous pressurised disc refining equipment (Bangor University).

2.4.1.4 Microwave Pre-treatment

Microwave pre-treatment was applied to the fine WS (< 250 µm) sample. The microwave pre-treatment experiment was conducted at the conditions specified by the

Design of experiments (DoE) v.11.1. In general, three vessels were used for each run. The samples were prepared by suspending 1 g of WS in the desired volume of distilled water in each one of the three reactor vessels. The slurry was stirred for 10 min before applying the microwave pre-treatment process. This step was essential to ensure that the WS absorbed the distilled water and to prevent the WS from forming a separate layer on top of the solution. Towards the end of the 10 min stirring, the three vessels were placed in the microwave holder. The pre-treatment temperature was monitored using a fibrotic sensor inside the reference vessel with the assumption that all the three vessels have the same conditions. During the microwave pre-treatment, the stirring percentage was fixed at 70%.

At the end of the microwave pre-treatment, the samples were left in the microwave to cool until the temperature below 100 °C was reached. The sample holder was then removed for the microwave and the vessels were opened slowly to prevent any loss in the samples due to build-up pressure. The cooling down time varied depending on the pre-treatment temperature and the water volume. In general, the cooling time was between 10 to 25 min. As for the low-temperature pre-treatment (lower than 100 °C), the samples were removed immediately from the microwave.

After the microwave pre-treatment was completed, the WS samples were passed through a 2 µm filter paper with a vacuum pump. The microwave reactor vessels were rinsed with distilled water to collect all the WS samples and the WS was then washed with distilled water. The final volume for the collected liquid was adjusted and used in the hemicellulose removal calculation. As for the solid fraction, it was dried at 35 °C before applying EH.

2.4.1.5 Steam Explosion Pre-treatment

Steam explosion (SE) experiments were carried out as a part of a two-week project in the National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA) facility in Italy. A pilot SE system with a batch reactor of 10 L, coupled with a 125 kW boiler was used to conduct the experiments. Two sets of experiments were carried out on the WS. The first set of the experiment was performed by pre-treating 500 g of WS at 224 °C for 10 min. Prior to the sample loading to the SE equipment, the WS was mixed with 450 g of distilled water to achieve a weight ratio of 1:1 (wt/wt) WS to distilled water (taking account the 10% (wt/wt) moisture content of the WS) for 5 min. The second set of the SE experiment was done by mixing 500 g of WS with 3% H_2SO_4 solution at a weight ratio of 1:1 (wt/wt). The mixture was loaded to the SE equipment and the pre-treatment was carried at 200 °C for 5 min. The SE pre-treatment conditions were chosen to match the severity of the liquid hot water (LHW) pre-treatment for comparison. Moreover, the SE pre-treatment conditions were based on a previous optimization investigation carried by ENEA on biomass. Figure 2-7 shows the SE system at ENEA facility.

The severity factor (Ro) was calculated using Equation 2.1 (Overend and Chornet, 1987b):

$$Ro = t \exp\left(\frac{T-100}{14.75}\right) \quad (2.1)$$

Where:

Ro is the severity factor

t time (min)

T temperature (°C).

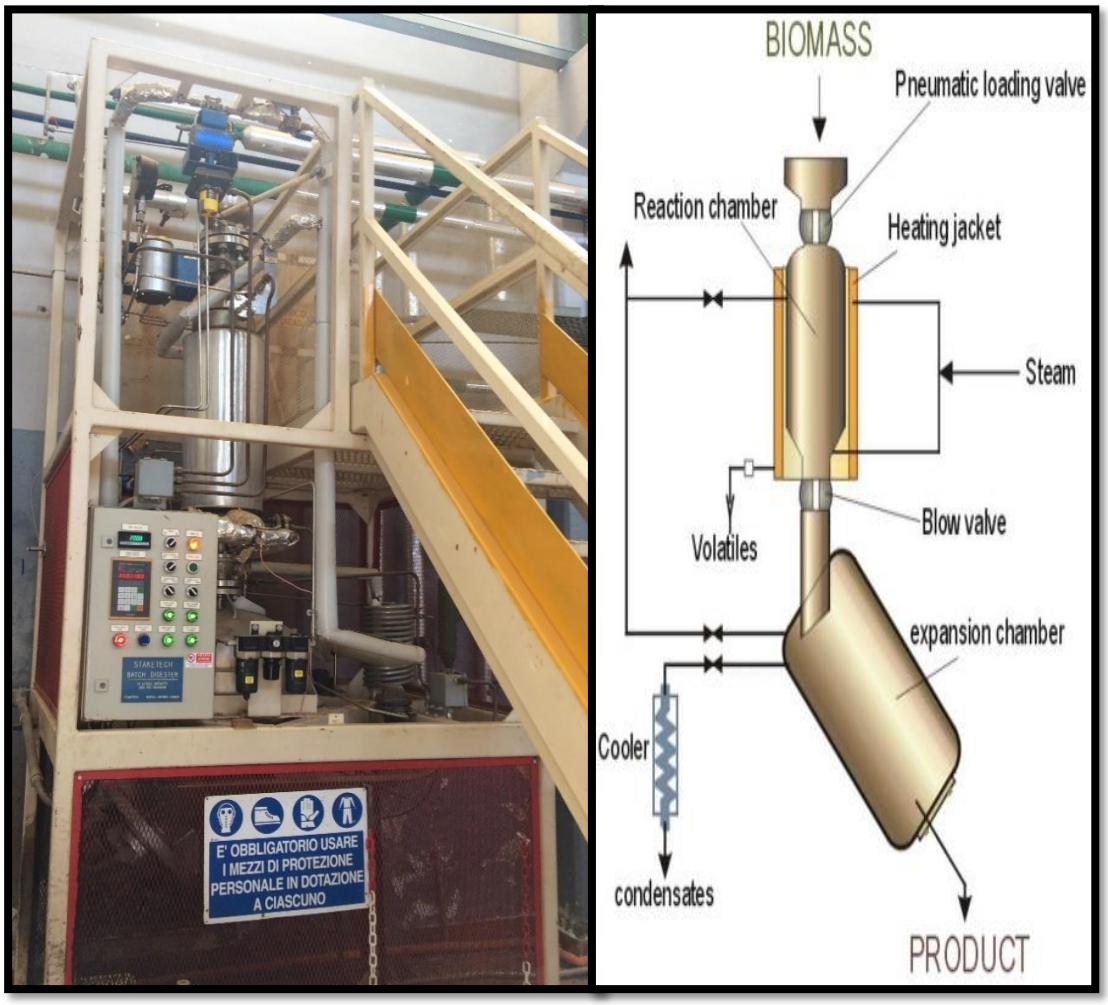


Figure 2-7. Steam explosion batch reactor (ENEA facility-Italy).

2.4.1.6 Liquid Hot Water pre-treatment

Similar to the SE, LHW pre-treatment was carried out as a part of the two-week project in ENEA facility. The LHW pre-treatment was carried out with a high pressure and temperature batch reactor (PARR-series) with a 0.5 L vessel and adjustable internal stirrer as well as heat control. Figure 2-8 shows the PARR reactor during the LHW pre-treatment operation.

Two experiments were conducted with LHW. The first experiment (LHW-H_2O) was done by pre-treating 5 g of WS with 100 mL distilled water at 204 °C for 30 min holding time. The time for heating up the reactor from 100 °C to the target temperature and the cooling done period after the end of the pre-treatment to 100 °C was 34 and

12 min, respectively. The second experiment (LHW-H_2SO_4) included pre-treating 5 g of WS with 100 mL of 3% H_2SO_4 (wt/wt %) solution. The pre-treatment was done at 164 °C with a holding time of 30 min. The heating and cooling as well as holding period temperature were recorded every 2 min. The temperature recording for the LHW-H_2O and LHW-H_2SO_4 experiments was initiated when the reactor temperature reached 100 °C and through the holding time (30 min) as well as during the cool-down stage until the reactor temperature dropped below 100 °C. For all the experiment, the stirring was at 400 rpm. The LHW-H_2O and LHW-H_2SO_4 pre-treatment severity target were 4.54 and 3.36, respectively.

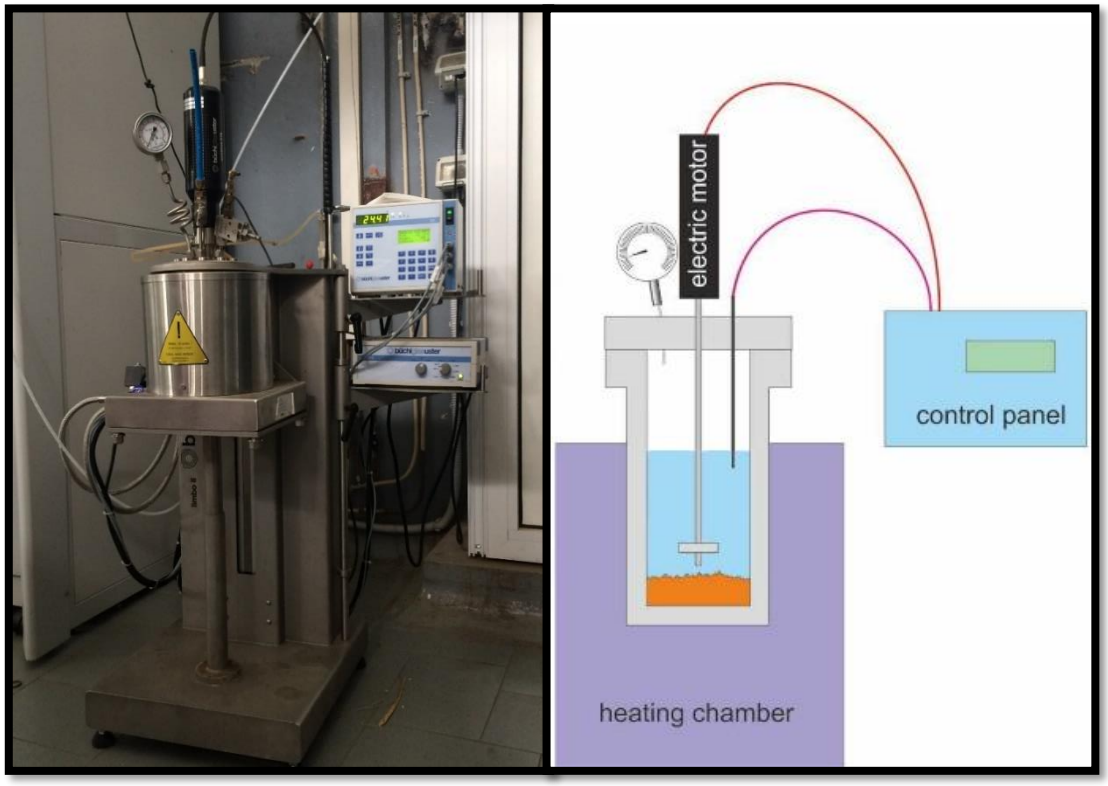


Figure 2-8. High pressure and temperature batch reactor (PARR) (ENEA facility-Italy).

2.4.2 Enzymatic Hydrolysis

2.4.2.1 Hydrolysis

The pre-treated WS collected from the different pre-treatment methods was enzymatically hydrolysed to release monomeric sugars from the WS. Through the

entire study, the enzymatic hydrolysis (EH) process was carried out at the same conditions unless otherwise mentioned. The EH was carried out by suspending 1g of pre-treated WS in 50 mL of buffer solution (sodium citrate 0.05 M). The EH was proceeded under mild conditions (50 ºC, 200 rpm) in the orbital incubator shaker for 72 hrs. The pH value of the solution was adjusted to the required pH value using 1M NaOH and 1M HCl. In general, for all EH experiment, the pH value was 5.8-6.0 unless otherwise mentioned.

Prior to adding the enzymes, 0.02% wt/wt NaN_3 was added to the aqueous solution and the solution was incubated for 60 min at (50 ºC, 200 rpm) to sterilize and inhibit the microbial growth as this may consume the monomeric sugar produced and inhibit the enzyme's activity (da Costa Lopes et al., 2013). After the 60 min sterilizing, different enzymes were added to the aqueous solution to start the EH.

The first enzyme cocktail of Celluclast from Trichoderma reesei (Celluclast® 1.5L) with a loading of 15 filter paper unit per gram of dry matter (FPU/g, DM) supplemented with Novozyme 188 with an activity loading of 22.5 cellobiose unit (CBU/g, DM) was used (unless otherwise mentioned).

The second set of EH experiment was done by adding xylanase enzyme (endo-1,4-β-Xylanase from Trichoderma longibrachiatum) with an activity loading of 1540 international unit (IU g^{-1}, DM) from Trichoderma. Finally, a commercial cellulose enzyme cocktail Cellic CTec2 with an activity loading of 15 FPU/g of DM from Novozyme was also used for EH.

The EH was carried out in a 50 mL conical flask. After adding the enzymes to the solution, the conical flask was carefully sealed with a glass stopper and parafilm. During the hydrolysis, samples were withdrawn every 24 hrs from the hydrolysis solution for the total reduced sugar measurements. During the sample withdraw, the

shaker was stopped for 5 min to allow the suspended WS to settle down before taking the sample.

2.4.2.2 Enzymes Activity Measurement (Enzymes Assay)

The activation of the cellulase enzymes Celluclast® 1.5L and Cellic CTec2 activate was measured using the National Renewable Energy Laboratory (NREL) standard procedure based on the International Union of Pure and Applied Chemistry (IUPAC) guidelines (Ghose, 1987, Adney and Baker, 1996). The activation was measured and expressed in terms of filter paper units (FPU) per mL of original (undiluted) enzyme solution. The FPU represents the concentration of the enzyme which releases 2 mg of glucose as a reduced sugar from 50 mg of filter paper in 60 min.

Three sets of experimental tubes were carried out in parallel:

- ❖ Glucose standards

The glucose standards were prepared by making a working stock solution of glucose with a concentration of 10 mg mL^{-1}. The stock solution was then used to prepare different solutions with concentrations using the following dilutions:

- 1.0 mL + 0.5 mL buffer = 1:1.5 = 6.7 mg mL^{-1} (3.35 mg 0.5 mL^{-1})
- 1.0 mL + 1.0 mL buffer = 1:2 = 5.0 mg mL^{-1} (2.50 mg 0.5 mL^{-1})
- 1.0 mL + 2.0 mL buffer = 1:3 = 3.3 mg mL^{-1} (1.65 mg 0.5 mL^{-1})
- 1.0 mL + 4.0 mL buffer = 1:5 = 2.0 mg mL^{-1} (1.00 mg 0.5 mL^{-1})

0.5 mL of each glucose dilution was mixed with 1.0 mL of sodium citrate buffer in a test tube.

- ❖ Assay mixture

The assay was prepared by adding 1.0 mL of sodium citrate buffer to Whatman filter paper No. 1 strip. The filter paper is 1.0 x 6.0 cm in dimension and weight about 50 mg. The filter paper was rolled and pushed down into the tube to ensure perfect

saturation with the buffer. Six different enzymes dilutions were made with the sodium citrate buffer. One dilution was made to release slightly more and another one slightly less than 2.0 mg (absolute amount) of glucose, respectively. 0.5 mL of the enzymes dilution was added to the buffer and the filter paper mixture.

All the six tubes were incubated for 60 min at 50 °C in the orbital incubator shaker to carry out the EH. At the end of the 60 min, the tubes were removed from the orbital incubator shaker and the DNS reagent was added to stop the activity of the enzyme.

- ❖ Blanks and Controls

The reagent blank was prepared by using 1.5 mL of sodium citrate buffer in a test tube. As for the assay controls, 1.0 mL of the buffer was added to 0.5 mL of the enzyme dilution (one control for each enzyme dilution). The reagent blank and the enzyme dilution controls were incubated with the assay mixture for 60 min at 50 °C.

After completion of the incubation for the assay mixture, enzymes dilution controls and the reagent blank, 3 mL aliquot of DNS reagent was added to all the glucose standards, assay mixture, enzymes dilution controls and the reagent blank.

All the test tubes were placed in a boiling water bath for 5 min to allow the DNS reagent to react with the sugars, followed by an ice bath to cool down to the room temperature before adding 20 mL of distilled water. The solutions were then mixed using a mini-shaker for 5 min to ensure uniform colour, the test tubes were left for 15 min to settle and centrifuged for 3 min.

The colour formed due to total sugar released was measured at 540 nm (Miller, 1959) using the spectrophotometer. The absorbance of each enzymes dilution was measured against its blank.

The standard curve was constructed by plotting the absolute glucose concentration (mg 0.5 mL^{-1}) versus the absorbance at 540 nm. The amount of the glucose released from each enzyme sample tubes was determined.

The enzyme concentration which realised exactly 2 mg of glucose was estimated by drawing the glucose concentration released after the EH against semi-logarithmic enzymes concentration. To find the exact enzymes dilution, two points very close to 2 mg used to draw a straight line between them and interpolate them. The exact concentration of the enzymes which release 2 mg of sugar was calculated using Equation 2.2 (Ghose, 1987):

$$\text{Filter Paper Unit} = \frac{0.37}{\text{enzymes concentration which released 2.0 mg glucose}} \text{ unite/mL} \quad (2.2)$$

β-Glucosidase is measured using cellobiose units (CBU), the β-glucosidase loading is often measured as a ratio to the FPU, the common ratio for biomass hydrolysis used by scholars is between 1:1 to 1:2 FPU/CBU. The cellobiose unit is based on the international unit (IU) where 1 IU = 1 μmole of the substrate converted per min (Ghose, 1987). The cellobiose is calculated using Equation 2.3.

$$\text{CBU} = \frac{0.0926}{\text{Enzyme concentration which released 1.0 mg of glucose}} \text{ units mL}^{-1} \quad (2.3)$$

The Xylanase (1,4-β-d-xylanase, EC 3.2.1.8) enzyme activity was measured in IU mL^{-1}, one activity unit represent the 1 μmol of reducing sugar liberated as xylose per min at pH 4.5 at 30 °C (Bailey et al., 1992).

2.4.3 Fermentation

2.4.3.1 Yeast Preparation

Saccharomyces Cerevisiae yeast was used in the fermentation process. The yeast was stored in the refrigerator at 2 °C. The yeast was hydrated (inoculum preparation) in a 250 mL conical flask by dissolved 1 g of yeast extract, 2 g of peptone and 2 g of

glucose in 100 mL distilled water. The mixture solution was stirred with a magnetic stirrer plate until the peptone, glucose and the yeast extract dissolved.

The growth media was sealed with foil and autoclaved for 5 min at 121 ºC to sterilize the solution before adding the yeast (Zhang et al., 2013). When the autoclave cycle ended, the flask was left to cool down to room temperature. The solution pH value was measured and adjusted (if necessary) to 6.0 (Karatay et al., 2016).

After adding the *S. Cerevisiae* yeast to the aqueous solution, an airlock was fitted to provide a semi-aerobic atmospheric for the yeast. The growth media was incubated in the orbital shaker incubator at 37 ºC and 80 rpm for 18 hrs. The cells were harvested by centrifuging the media at 4400 rpm for 5 min and then washed with distilled water twice (da Silva et al., 2010).

2.4.3.2 Fermentation

All the fermentation processes in the current study were carried out under the same conditions (unless otherwise mentioned) using the separate hydrolysis and fermentation (SHF) approach. The fermentation was conducted in 250 mL conical flask with a working volume of 100 mL. The glucose concentration after EH was adjusted to 20 g L^{-1} before adding the harvested yeast. The fermentation was performed at pH 6.5 and 35 ℃ for 72 hrs in the orbital shaker incubator at 100 rpm in a semi-aerobic media (Saha et al., 2005, Saha and Cotta, 2007).

2.4.3.3 Mass balance

The ethanol yield was calculated based on the glucose reaction to produce ethanol and carbon dioxide.

$$C_6H_{12}O_6 \rightarrow 2\ CO_2 + 2\ C_2H_5OH$$

The hexose sugars moles was calculated using Equation 2.4.

$$\text{Mole of (C6)} = \text{Mass} / \text{MW} \qquad (2.4)$$

Where mass is in g and MW is the glucose molecular weight in g $mole^{-1}$

And since 1 mole of C6 will gives 2 moles of ethanol, then ethanol mass can be calculated using Equation 2.5.

$$\text{Mass of ethanol} = (\text{C6 mole}) \times 2 \times \text{MW} \quad (2.5)$$

Where the mass is in g and MW is the ethanol molecular weight in g $mole^{-1}$.

The ethanol percentage yield was calculated based on the actual and theoretical ethanol yield. Equation 2.6 was used for ethanol yield calculation.

$$\text{Ethanol yield (\%)} = \frac{\text{Ethanol produced from the glucose released after EH}}{\text{Theoretical ethanol yield if all the glucose released after EH was fermented}} \times 100 \quad (2.6)$$

2.4.4 Distillation

The fermented samples were distilled to separate the ethanol from the rest of the solution. The distillation process was carried out in the Radleys Carousel reactor. Figure 2-9 shows the Radleys with the azeotropic round bottom flasks during distillation. The reactor is equipped with 6 round bottom azeotropic distillation flasks. The azeotropic distillation flasks have a drop lock to collect the distilled ethanol and separate from the mixture. The reactor heater was set at 80 °C to allow the ethanol to evaporate.

The distilled ethanol was collected in a test tube submerged in an ice-water bath to cool down the condensed ethanol and reduce ethanol lost. The distillation process was carried out until there were no more ethanol drops forming. The distillation process took approximately 3 to 6 hrs.

Figure 2-9. Radleys with the Azeotropic round bottom flasks during distillation.

2.5 Analysis

2.5.1 Raw Material Composition Analysis

The WS moisture content was calculated by using the National Renewable Energy Laboratory (NREL) standard protocol for total solids determination in the biomass using High Performance Liquid Chromatography HPLC (Sluiter et al., 2008b). The moisture percentage content represents the weight difference between the WS before and after drying. Initially, the raw WS weight was recorded to the nearest 0.1 mg. The WS was then placed inside the oven set at 105 °C. The sample weight was monitored every four hrs until there was no change in weight. The moisture percentage content was calculated using Equation 2.7:

$$\text{Moisture}\ (\%) = 100 - \frac{\text{Weight (dry WS)}}{\text{Weight (raw WS)}} \times 100 \qquad (2.7)$$

The carbohydrate composition of the raw WS was determined by NREL standard protocol (Sluiter et al., 2008c). Oven-dried WS (0.3 g) was hydrolyzed using 3 mL of

72% H_2SO_4. The WS (0.3 g) was suspended in 3 mL of 72% H_2SO_4. The mixture was stirred with a spatula until all the WS was dissolved in the H_2SO_4. Separate spatulas were used for each test tube. After mixing the WS with the acid, the test tubes with the spatula were incubated for 60 min at 30 ºC in a shaker incubator. The orbital incubator shaker was set at 80 rpm to ensure good mixing. Moreover, every 10 min, the samples were given extra mixing by using the spatula.

At the end of the 60 min, the test tubes were removed from the incubator and then diluted with 84 mL of deionized water to achieve an acid concentration of 4%. After the dilution, the samples were autoclaved for another 60 min at 121 ºC. Towards the end of autoclaving, the solution was filtered and 50 mL of the solution was taken for analysis of the carbohydrates and soluble lignin. Around 150 mL of deionized water was used to wash the test tube and remove all the solid particle to the filtering crucible. To determine the insoluble lignin, the filtered solid in the filtering crucible and the crucible were dried in the oven at 105 °C for 5-6 hrs until a constant weight was achieved. The weight of the dried crucible with the dry residue was recorded. The crucible with the residue was placed in the muffle furnace for 24 hrs at 575 °C. After cooling down to the room temperature, the crucible with ash weight was recorded. The acid insoluble residue (AIR) was calculated using Equation 2.8.

$$\text{AIR}\ (\%) = \frac{\text{Weight (crucible plus AIR)} - \text{Weight (crucible)}}{\text{Sample weight (oven dry)}} \times 100 \qquad (2.8)$$

To determine acid-soluble lignin, the solution absorbance was measured using a UV-Visible spectrophotometer at 240 nm. The sample was diluted with 4% (wt/wt) H_2SO_4 to get the absorbance between 0.7 and 1.0. As for blank, 4% (wt/wt) H_2SO_4 was used. The measurement was done within 6 hrs after collecting the end of the autoclave step. The amount of the Acid-Soluble Lignin (ASL) was determined according to the following Equation 2.9:

$$\% \text{ ASL} = \frac{\text{UV abs} \times \text{Volume filtrate} \times \text{Dilution}}{\varepsilon \times \text{ODW sample} \times \text{Pathlength}} \text{ x } 100 \quad (2.9)$$

Where:

UV abs = UV-absorbance for the sample at 240 nm

Volume $_{\text{hydrolysis liquor}}$ = volume of filtrate, 86.73 mL

ε = absorptivity of biomass at a specific wavelength

ODW sample = weight of sample in mg

Path length = path length of UV- Vis cuvette in cm

The dilution was calculated by Equation 2.10:

Dilution =

[(Volume (sample) + Volume (diluting solvent)) / Volume (sample)] (2.10)

For carbohydrate determination, the hydrolysis liquor was neutralized with $CaCO_3$. The $CaCO_3$ was added slowly to the hydrolysis liquor with continuous stirring. When the solution pH value reached between 5.0 and 6.0, the addition of $CaCO_3$ was stopped and the sample was left for 10 min to settle. The sample was then centrifuged for 10 min at 4400 rpm. The supernatant was filtered by passing through a 2 µm filter paper and collected for the determination of the carbohydrate composition. A standard calibration curve was done by preparing five standard dilutions 0.125, 0.25, 0.5, 1.0, 2.0 and 4.0 mg mL^{-1} of all the standard sugars that might appear in the biomass (D (+) glucose, D (+) xylose, D (+) galactose, D (+) mannose, L (+) arabinose, D (-) cellobiose). High-performance liquid chromatography (HPLC, Nexera-1, Shimadzu) with a UV detection at 280 nm was used to determine the composition of the carbohydrate. The instrument was equipped with a Shodex sugar SP0810 column. The separation was carried out at 80º C. Deionized water was used as eluent in a flow rate of 0.6 mL min^{-1} and 20 mL inject the sample volume.

2.5.2 Total Reduced Sugar Analysis Using DNS- Method

The DNS-method were used to determine the total reduced sugars after EH (Miller, 1959). The DNS indicator was prepared by mixing 10 g of 3,5-dinitro-2-hydroxybenzoic acid dissolving in 200 mL of NaOH (2 M) with 300 g sodium potassium tartrate (Rochelle salt) dissolved in 500 mL of distilled water. The final volume was adjusted to 1 L. The DNS indicator was stored in a round glass bottle.

The concentration of the total reduced sugar liberated during and at the end of the EH was measured by taking 1 mL from the hydrolysis solution. The sample was boiled for 1 min to stop the activities of the enzymes. To determine the total reduced sugar concentration, 3 mL of the DNS indicator was mixed with 1 mL of sodium citrate buffer (0.05M) and 0.5 mL of hydrolysis solution supernatant. The blank solution was prepared by mixing 1.5 mL of sodium citrate buffer with 3 mL of DNS indicator. The sample and blank tubes were submerged in a water bath at 100 °C for 5 min, then they were cooled down using an ice bath to room temperature. The DNS indicator will react with the sugar present in the hydrolysis solution (Equation 2.11):

$$\text{3,5-dinitrosalicylic acid} \rightarrow \text{3-amino,5-nitrosalicylic acid} \qquad (2.11)$$

After cooling the sample to room temperature, 20 mL of deionized water was added to the sample and the blank. The solution was mixed for 5 min to ensure a uniform colour. During the reaction, the DNS indicator colour changed from yellow to red with the present of the monosaccharide sugars. The colour intensity depends on the total reduced sugar concentration present in the hydrolysis solution. The colour intensity was measured by using the UV-spectrophotometer at 540 nm wavelength.

Before measuring the total reduced sugar concentration, a set of glucose standards were prepared to construct the standard calibration curve. A working stock solution of

glucose with 10 mg mL^{-1} concentration was prepared with a fresh working solution being prepared each time the calibration curve was measured.

Four solutions with different glucose concentrations were prepared using the stock solution and the sodium citrate buffer for dilution, the different dilutions are shown in Table 2.2.

Table 2-2. Preparation of different glucose standard solutions with different concentrations

Stock Solution	Sodium Citrate Buffer	Dilution	Concentration
1 mL	0.5 mL	1:1.5	3.35 mg 0.5 mL^{-1}
1 mL	1.0 mL	1:2	2.50 mg 0.5 mL^{-1}
1 mL	2.0 mL	1:3	1.65 mg 0.5 mL^{-1}
1 mL	4.0 mL	1:4	1.00 mg 0.5 mL^{-1}

The standard calibration curve was prepared by mixing 0.5 mL of each glucose concentration with 1 mL of sodium citrate buffer in a test tube. 3 mL of DNS indicator was then added. The blank solution was prepared by mixing 1.5 mL of sodium citrate buffer with 3 mL of DNS indicator. All the glucose standards and blank were placed in a boiling water bath for 5 min. After the end of the reaction, immediately the tubes were removed from the boiling water bath and placed in an ice bath and left to cool down to the room temperature. 20 mL of deionized water was added to all the tubes and then they were shacked for 5 min to ensure uniform colour development. The colour intensity of each the different glucose concentration solutions was measured against the blank at 540 nm.

The glucose calibration curve was used to determine the total reduced sugar concentration since the glucose is the major monosaccharide product after EH. The calibration curve Equation 2.12 is:

$$Y = 0.3098\ X + 0.0618 \quad (2.12)$$

The $R^2 = 0.9957$

Where the Y represents the absorbance and the X represent the total reduced sugar concentration (mg 0.5 mL^{-1}).

The same procedure was carried out using xylose instead of glucose since xylose is obtained from the hemicellulose. The results show almost the same absorbance and therefore the total reduced sugar yield after EH of all the pre-treated WS in the current study was measured by using the glucose standard curve (unless otherwise mentioned).

2.5.3 Sugar Analysis after hydrolysis

At the end of EH, the samples were centrifuged and filtered through 0.2 µm filter paper. The liquid collected was used to determined monosaccharide sugars released during EH. 300 µl of the supernatant was transferred to the GC-MS vessels and evaporated to dryness by placing the vessels in an air dryer at 25 ºC. The dried sugars were suspended with 300 µl of methoxyamine hydrochloride in pyridine solution (concentration of 20 mg mL^{-1}), the solution was then incubated at 37º C for 90 min with gentle shaking at 60 rpm. At the end of the 90 min, 300 µl of n-methyl-n-(trimethylsilyl) trifluoroacetamide (MSTFA) was added to the solution and the samples were incubated for another 60 min at the same temperature and shaking speed (Yang et al., 2013).

The monosaccharide sugars were analyzed using GC-MS (Agilent 6890 with 5973 N MS, Agilent Technologies, Palo Alto, CA, USA) equipped with restek MS column (30 m × 0.25 mm × 0.25 µm, Rxi-5Sil, USA). The GC oven temperature was kept constant for 1 min at 70 °C and gradually increased at a fixed rate (5 °C min^{-1}) until it reached 320 °C. The injection port and transfer line temperatures were at 260 °C and

280 °C, respectively. The carrying gas (helium) flow rate was 1 mL min^{-1} with a split injection ratio 50:1. The data were recorded in the mass range of 50–500 m/z and the results were specified by comparison (cross-matching) with GC-MS library.

Solutions of the standard sugars (D (+)-glucose, D (+)-xylose, D (+)-galactose, D (+) arabinose, L (+) mannose and D (-) cellobiose) were made at concentration of 30 mg mL^{-1} to identify the peak and the retention time of each monosaccharide (Yang et al., 2013).

The sugar yield for each monosaccharide was calculated using Equation 2.13 (Hideno et al., 2009).

Sugar yield (%)

$$= \frac{\text{weight of monomeric sugars after EH (g)}}{\text{weight of maximum monomeric sugars after H2SO4 (72 \%)hydrolysis}} \times 100 \quad (2.13)$$

2.5.4 Ethanol Analysis after Fermentation

The ethanol concentration after the fermentation process was measured using a GC equipped with Restek stabilwax (column 30 m × 0.25 mm × 0.25 μm). A standard ethanol calibration curve was established in order to evaluate the ethanol concentration after fermentation. The standard ethanol solution was prepared by mixing different ethanol concentrations with internal standard propanol in 10 mL volumetric flasks. The final volume was made up to 10 mL by adding deionized water. Table 2.3 shows the different ethanol concentration.

Table 2-3. Ethanol solutions with different concentrations for constructing the calibration curve.

No.	Propanol (mL)	Ethanol (mL)	Water (mL)
1	1	0.1	8.9
2	1	0.2	8.8
3	1	0.4	8.6
4	1	0.6	8.4
5	1	0.8	8.2
6	1	1.0	8.0
7	1	1.2	7.8
8	1	1.4	7.6

The different ethanol concentration solution was tested twice with GC to create the standard calibration curve. Table 2.4 shows the ethanol and propanol peak area for the two tests. The average ratio and the ethanol (%) in Table 2-4 were to generate the standard calibration curve and Equation 2.14 represent the calibration linear equation.

$$Y=0\ .0792\ X - 0.0155 \tag{2.14}$$

Where Y is representing the Ethanol/propanol peak area ratio and X represent the ethanol percentage concentration. The R^2 was found to be 0.9994. By using the above equation, the ethanol yield after fermentation was quantified.

Table 2-4. Ethanol solutions concentrations and the ethanol - propanol peak areas.

Ethanol %	Ethanol peak area 1	Ethanol peak area 2	Propanol peak area 1	Propanol peak area 2	Peak Area Ratio 1	Peak Area Ratio 2	Ave Ratio
1	8.84	18.02	125.14	261.89	0.071	0.069	0.070
2	14.49	56.42	98.8	384.41	0.150	0.147	0.147
4	40.51	91.20	133.59	323.95	0.303	0.282	0.292
6	121.03	136.91	275.48	301.44	0.440	0.454	0.447
8	61.83	72.67	99.23	118.31	0.623	0.614	0.619
10	61.43	191.34	77.32	241.94	0.795	0.791	0.793
12	77.01	126.85	85.62	131.03	0.899	0.968	0.934
14	115.45	115.45	106.02	106.02	1.089	1.089	1.089

Chapter Three: Analysis and Grinding Pre-treatment

3.1 Analysis

In this thesis, wheat straw (WS) obtained from assorted sources was used:

a- WS from a local farm near Hull, East Riding of Yorkshire, UK (harvest in winter 2014).

b- WS from East of England, UK (harvest in winter 2015).

c- WS from a local farm near Driffield, East Riding of Yorkshire, UK (harvest in summer 2017).

The WS harvest near Hull in winter 2014 was initially used to study the grinding pre-treatment effect on enzymatic hydrolysis (EH). The grinding pre-treatment experiments were performed again using the WS (harvest in summer 2017).

Furthermore, WS (harvest in summer 2017) was used for the microwave, steam explosion (SE), liquid hot water (LHW) pre-treatment as well as for the pH study. The results reported in the current study refers to the WS (harvest in summer 2017).

WS from East of England, UK (harvest in winter 2015) was used in atmospheric disk refiner (ADR) and pressurized disc refiner (PDR) pre-treatment.

3.1.1 Raw Wheat Straw Composition Analysis

The moisture content of the WS (harvest in summer 2017) was determined according to the National Renewable Energy Laboratory (NREL) standard procedure (Sluiter et al., 2008a). In summary, approximately 5.0 g of WS was weighed and placed in the oven at 105 °C until a constant weight was achieved. The sample weight before and after dryness was recorded and the moisture content for WS was found to be around 10%. Prior to WS analysis, a series of standard sugars (glucose, xylose, galactose, arabinose, mannose and cellobiose) concentrations were made. The different sugars concentrations are shown in Table 3-1.

Table 3-1. Standards sugars solutions with a range of concentrations for the calibration curve.

Component	Concentration (mg mL^{-1})					
D (+) glucose	0.125	0.250	0.500	1.000	2.000	4.000
D (+) xylose	0.125	0.250	0.500	1.000	2.000	4.000
D (+) galactose	0.125	0.250	0.500	1.000	2.000	4.000
L (+) arabinose	0.125	0.250	0.500	1.000	2.000	4.000
D (+) mannose	0.125	0.250	0.500	1.000	2.000	4.000
D-cellobiose	0.125	0.250	0.500	1.000	2.000	4.000

For each standard sugar, the six solutions with the different concentrations were tested with the high performance liquid chromatography (HPLC) to create the standard calibration curve. Figure 3-1 shows the standard calibration curve for each monosaccharide sugar.

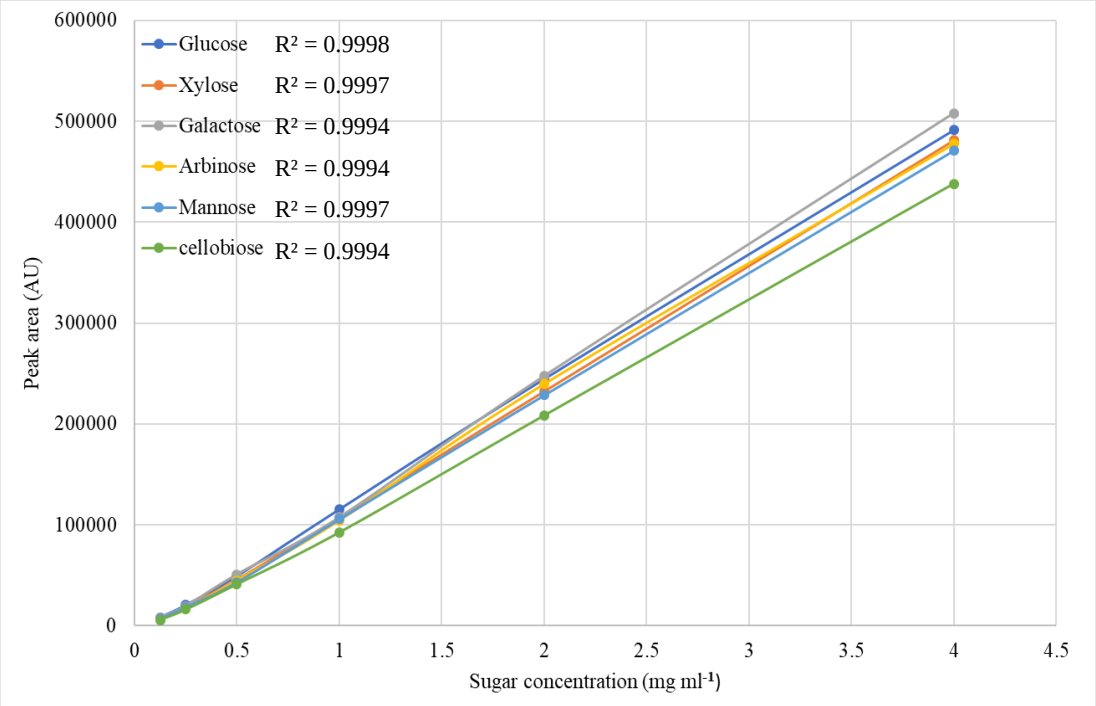


Figure 3-1. Standard sugars calibration curve in the HPLC.

As can be seen from Figure 3-1, the coefficient of determination (R^2) was very high which indicate high precision in the calibration curves for all the sugars. The carbohydrate composition analysis was carried out using the NREL standard procedure (Sluiter et al., 2010). In abridgement, 300 mg of dried WS was hydrolyzed using 3 mL of 72% H_2SO_4 for 60 min at 30 ºC. The samples were then diluted with 84 mL of deionized water to obtain a 4% concentration of H_2SO_4. The diluted samples were then autoclaved at 121 ºC for another 60 min. After autoclaving finished, 50 mL of the hydrolysis liquor was used to measure the acid soluble lignin by UV-visible. As for the carbohydrates, the hydrolysis liquor pH was neutralized using calcium carbonate ($CaCO_3$) to pH (5.0 – 6.0). The samples were then centrifuged and filtrated prior to HPLC analysis. The WS was analysed twice and the monosaccharide sugars mass are illustrated in Table 3-2.

Table 3-2. Wheat straw monosaccharide sugars mass in the 300 mg of the raw WS (harvest in summer 2017).

Monosaccharide sugars	**Mass (mg)**		
	Run 1	**Run 2**	**Average**
Glucose	103.2	104.4	103.8 ± 0.84
Xylose	64.7	63.1	63.9 ± 1.10
Galactose	12.8	11.8	12.3 ± 0.70
Arabinose	33.1	32.7	32.9 ± 0.28
Mannose	13.3	16.1	14.7 ± 1.90
cellobiose	0	0	0
Total	227.1	228.1	227.6 ± 0.7

The sugars mass values were reported based on the original 300 mg of raw WS used for analysis. The results indicate that glucose is the main sugar in the WS followed by xylose. On the other hand, there is no cellobiose appeared in the WS analysis.

Insoluble lignin, acid soluble lignin and ash content were calculated as described in Chapter 2 (Section 2.5.1).

To specify the WS structure, the hexoses sugars (glucose, galactose and mannose) were counted as cellulose, whilst pentose sugars (xylose and arabinose) were counted as hemicellulose. The WS compositions analysis is reported in Table 3-3 as weight percentages. Figure 3-2 demonstrate the HPLC analysis of the standard sugars and the WS sample retention times.

Table 3-3. Composition analysis of the wheat straw (harvest in summer 2017).

Component	**(wt/wt %)**
Cellulose	43.6 ± 2.4
Hemicellulose	32.2 ± 4.5
Lignin	18.8 ± 3.1
Ash and others	5.4 ± 3.9
Total	100

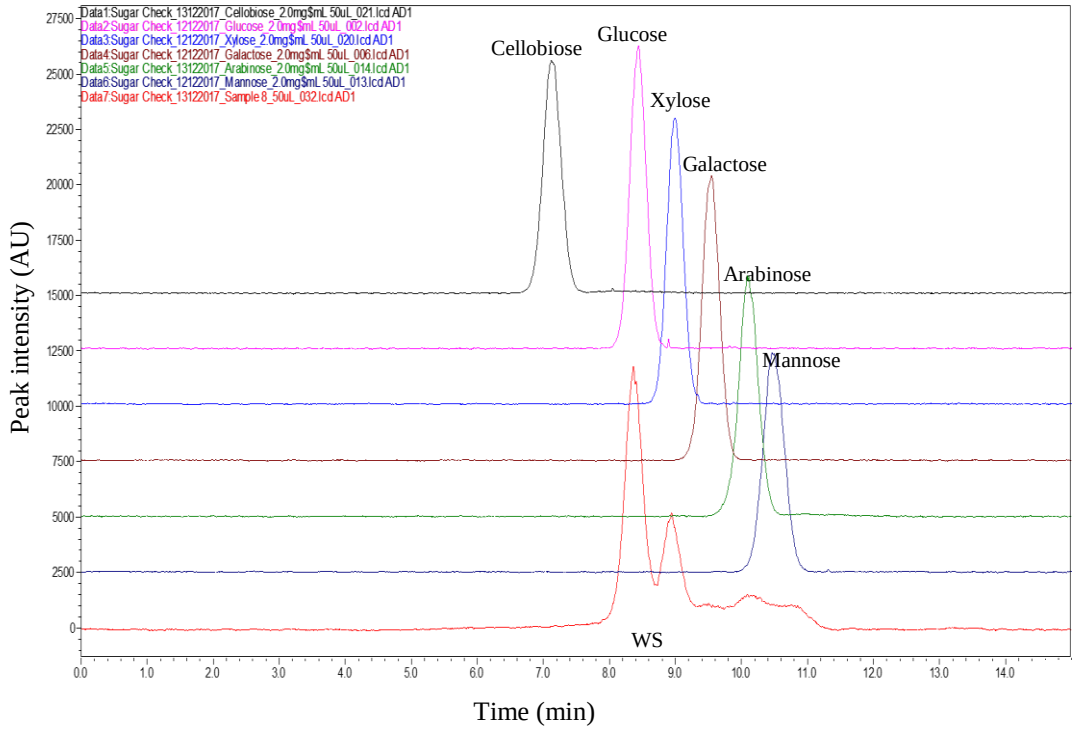


Figure 3-2. Standard sugars and the WS chromatography.

3.1.2 Total Reduced Sugar yield and Carbohydrate Analysis after Enzymatic Hydrolysis

The total reduced sugar concentration after EH was determined by the means of 3,5-Dinitrosalicylic acid (DNS) indicator (Miller, 1959). The DNS method as described previously (see Chapter 2). In summary, 0.5 mL from the EH aqueous solution was taken every 24 hrs. The sample was boiled for 5 min, cooled down to room temperature before adding 3 mL of DNS indicator and 0.5 of sodium citrate buffer. The solution was then boiled for 5 min to allow the DNS to react with the reduced sugars followed by cooling down with an ice bath to the room temperature.

Prior to EH, calibration curve equation for both glucose and xylose was established by plotting known concentrations against absorbance 540 nm. Figures 3-3 and 3-4 display the standard calibration curve with the linear equation for glucose and xylose, respectively.

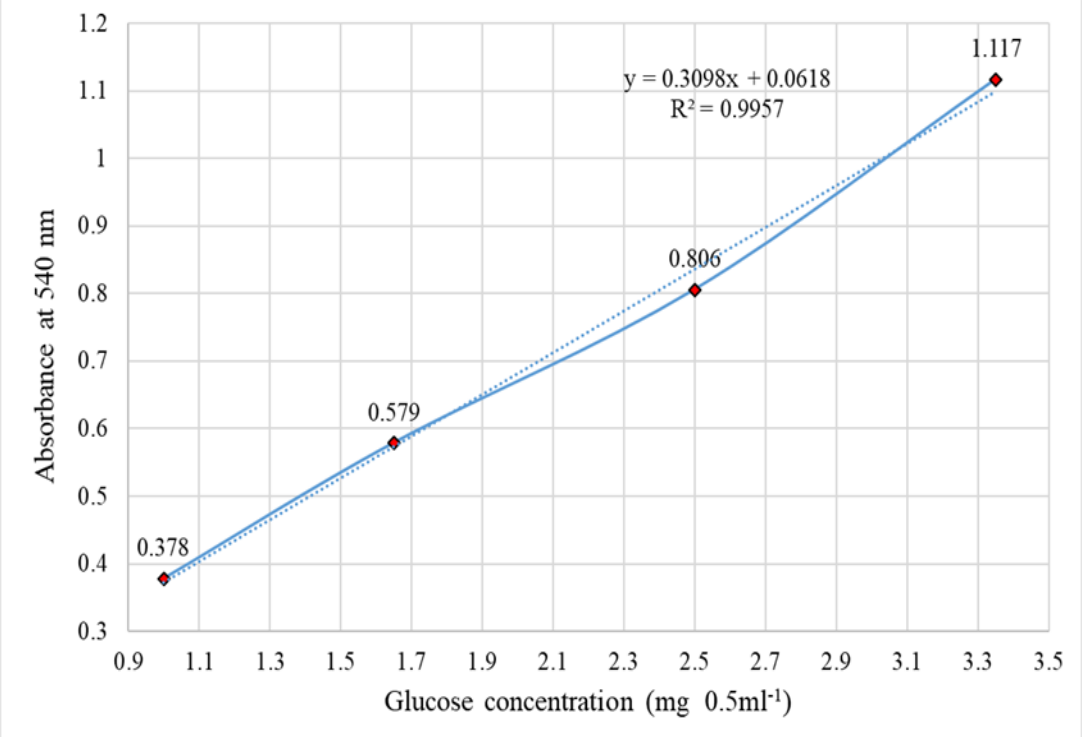


Figure 3-3. Standard calibration curve for the glucose.

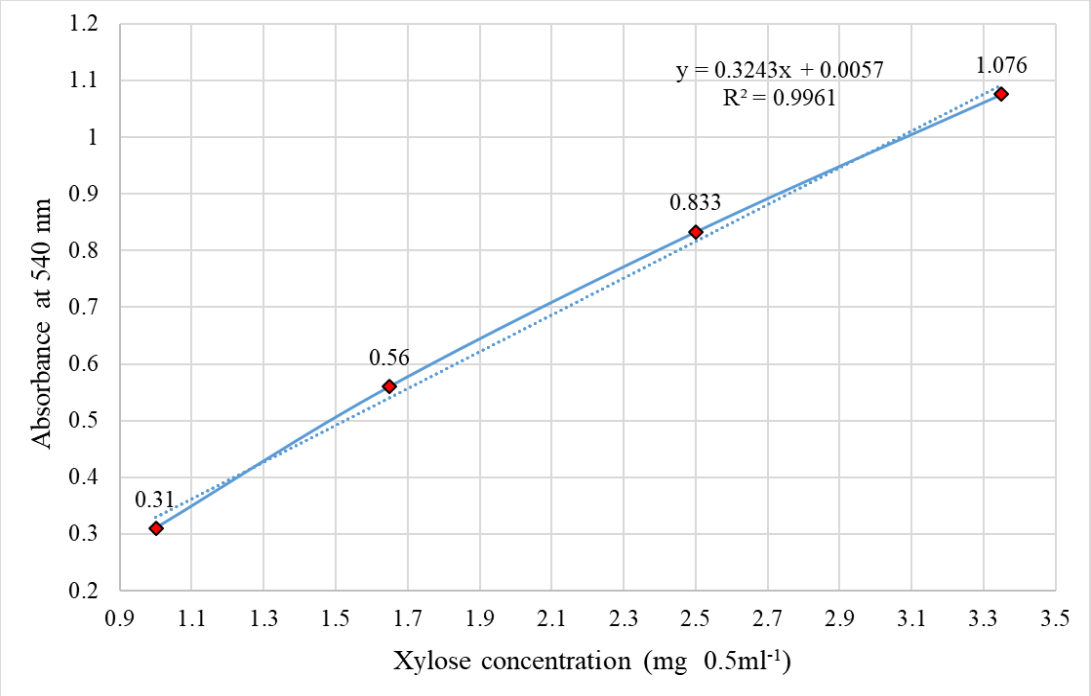


Figure 3-4. Standard calibration curve for the xylose.

As it can be seen from Figures 3-3 and 3-4 that the R^2 was near 1 which indicates a reliably best fit a linear equation to be used to quantify the total reduced sugars liberated during EH. Furthermore, both glucose and xylose linear calibration equation showed a great similarity. Since glucose is the major monosaccharide sugar produced during EH, therefore glucose standard calibration curve was used to quantify the total reduced sugars for all the samples. Xylose standard calibration equation was only used when EH was carried by using endo-1, 4-β-Xylanase enzyme in the pH effect section (see Chapter 4). In general, the DNS method was used to evaluate the pre-treatment methods by monitoring the total reduced sugar yield produced after EH. Selected samples were further analysed to identify the monosaccharides sugars with GC-MS.

3.1.3 Ethanol Yield

After pre-treatment and EH, the liberated hexose sugars were fermented to ethanol using *Saccharomyces Cerevisiae* yeast. Ethanol concentration after fermentation was measured using GC (see Chapter 2).

Various concentrations of ethanol in propanol solution were made to create the ethanol/propanol calibration curve. The solutions were tested with GC and the peak area for ethanol, propanol and the peak area ratio between them was calculated. The different ethanol/propanol solution concentrations were tested twice and the average ratio was calculated. Figure 3-5 shows the standard calibration curve of ethanol virus average peak area ratio and the line equation.

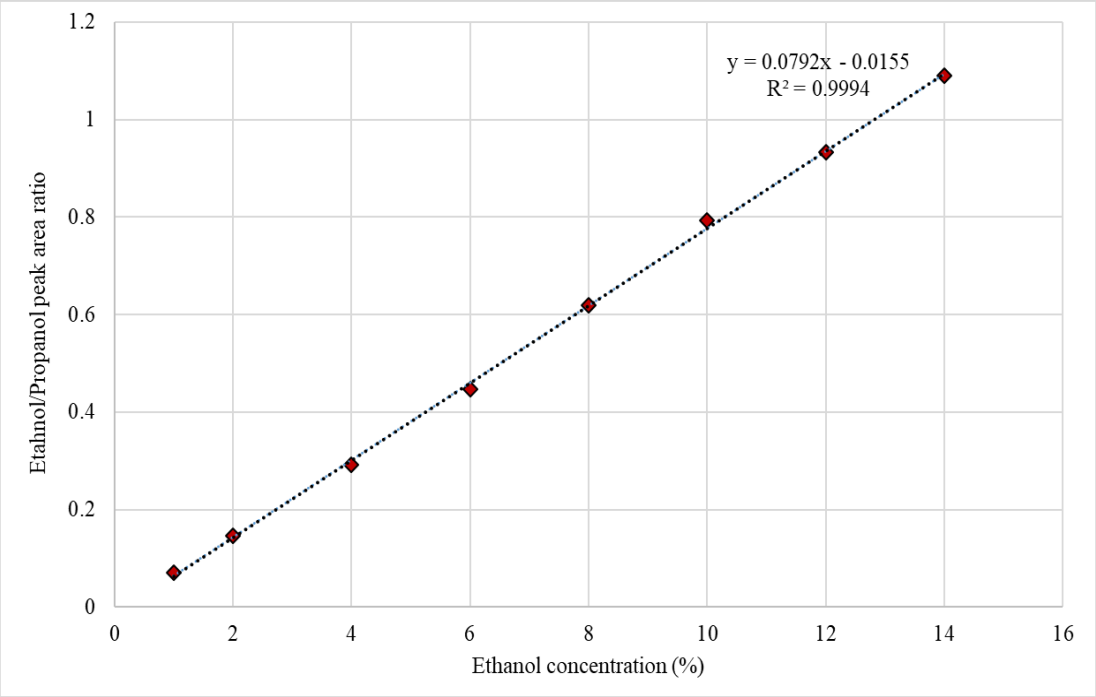


Figure 3-5. Ethanol VS Ethanol/Propanol peak area ratio.

It can be noticed from Figure 3-5 that R^2 is 0.9994, which indicates a high reliability for the linear equation which were used to calculate the ethanol concentration after fermentation.

3.2 Grinding Pre-treatment

3.2.1 Grinding and Sieving

To investigate the particle size and surface area effects on total reduced sugar concentration during the EH process, the WS was ground using WALDNER grinder (see Chapter 2). The gap between the two ceramic disks was adjusted to obtain a

particle size ranged between < 250 µm to > 2000 µm. The ground WS was sieved to establish homogenise sample. Figure 3-6 presents the range of WS with a different particle sizes after grinding and sieving.

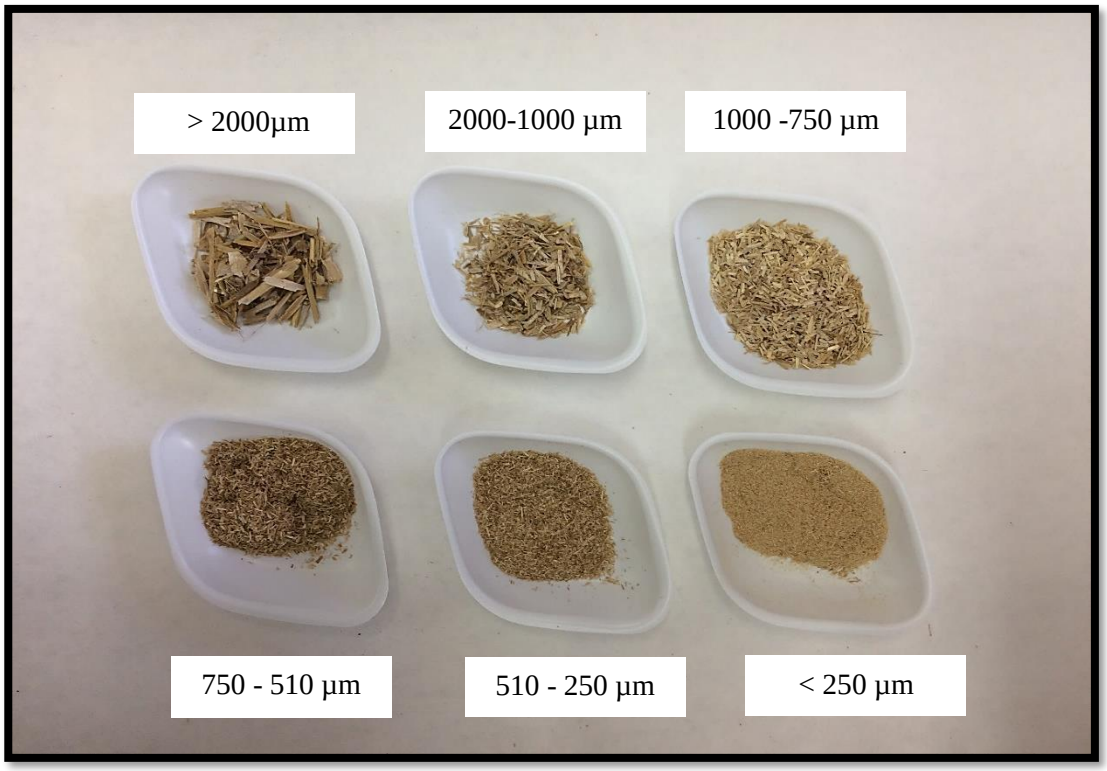


Figure 3-6. Different particle sizes of the grounded wheat straw.

3.2.2 Enzymatic Hydrolysis

The ground WS samples were subjected to EH according to the procedure described in Chapter 2. In summary, 1 g of the WS was submerged in 50 mL of sodium citrate buffer 0.05 M and pH 4.8. Three sets of enzymes including Celluclast 1.5L (15 FPU g^{-1} DM) with 22.5 CBU g^{-1} Novozymes 188, endo-1, 4-β-Xylanase (1540 IU g^{-1} DM) and Cellic CTec2 15 FPU g^{-1} DM were used to perform the EH. The EH process was carried out for 94 hrs. Samples from the hydrolysis solution were taken every 24 hrs to measure the total reduced sugars concentration. All the EH experiments were carried out in triplicate to ensure reproducibility. Figures 3-7, 3-8 and 3-9 show the total reduced sugar concentration with time obtained for the diverse particle sizes

hydrolysed by Celluclast 1.5L with Novozymes 188, endo-1, 4-β-Xylanase and Cellic CTec2, respectively.

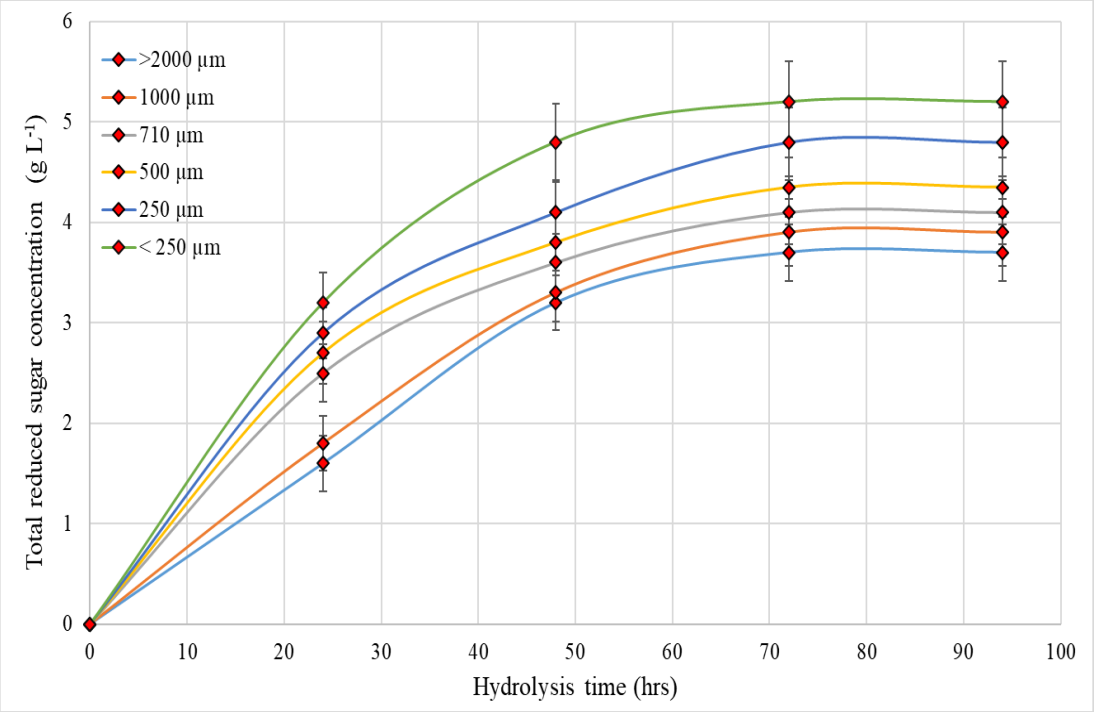


Figure 3-7. Total reduced sugar concertation during EH (using Celluclast 1.5 L supplements with Novozymes 188 enzymes).

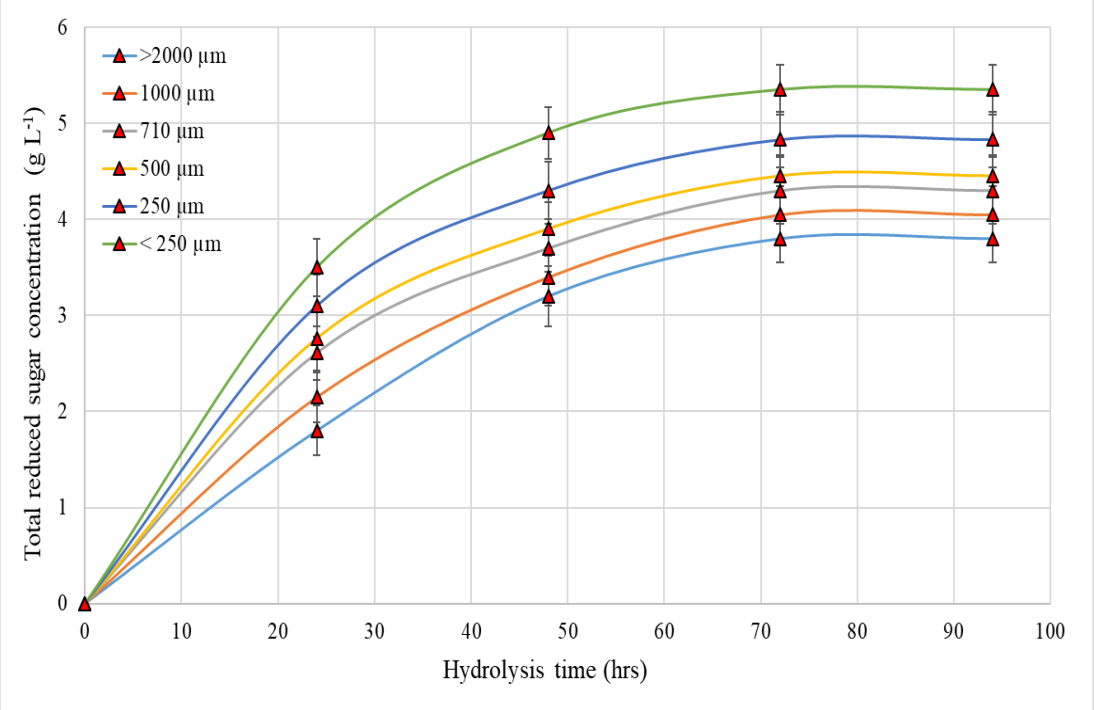


Figure 3-8. Total reduced sugar concentration during EH (using endo-1, 4-β-Xylanase enzyme).

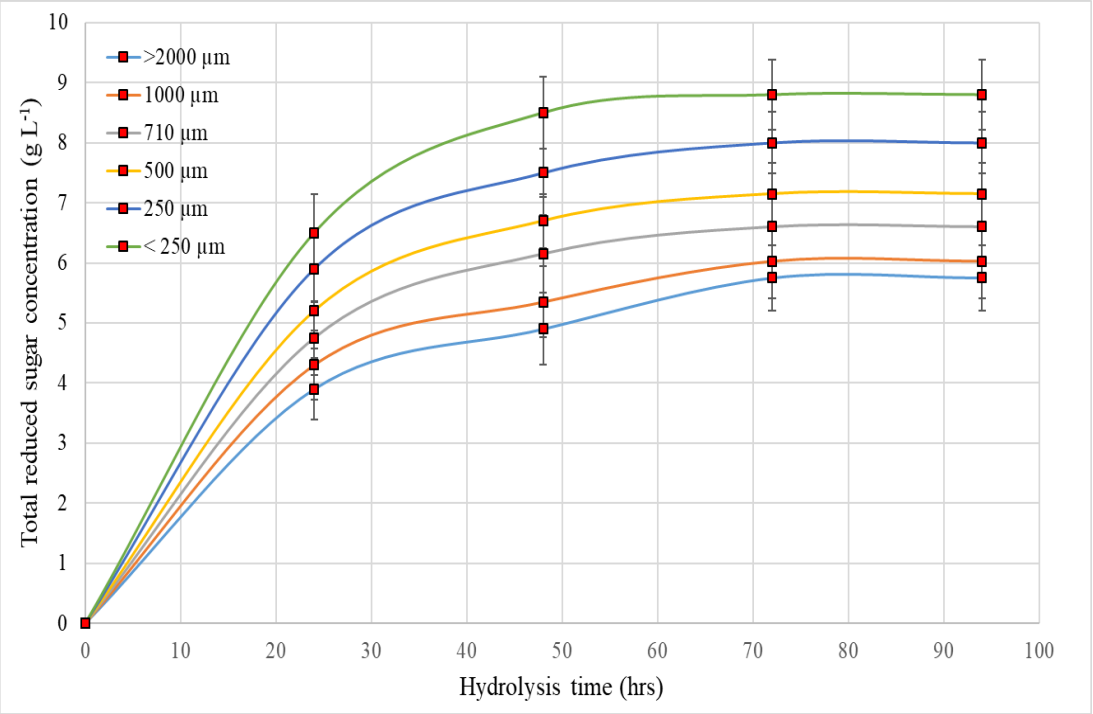


Figure 3-9. Total reduced sugar concentration during EH (using Cellic CTec2 enzyme).

As it can be observed from Figures 3-7, 3-8 and 3-9, similar behavior in all samples was observed where the total reduced sugar concentration increased rapidly with time up to about 50 hrs then it begins to level out. After 72 hrs, there was no significant increase in the total reduced sugar concentration for all the samples. 72 hrs is commonly reported as the end of EH (Lan et al., 2013). Furthermore, the total reduced sugar concentration was found to increase with WS particle size reduction as shown in Figures 3-7, 3-8 and 3-9. Grinding and milling the WS to smaller particle sizes results in increasing the WS surface area which consequently reduces the crystallinity degree of the WS. As a result, enzymes will have more accessibility to cellulose and hemicellulose during EH and therefore the total reduced sugar yield enhanced (Silva et al., 2012).

The total reduced sugar yield percentage was calculated based on the theoretical sugars available in the WS using Equation 3.1. The total reduced sugar yield at the end of EH for all the samples are listed in Table 3-5.

Total reduced sugar yield (%) =

$$\frac{\text{wieght of sugars released after EH}}{\text{wieght of theoretical sugars availabe in WS}} \times 100\ \% \qquad (3.1)$$

Table 3-4. Total reduced sugar yield at the end of the EH process (72 hrs) at pH 4.8.

Enzymes type	**Particle size (µm)**	**Total reduced sugar yield (wt/wt %)**
Celluclast 1.5L with Novozymes 188	> 2000	24.4 ± 2.8
	200-1000	25.7 ± 3.4
	1000-710	27.0 ± 3.2
	710-500	28.7 ± 3.1
	500-250	31.7 ± 3.4
	< 250	34.0 ± 4.1
endo-1, 4-β-Xylanase	> 2000	25.0 ± 2.5
	200-1000	26.7 ± 3.4
	1000-710	28.4 ± 3.5
	710-500	29.4 ± 2.2
	500-250	31.9 ± 2.9
	< 250	35.3 ± 2.6
Cellic CTec2	> 2000	37.9 ± 5.4
	200-1000	39.8 ± 6.2
	1000-710	43.5 ± 5.8
	710-500	47.2 ± 5.2
	500-250	52.8 ± 5.1
	< 250	58.0 ± 5.5

Table 3-5 demonstrated that reducing the WS particle size will increase the total reduced sugar yield for all enzymes cocktails. Moreover, Cellic CTec2 gives the

highest total reduced yield compared to Celluclast 1.5L with Novozymes 188 and endo-1, 4-β- Xylanase enzymes. WS particle size is a fundamental parameter that affects biomass digestion efficiency. Hu et al (2017) suggested that grinding the WS to a fine size will breaks down the lignin as well as increase the surface area of the sample, therefore, the enzymes will have more accessibility and facilitate biomass digestion.

The results in Table 3-5 showed that the total reduced sugar yield have an inverse relationship with the particle size. These findings are consisted with a similar study carried by Silva et al (2012) where a yield of 36% was gained from WS ground to 100 µm. In Table 3-5, a higher yield of 58% was observed from < 250 µm sample when it was subjected to EH by using Cellic CTec2 enzyme. Moreover, (Silva et al., 2012) concluded that further grinding and size reduction will not improve the total sugars yield unless the WS internal structure is altered by using ball milling.

In another study conducted by Pedersen and Meyrer (2009), glucose and xylose yield after EH increased with the WS size reduction. For particle size ranged (53 – 149) µm, glucose and xylose yield increased by 39% and 20%, respectively compared to ungrounded WS (2 – 4) mm. However, it was suggested that a combination of grinding with wet oxidized pre-treatment will further enhance EH. The sample with a particle size of (53 – 149) µm was pre-treated with wet oxidized and the yield of glucose and xylose was found to be 90% and 39%, respectively (Pedersen and Meyer, 2009).

In general, mechanical pre-treatment have the ability to increase the total reduced sugar yield up to 50%. Moreover, the energy required to mill the biomass to very fine particle sizes is considered high (Mani et al., 2004). Therefore, it is common to combine mechanical pre-treatment with other types of pre-treatment such as acid,

alkaline, microwave, LHW, wet oxidized to establish higher sugar yield and reduce grinding energy consumption (Pedersen and Meyer, 2009, Vidal et al., 2011).

The results in Table 3-5 clearly indicate that size reduction increases the sugars yield. The WS with the lowest particle size of (< 250 μm) which gave the highest yield was selected to be used as raw material in Chapter 4 for the pH optimization during EH and in Chapter 5 for the microwave pre-treatment.

3.2.3 Fermentation

The EH solution obtained from using Celluclast 1.5L with Novozymes 188, endo-1, 4-β- Xylanase and Cellic CTec2 enzymes on the < 250 μm sample was fermented.

After EH, the solution pH was adjusted to 6.0 before adding *Saccharomyces Cerevisiae* yeast. The fermentation process is described in details (see Chapter 2). Since the *S. Cerevisiae* yeast has the ability to ferment hexose (C6) sugars only. Therefore, the aqueous solutions after EH were analysed with GC-MS to determine the monosaccharide sugars. The main hexose sugar was found to be glucose. The total reduced sugars and hexose sugars concentration after EH as well as the ethanol concentration after fermentation obtained from different enzymes cocktails are listed in Table 3-6.

Table 3-5. The total reduced sugar and hexose sugars concentrations after EH and the ethanol production from the WS with particle size of < 250 μm.

Enzymes	**Total reduced sugar concentration ($g\ L^{-1}$)**	**Hexose sugars concentration ($g\ L^{-1}$)**	**Ethanol concentration ($g\ L^{-1}$)**
Celluclast 1.5L with Novozymes 188	5.2 ± 0.4	4.0 ± 0.1	1.84 ± 0.1
endo-1, 4-β-Xylanase	5.4 ± 0.26	3.7 ± 0.4	1.73 ± 0.2
Cellic CTec2	8.8 ± 0.58	6.6 ± 0.2	3.2 ± 0.2

Since *S. Cerevisiae* yeast can ferment C6 sugars only, ethanol yield calculation was based on the available hexose after EH. As shown in Table 3-6, the ethanol produced from Celluclast 1.5L with Novozymes 188 enzymes sample is 1.84 g L^{-1} which represent 90% of the available hexose liberated after EH to ethanol. Similar ethanol yield was reached from endo-1, 4-β- xylanase with 91%. The highest ethanol yield of 95% was established when Cellic CTec2 was used for EH in which hexose and ethanol concentration after EH and fermentation are 6.6 g L^{-1} and 3.2 g L^{-1}, respectively.

It was reported in several studies that *S. Cerevisiae* yeast is the best microorganism to ferment hexoses sugars and the ethanol yield can be as high as 90- 99% (Claassen et al., 1999, Jørgensen, 2009). On a study conducted by Da Silva et al (2010), *S. Cerevisiae* and modified C6/C5 yeast were used for fermentation. The ethanol yield from *S. Cerevisiae* and modified C6/C5 were found to be 91.8% and 78%, respectively (da Silva et al., 2010). In other studies done by Saha et al (2005) and Saha et al (2007), the ethanol yield from WS pre-treated with lime and acid using *S. Cerevisiae* ranged in 85-92%.

The aqueous solution after EH and after fermentation was analysed for sugars trace with GC-MS. Figure 3-10 shows the GC for sugars trace after EH and after fermentation of the sample hydrolysed with Cellic CTec2 enzyme. The GC clearly shows that most of glucose and galactose were fermented since their peaks disappeared after fermentation. On the other hand, it can be seen that the xylose is still in the solution after fermentation due to *S. Cerevisiae* yeast fermenting limitation.

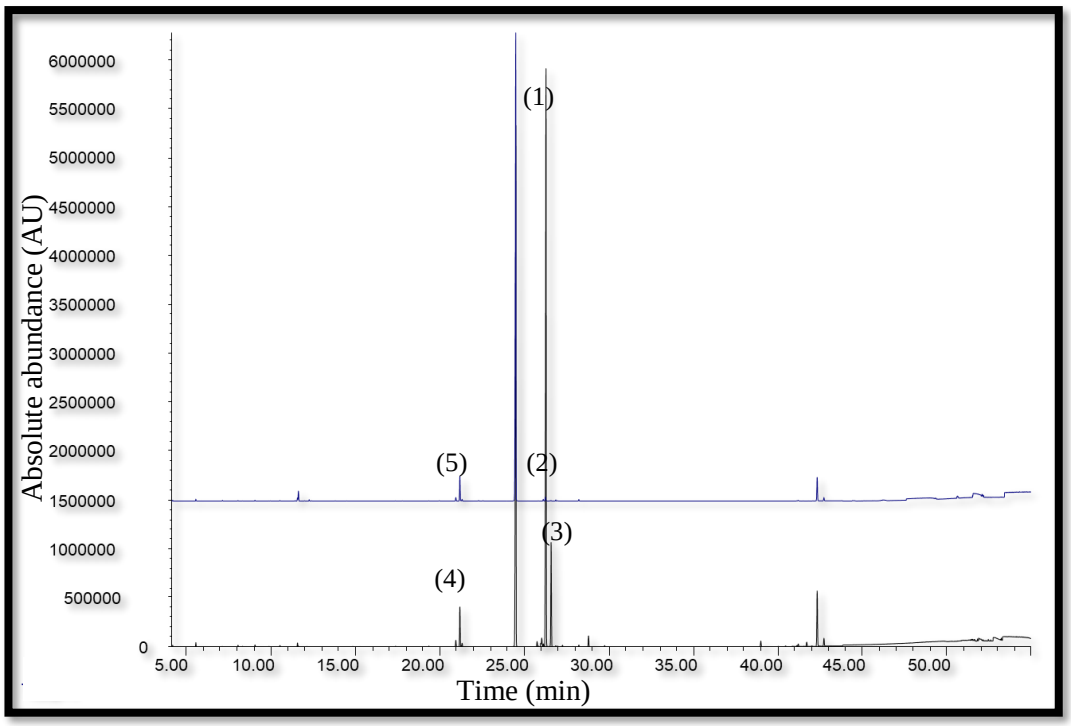


Figure 3-10. Monosaccharide sugars peak after EH using Cellic CTec2 enzyme @ pH 4.8 and after fermentation for (<250 µm) sample. (1) glucose after EH, (2) glucose after fermentation, (3) galactose after EH, (4) xylose after EH and (5) xylose after fermentation.

3.2.4 Summary

In this chapter, the effect of WS particle size on the total reduced sugar yield after EH was investigated. The WS was ground to a range of particle sizes (> 2000 - < 250 µm). The total reduced sugar yield showed a proportional relationship with the particle size. The EH process was carried out using Celluclast 1.5L supplement with Novozymes 188, endo-1, 4-β-Xylanase and Cellic CTec2 enzymes. The WS with a particle size of < 250 µm showed the highest total reduced sugar yield of 34.0 ± 4.1%, 35.3 ± 2.6% and 58.0 ± 5.5% after applying EH using Celluclast 1.5L supplement with Novozymes 188, endo-1, 4-β-Xylanase and Cellic CTec2 enzymes, respectively. The lowest total reduced sugar yield was obtained for the WS with a particle size of > 2000 µm. Furthermore, it was found and concluded that carrying out the EH beyond 72 hrs has no significant effect on the final total reduced sugar yield because all the accessible cellulose and hemicellulose to enzymes were hydrolysis. The EH solutions of the WS

with a particle size of < 250 µm was fermented using *S. Cerevisiae* yeast. The glucose conversation to ethanol yield of 90%, 91% and 95% were achieved from the EH solution of the Celluclast 1.5L supplement with Novozymes 188, endo-1, 4-β-Xylanase and Cellic CTec2 enzymes, respectively.

Chapter Four: Study The Effect of pH on the Sugar Yield during Enzymatic Hydrolysis

4.1 Introduction

The pH effects on the enzyme's activity were chosen to be evaluated due to the possibility of improving the EH in terms of the sugars yield. For the pH effects study, the finest WS (< 250 µm) sample in Chapter 3 was selected since it produced the highest sugars yield with all the three enzymes cocktails comparing to the other samples. The three enzymes cocktails Celluclast 1.5L supplement with Novozymes 188, endo-1, 4-β-Xylanase and Cellic CTec2 were subjected to the pH evaluation.

The majority of studies conducted on the EH of lignocelluloses using Trichoderma reesei (i.e Celluclast 1.5L) are performed at pH 4.8 and at a temperature of approximately 50 °C. These conditions were considered as the optimum condition for hydrolysis based on laboratory enzyme activities using model substrates, i.e., pure cellulose (Lan et al., 2013). The condition used for EH with endo-1, 4-β-Xylanase are quite similar to those commonly reported for Celluclast 1.5L with Novozymes 188, which include a temperature of 50 °C and pH 4.8-5.0 (Yang et al., 2015, Maitan-Alfenas et al., 2015). Similarly, although the recommended pH range for Cellic CTec2 by Sigma Aldrich (Novozymes) is 5.0 – 5.5, pH 4.8 or 5.0 is the most commonly reported in the literature (Procentese et al., 2017, Sun et al., 2018).

Celluclast 1.5L with Novozymes 188 and Cellic CTec2 are among the most used enzymes for cellulose hydrolysis, Whilst endo-1, 4-β-Xylanase is for hemicellulose hydrolysis (Avci et al., 2013, Oladi and Aita, 2018, Jørgensen et al., 2007, Kumar et al., 2008).

Since the main objective in this section is to investigate the effect of changing the pH on EH, therefore pH values of the EH solution were measured before and after addition of the enzymes to ensure a fair comparison. Sodium Citrate (0.05 M) buffer with different pH values was prepared and the required pH was obtained by using HCl (1 M) and NaOH (1 M). The WS was suspended with the buffer solution and 0.02% wt/wt NaN_3 was added. The pH was measured before and after adding the enzymes. EH was done according to the same method described previously in Chapter 2. The experiment was done three times and the average results with the STD were reported. The measured pH values are reported in Table 4-1 for both before and after the addition of the enzymes to the aqueous solution (buffer solution and WS). Since the pH increased as a result of adding the enzymes, the solution's pH was adjusted back to the original pH value. This is reported as pH-adjusted in Table 4-1.

It was found that at low pH values, the shifting in the pH value was higher after adding the enzymes than at high pH values for all the enzymes cocktails due to the low acidity of the enzymes (pH 6.0-6.5). The highest increase in the pH value was recorded after adding Cellic CTec2 to pH 3.0 solution in which the pH value increased from 3.0 to 3.61. On the other hand, the lowest change recorded after adding the Cellic CTec2 to pH 7.0 solution, the increase was very small and was neglected. The adjusted pH values were used as the pH at the start of the EH (0 hr).

Table 4-1. pH values before adding the enzymes, after adding the enzymes and after adjusting.

	pH- After adding the enzymes			pH- Adjusted		
pH before adding the enzymes	**Celluclast 1.5L + Novozymes 188**	**endo-1, 4-β-Xylanase**	**Cellic CTec2**	**Celluclast 1.5L + Novozymes 188**	**endo-1, 4-β-Xylanase**	**Cellic CTec2**
3.00	3.55 ± 0.1	3.41 ± 0.07	3.61 ± 0.08	3.00 ± 0.01	2.99 ± 0.01	3.00 ± 0.01
3.50	3.96 ± 0.07	3.85 ± 0.05	4.00 ± 0.1	3.49 ± 0.01	3.5 ± 0.01	3.50 ± 0.01
4.00	4.40 ± 0.08	4.32 ± 0.08	4.42 ± 0.07	4.05 ± 0.01	4.02 ± 0.01	4.00 ± 0.01
4.50	4.79 ± 0.05	4.79 ± 0.06	4.70 ± 0.09	4.52 ± 0.01	4.49 ± 0.01	4.50 ± 0.01
4.80	5.10 ± 0.07	5.10 ± 0.06	5.20 ± 0.05	4.80 ± 0.01	4.79 ± 0.01	4.80 ± 0.01
5.00	5.24 ± 0.07	5.20 ± 0.06	5.18 ± 0.05	5.00 ± 0.01	5.00 ± 0.01	5.03 ± 0.01
5.50	5.70 ± 0.05	5.68 ± 0.04	5.63 ± 0.03	5.50 ± 0.01	5.49 ± 0.01	5.48 ± 0.01
5.70	5.88 ± 0.05	5.81 ± 0.02	5.80 ± 0.03	5.69 ± 0.01	5.70 ± 0.01	5.70 ± 0.01
6.00	6.15 ± 0.02	6.15 ± 0.02	6.13 ± 0.03	6.01 ± 0.01	6.00 ± 0.01	5.99 ± 0.01
6.30	6.43 ± 0.04	6.40 ± 0.02	6.39 ± 0.02	6.30 ± 0.01	6.29 ± 0.01	6.29 ± 0.01
6.50	6.60 ± 0.02	6.59 ± 0.01	6.55 ± 0.02	6.49 ± 0.01	6.51 ± 0.01	6.49 ± 0.01
6.70	7.10 ± 0.02	6.77 ± 0.01	6.75 ± 0.03	7.00 ± 0.01	6.69 ± 0.01	7.00 ± 0.01
7.00	7.20 ± 0.03	7.05 ± 0.01	7.03 ± 0.01	6.99 ± 0.01	7.00 ± 0.01	7.03 ± 0.01

4.2 pH Effect on Celluclast 1.5 L with Novozymes 188

To assign the pH value to its corresponded total reduced sugar, the pH at the start (0 hr) and at the end (72 hrs) of the EH were measured and reported in Figure 4-1.

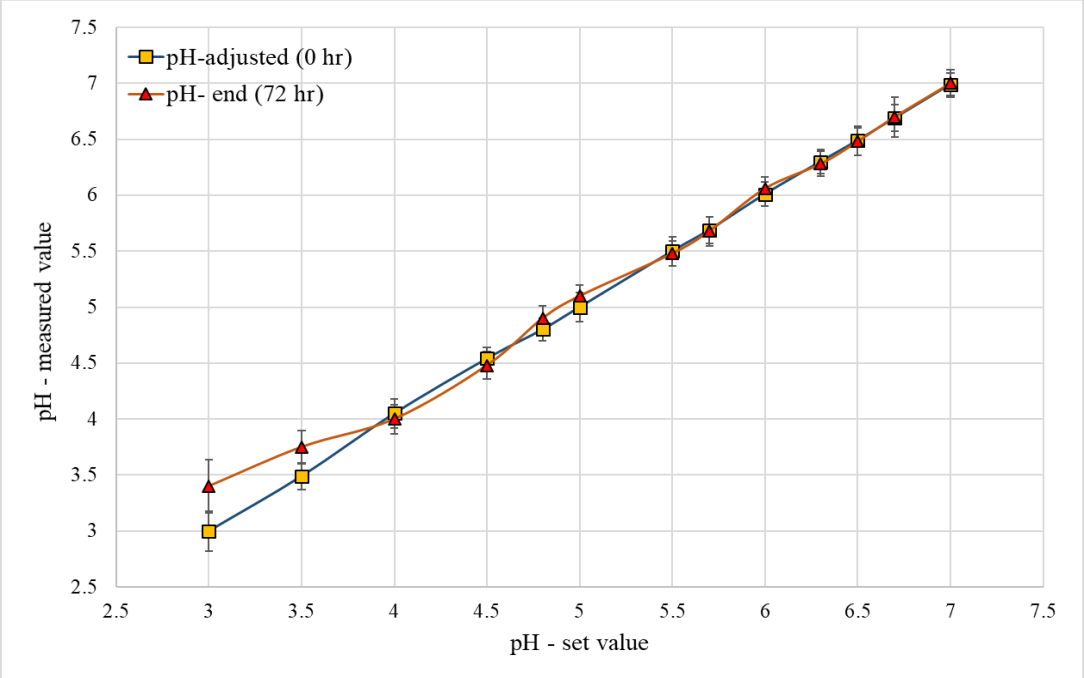


Figure 4-1. pH data at the beginning (0 hr) and the end (72 hrs) of EH using (Celluclast 1.5L with Novozymes 188) enzymes.

As it can be noticed for Figure 4-1 that for Celluclast 1.5 l with Novozymes 188 enzymes, there was a minor increase in pH at the end of the EH for most of the samples except the samples with pH 3.0 and 3.5. The pH 3.0 sample shows the maximum difference in the pH at the end of EH with only were less than 0.17%.

Although the pH 3.0 and 3.5 samples show an increase in the pH value however the difference is not very high and is within the error bar. Therefore, the adjusted pH value at (0 hr) and the final pH measured at each different pH point studied show no significant difference and can be assumed to be the same.

The EH was carried for 72 hrs and the total reduced sugar concentration for each pH are shown in Figure 4-2.

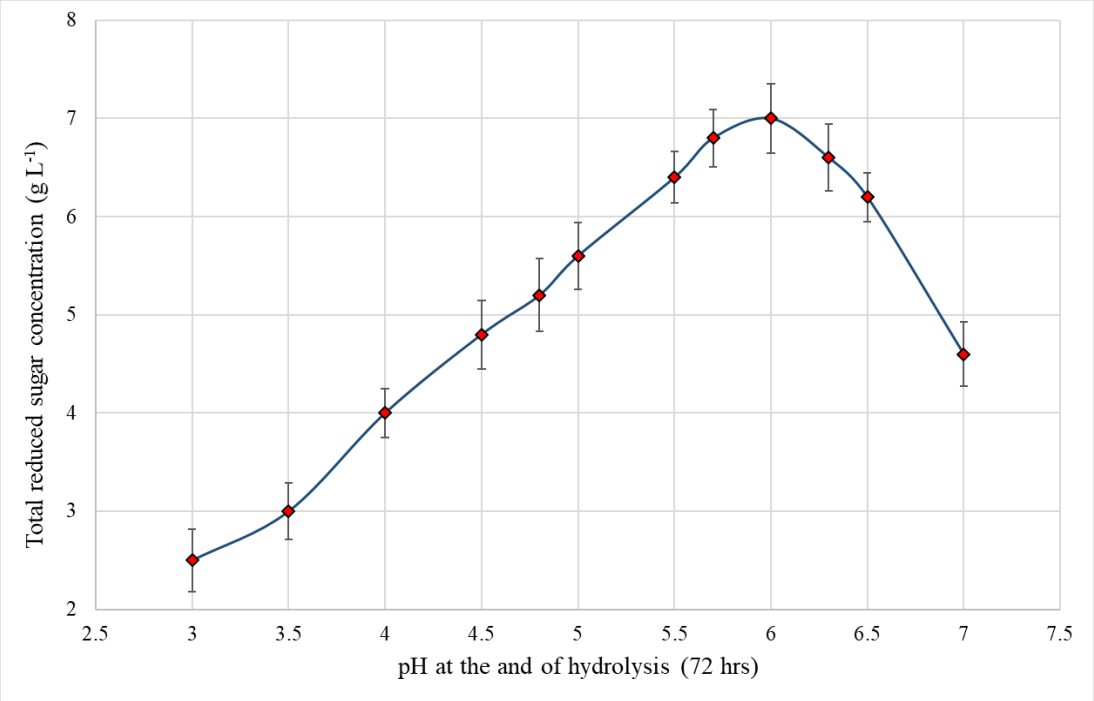


Figure 4-2. Total reduced sugars concentration for different pH solution at the end of the EH using (Celluclast 1.5L with Novozymes 188).

As can be seen from Figure 4-2, the highest reduced sugar yield was achieved between pH 5.5 – 6.3 rather than at 4.8 as cited by most researchers (Lan et al., 2013). The total reduced sugar concentration after EH increased from 5.2 g L^{-1} to 7.0 g L^{-1} by changing the pH value from 4.8 to 6.0, respectively.

To give further confirmation, the total reduced sugar concentration was observed between 0 – 72 hrs at pH 4.8 and 6.0 and shown in Figure 4-3. The results clearly indicate that the total reduced sugar concentration after EH for the WS substrate increased from 5.1 g L^{-1} to 7.1 g L^{-1} (approximately 28%) when the EH pH increased from 4.8 to 6.0, respectively.

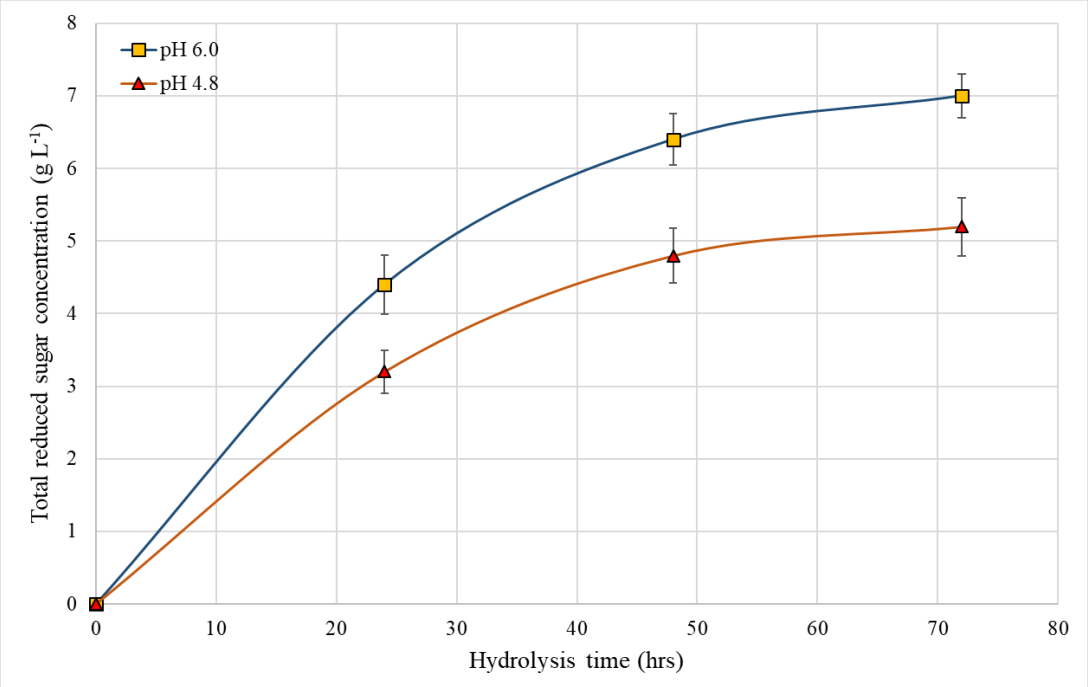


Figure 4-3. Total reduced sugar concentration at the end of hydrolysis process (72 hrs) for pH 4.8 and 6.0 using (Celluclast 1.5L with Novozymes 188).

Similar results were reported by Lan et al (2013). They pre-treated lodgepole pine trees with different pre-treatment methods as a raw material to study the pH effect. They used a mixture of Celluclast 1.5L supplemented with Novozyme 188 (β-glucosidase) and Cellic CTec2 cocktail with a pH range (4.0 – 7.0). The pH effect was evaluated based on the glucose produced after EH. Lan et al (2013) reported that the Celluclast 1.5L and Novozyme 188 enzymes mixture optimum pH is between 5.2 – 5.5 which is slightly lower than pH 5.5 – 6.3 which was reported in the current study. The difference in the optimum pH can be explained by the fact that Lan et al (2013) used lodgepole and reported the glucose yield after EH. Whilst in the current study, WS straw was used and the total reduced sugar yield after EH is reported (Lan et al., 2013).

In contrast, Saha et al (2007) reported that the optimum pH of EH is 5.0 for WS. They studied the effects of both pH (3.5 – 6.5) and temperature (25 – 70 °C) during EH on the total reduced sugars. In their study, enzymes mixture containing Celluclast 1.5L,

Novozyme 188 and Viscostar 150 L (as a xylanase enzyme) were used to perform EH. Based on their results, it was suggested that the optimum pH for EH is 5.0 (Sun et al., 2016b). Adding Viscostar 150 L enzyme to Celluclast 1.5L and Novozyme 188 mixture during EH might be one of the reasons why Saha et al (2007) concluded that the optimum pH is 5.0 instead of 5.8-6.0 which is the finding in the current study. Adding a third enzyme can cause interaction between all the enzymes and might lower the optimum pH to 5.0. Furthermore, Saha et al (2007) pre-treated the WS using lime pre-treatment while in the current study, only size reduction pre-treatment was used to pre-treat the WS. Applying chemical pre-treatment, such as lime during the pre-treatment process may cause lignin and hemicellulose dissolving and altering the biomass structure (Sun et al., 2016b). Therefore there is a possibility for the EH optimum pH to be shifted to a different range.

4.3 pH Effect on endo-1, 4-β-Xylanase

Similar to the previous enzymes, endo-1, 4-β-Xylanase was used for pH evaluation. The EH was carried over a range of pH (3 – 7). The pH of the aqueous solutions were measured at the beginning (0 hr) and the end (72 hrs) of EH. The results are reported in Figure 4-4.

As can be seen in Figure 4-4, the difference in the pH values at (0 hr) and (72 hrs) was very low and can be neglected. The highest increase in pH at (72 hrs) was recorded for the pH 3.0 sample. Nevertheless, the difference in the pH is still very small and is within the error bar and can be neglected.

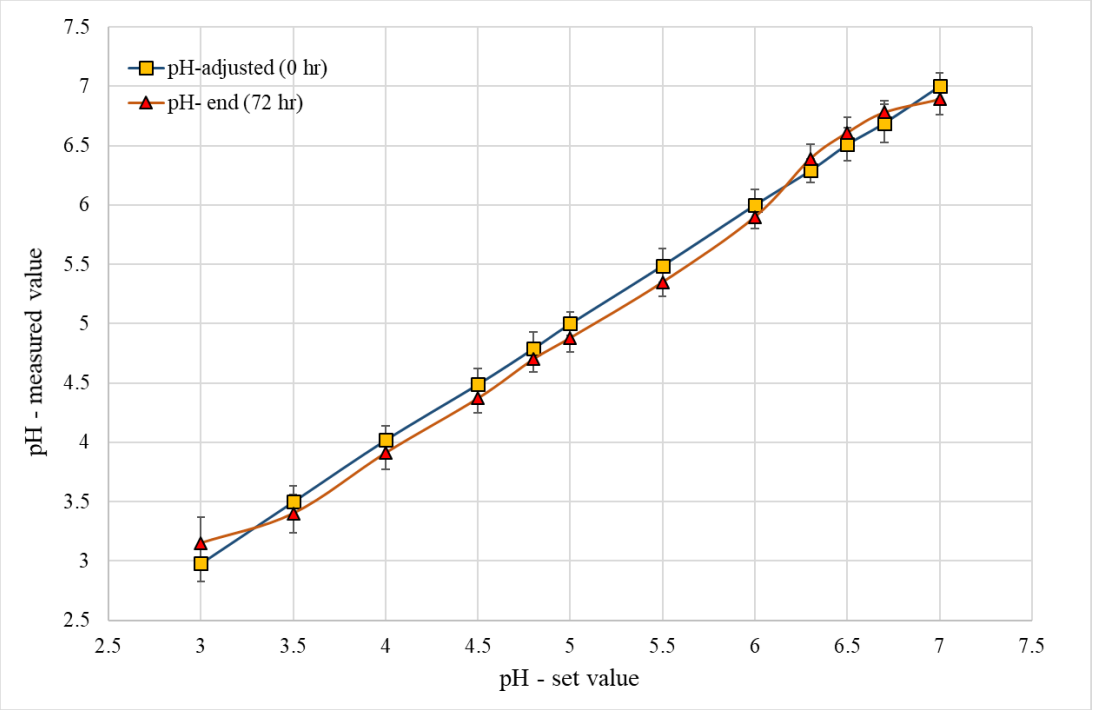


Figure 4-4: pH data at the beginning (0 hr) and the end (72 hrs) of EH using endo-1, 4-β-Xylanase enzyme.

The EH experiments were repeated using the same conditions for the endo-1, 4-β-Xylanase enzyme and the total reduced sugar concentration after EH are as shown in Figure 4-5. Similarly to the previous enzyme cocktail, pH 4.8 – 5.0 is currently the preferred value for EH (Avci et al., 2013). It can be seen that there was a detectable increase in total reduced sugar concentration efficiency from 3.1 g L^{-1} to 7.4 g L^{-1} in the pH range of 3.0 – 6.0. The optimum range was found to be pH 5.7 – 6.0 instead of 4.8 as widely used by researchers.

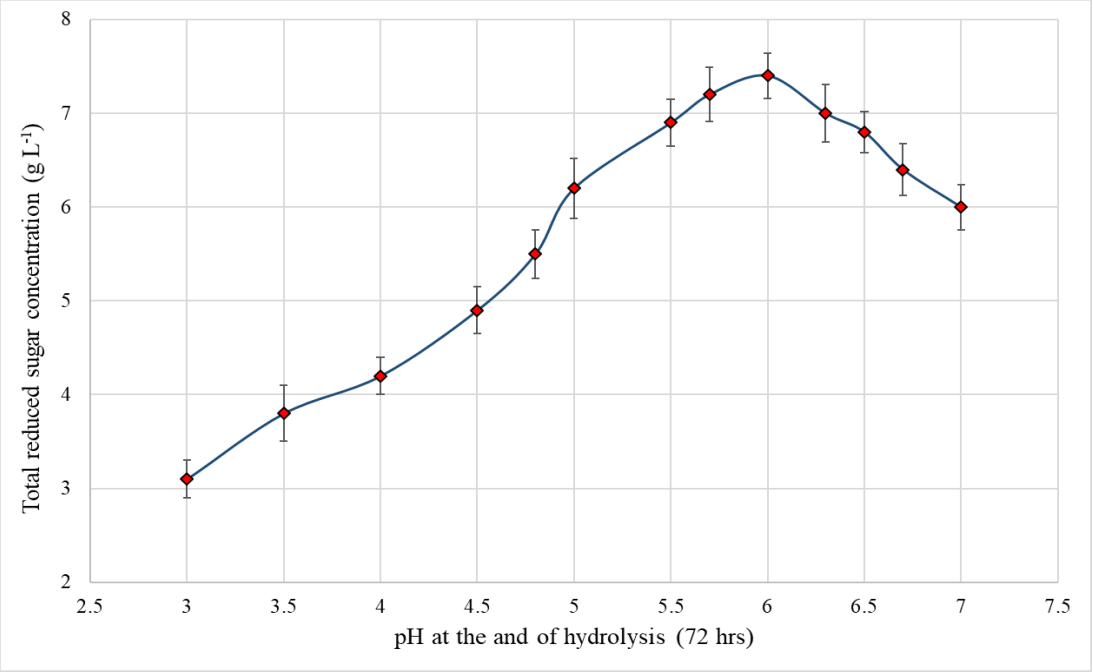


Figure 4-5. Total reduced sugars concentration for different pH solution at the end of the hydrolysis (72 hrs) using endo-1, 4-β-Xylanase.

Figure 4-6 shows the total reduced sugar concentration with time by using endo-1, 4-β- Xylanase for pH 4.8 and 6.0 samples. The total reduced sugar concentration increased from 5.5 g L^{-1} to 7.4 g L^{-1} at the end of EH when the aqueous solution pH increased from 4.8 to 6.0, respectively.

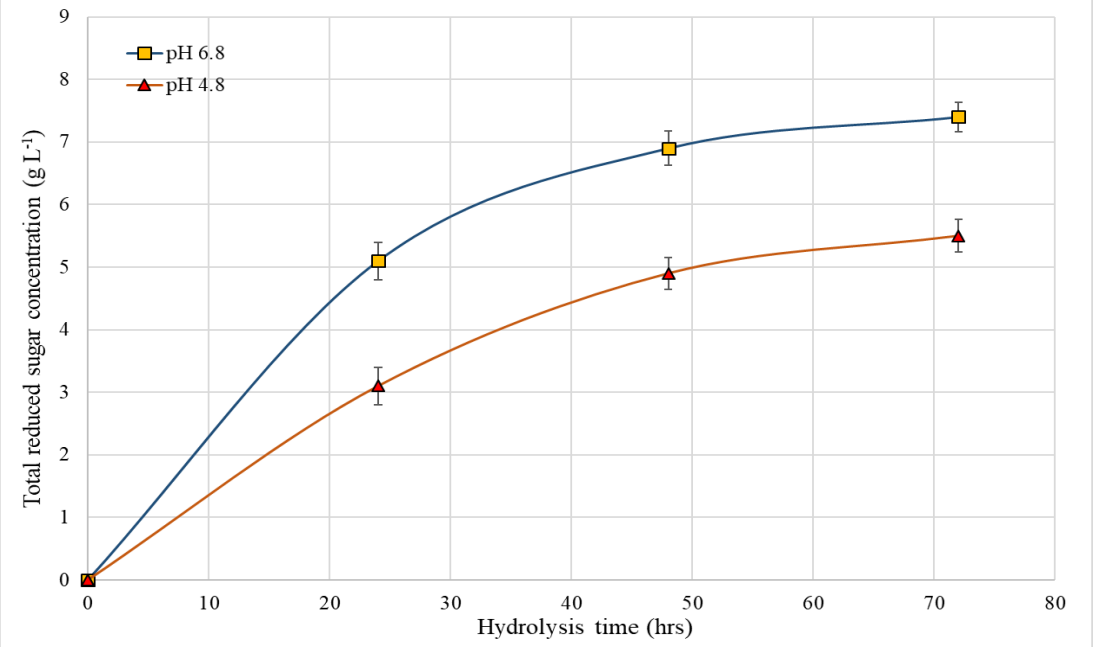


Figure 4-6. Total reduced sugar concentration at (72 hrs) for pH 4.8 and 6.0 using endo-1, 4-β-Xylanase.

4.4 pH Effect on Cellic CTec2

Cellic CTec2 enzyme is a commercial cocktail contains cellulases, ß-glucosidases, and hemicellulose (Sheet). Cellic CTec2 was also subjected to the optimum pH investigation. The WS was enzymatically hydrolyzed under the same experimental conditions as for the previous enzymes (Celluclast 1.5L with Novozymes 188 and endo-1, 4-β-Xylanase). The aqueous solutions pH were also measured at the beginning (0 hr) and the end (72 hrs) of EH. The results are reported in Figure 4-7.

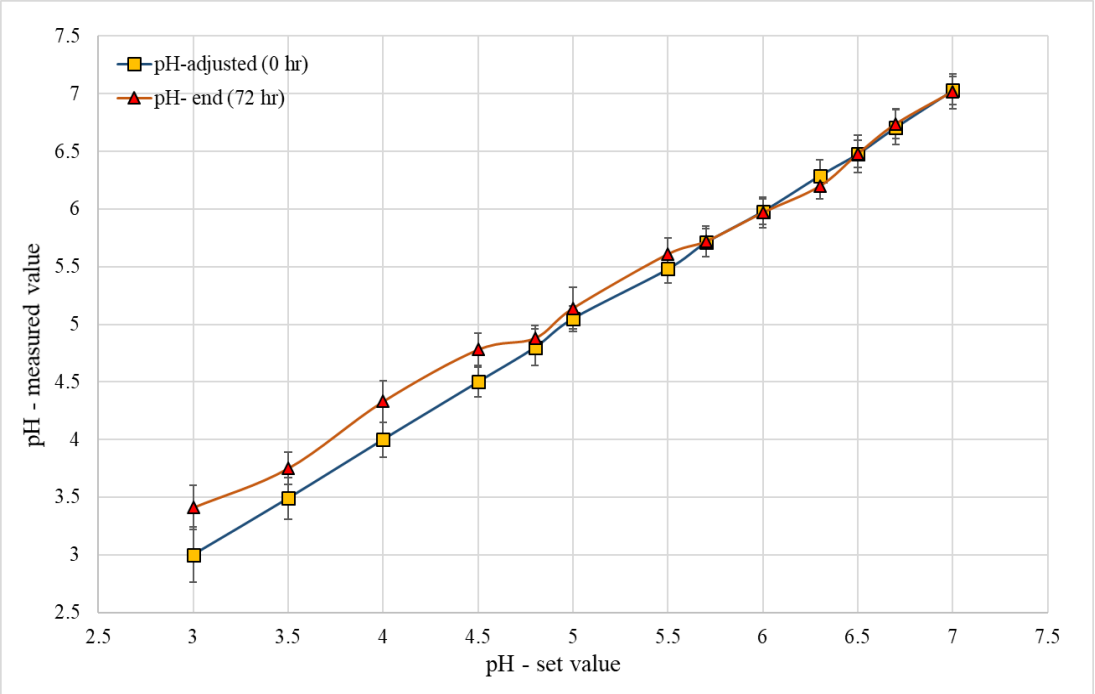


Figure 4-7. pH data at the beginning (0 hr) and the end (72 hrs) of EH using Cellic CTec2.

Cellic CTec2 shows a higher change in the pH at the end of the EH comparing to the previous two cocktails. The greatest change was seen between pH 3.0 – 4.8. In which the highest increase was found at pH 3.0 where the pH increased from 3.0 at (0 hr) to 3.41 at (72 hrs). This means that there was approximately 13% increase in the pH value at the end of hydrolysis.

Although Cellic CTec2 shows the highest difference in pH value at pH 3.0, 3.5, 4.0, 4.5, the difference is not very high and is within the error bar. Therefore, the adjusted

pH value at (0 hr) and the final pH measured at each different pH point studied show no significant difference and can be assumed to be the same.

Figure 4-8 illustrates the total reduced sugar concentration at the end of EH (72 hrs) against the pH value. It can be noted that the highest total reduced sugar yield was achieved at pH 5.0 – 6.3 and with pH as the optimum pH were the total reduced sugar was maximized.

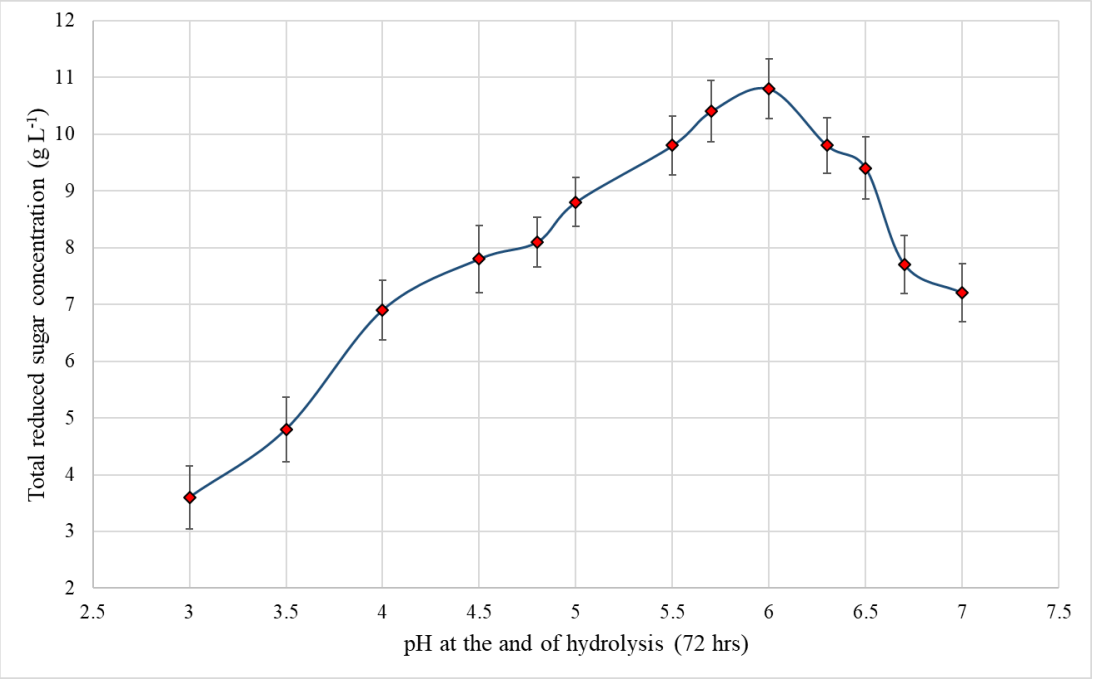


Figure 4-8. Total reduced sugars concentration at the end of the EH (72 hrs) obtained from different pH solution.

The total reduced sugar concentration for both pH 4.8 and 6.0 was also monitored with time during the EH and the results are shown in Figure 4-9. The total reduced sugars concentration from pH 4.8 and 6.0 behave similarly with time. The gap between the reduced sugar concentrations was almost constant at 2.2 g L^{-1} during the hydrolysis. By increasing the pH from 4.8 to 6.0, the total reduced sugar concentration increased from 8.5 g L^{-1} to 10.8 g L^{-1}.

The results are with an agreement with the finding of a similar study done by (Lan et al., 2013). They found that the optimum pH for lodgepole pine trees biomass pre-

treated with several pre-treatment methods 5.5 – 6.2 when Cellic CTec2 was used. Their results show approximately 70% increase in glucose yield when the pH was increased from 4.9 to 6.2. Therefore, it is recommended to use pH 6.0 to achieve high reduced sugar yield from WS.

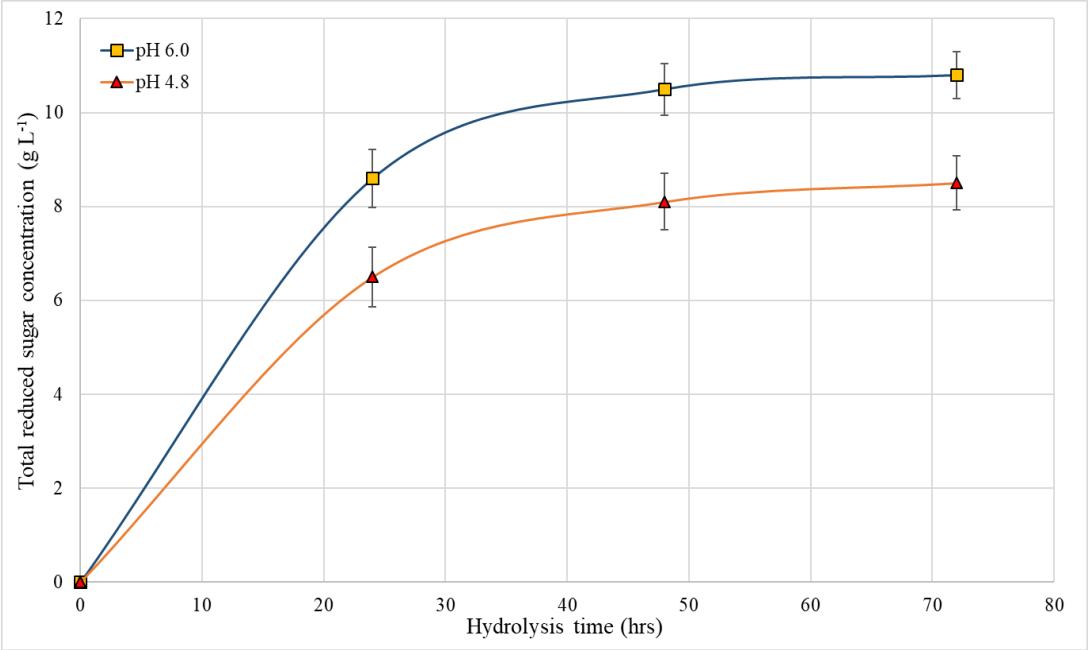


Figure 4-9. Total reduced sugar yield during EH (72 hrs) for pH 4.8 and 6.0 samples.

To summarize, all the enzymes subjected to the pH study showed an improvement after changing the pH from 4.8 to a higher value. Figure 4-10 illustrates the total reduced sugar concentration after EH for all enzymes at pH 4.8 and 6.0. By changing the pH of the solution from 4.8 to 6.0, Celluclast 1.5L with Novozymes 188 and endo-1, 4-β-Xylanase show an increase in the total reduced sugar yield from 5.2 g L^{-1} to 7.0 g L^{-1} and 5.5 g L^{-1} to 7.4 g L^{-1}, respectively. In the case of Cellic CTec2 , the total reduced sugar increased from 8.5 g L^{-1} to 10.8 g L^{-1}.

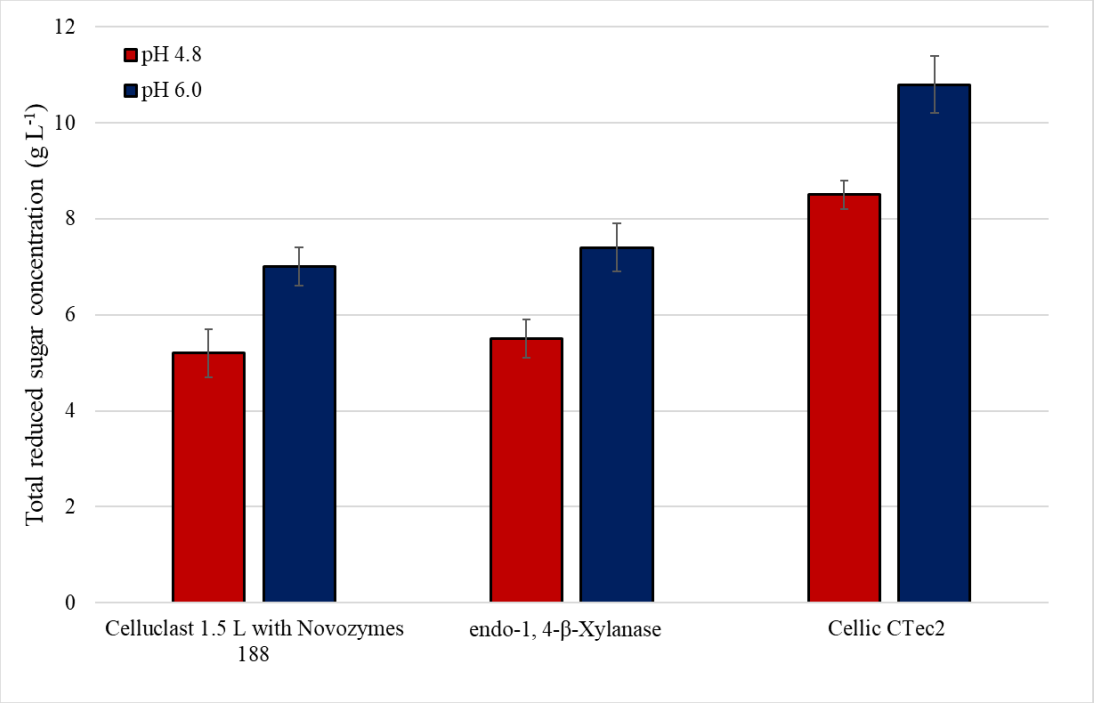


Figure 4-10. Total reduced sugar yield @ pH 4.8 and 6.0 obtained from Celluclast 1.5L with Novozymes 188 and endo-1, 4-β-Xylanase and Cellic CTec2 enzymes.

4.5 Fermentation

The fermentation was carried out on the samples with pH 4.8 and 6.0 for the three enzymes cocktails used. The results are listed in Table 4-2.

Table 4-2. Ethanol yield for the samples hydrolysed at 4.8 and 6.0.

Enzymes	**Ethanol concentration ($g\ L^{-1}$) @ 4.8**	**Ethanol concentration ($g\ L^{-1}$) @ 6.0**
Celluclast 1.5L with Novozymes 188	1.84 ± 0.1	2.43 ± 0.4
endo-1, 4-β- Xylanase	1.73 ± 0.2	2.32 ± 0.3
Cellic CTec2	3.2 ± 0.2	3.85 ± 0.5

The increase in the ethanol yield is due to the increase in hexose sugars liberated during EH for the solution of pH 6.0. All the samples showed an ethanol yield up to 92 ± 3% of the theoretical yield.

Figure 4-11 shows the GC for sugars trace after EH and after fermentation of the sample hydrolysed with Cellic CTec2 enzyme @ pH 6.0. It is clear that almost all hexose sugars were fermented.

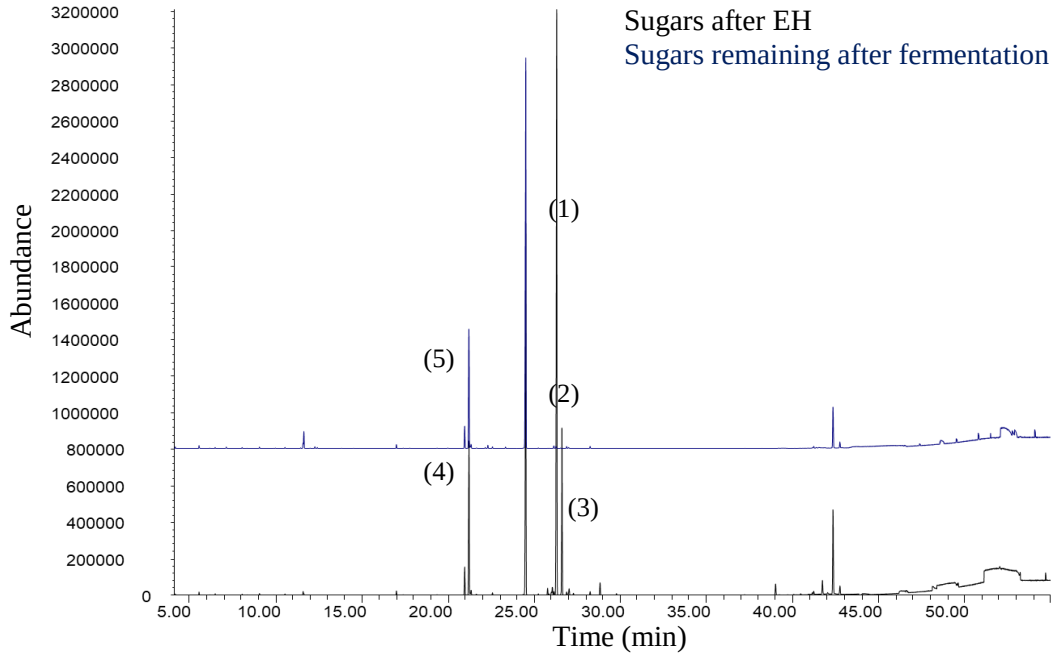


Figure 4-11. Monosaccharide sugars peak after EH using Cellic CTec2 enzyme @ pH 6.0 and after fermentation for (vws12-10) sample. (1) glucose after EH, (2) glucose after fermentation, (3) galactose after EH, (4) xylose after EH and (5) xylose after fermentation.

4.6 Summary

The results of the pH evaluation indicate that the optimum pH for EH using (Celluclast 1.5L with Novozymes 188, endo-1, 4-β-Xylanase and Cellic CTec2) enzymes is different from the range pH 4.8 – 5.0 which is exclusively used by almost all the existing literature. The enzymes activity test based on using pure cellulose substrate (Whatman paper) at pH 4.8 as an optimum pH suggested by cellulase manufacturers is not necessarily the same optimum value for lignocellulosic (i.e. WS) substrate.

Reducing the acidity in lignocellulosic substrates during EH might have an effect on reducing lignin inhibition to the enzyme activity. As a result, lignin absorption of

enzymes or affecting the lignin-cellulose binding and interaction might be reduced by affecting the electrostatic charge and hydrophilicity of the lignin (Lou et al., 2013). Furthermore, increasing the pH could decrease the lignin-derived inhibitors (Qin et al., 2016).

All the enzymes which were used show a significant improvement in the total reduced sugar yield after changing the pH from 4.8 to 6.0. Both Celluclast 1.5L with Novozymes 188, endo-1, 4-β-Xylanase show an increase of (25%) while Cellic CTec2 shows an increase of (21%). Since Cellic CTec2 gave the highest total reduced sugar yield, it was chosen for EH for the result of this study and the EH was carried at pH 5.8 – 6.0.

Chapter Five: Microwave Pre-treatment

5.1 Introduction

The grounded WS with a particle size of < 250 µm was chosen to perform the microwave pre-treatment. The effect of the time, temperature, distilled water volume and power of the microwave pre-treatment on the overall sugar recovery were investigated. Overall sugar recovery includes the hemicellulose extracted in the liquid fraction after the microwave pre-treatment and the total reduced sugar produced after EH. Design of experiments v.11.1 (DoE) was used to evaluate the microwave pre-treatment conditions time, temperature, power and distilled water volume) on the overall sugar recovery. Furthermore, the microwave pre-treatment conditions were optimized by DoE with the aim of reducing the microwave pre-treatment time and increasing the overall sugar recovery yield.

5.2 Microwave pre-treatment Conditions Specification using Design of experiments

DoE software with the central composite design (CCD) techniques (as known as face-centred cubic) was used to describe the quadratic models of the microwave pre-treatment. The maximum values for the microwave pre-treatment temperature, power and distilled water volume were 200 °C, 900 W and 40 mL, respectively. These values were selected based on literature and preliminary experiments. Furthermore, from the preliminary results (not shown in this report) it was found that pre-treating WS beyond 120 min will have no significant effect on the overall sugar recovery yield. Therefore, 120 min was set as the upper limit for the microwave pre-treatment time. On the other hand, the lower values for the microwave pre-treatment conditions were chosen based on literature. The lower, the mean and the upper values for microwave pre-treatment conditions (factors) and their codes are given in Table 5-1.

Table 5-1. The lower, the upper and the mean values of the microwave pre-treatment factors.

Factor	Name	Units	Coded Low (-1)	Mean (0)	Coded High (+1)
A	Time	(min)	5	62.5	120
B	Temperature	(°C)	50	125	200
C	Distilled water volume	(mL)	20	30	40
D	Irradiation Power	(W)	200	550	900

A numerical simulation approach was adopted for all the factors (A, B, C and D). The overall sugar recovery yield was selected as the response. The microwave pre-treatment procedure is described in Chapter 2. The outline of the pre-treatment includes immersing 1 g of the ground WS with a particle size of < 250 µm in the desired value of distilled water stirring solution with a magnetic stirrer for 5 min before applying the microwave pre-treatment.

After specifying the factor's upper and lower values, DoE will suggest a set of experiments to be carried out at different conditions to evaluate the effect of the factors on the response. When the microwave pre-treatment was carried out, it was found that there is a difference between the experiment design values suggested by the DoE and their actual values during the experiments performing. During the microwave pre-treatment, the time consumed to reach the set temperature was monitored and nominated as (reaching target). Moreover, it was noticed that at the start of experiments, the microwave pre-treatment temperature exceeded the designed temperature for a short period (~1 min). The maximum temperature reached by the microwave at the starting of the pre-treatment was reported as (overshot). Finally,

although the microwave power was set on the desired value, however during the pre-treatment the microwave power fluctuated to maintain the pre-treatment temperature stable. Therefore, the average microwave power during the pre-treatment was calculated and reported as (average power). The design and the actual values for the microwave pre-treatment factors (time, temperature and power as well as the distilled water volume) are reported in Table 5-2. As it can be seen from Table 5-2, the experiments with a microwave power of (550 and 900 W) consumed less time to reach the designed pre-treatment temperature compared to the experiments conducted at 200 W. It can be also noticed from Table 5-2 that run number 1 and 17 has no values for the (Reaching Target). The reason was that the microwave did not reach the designed temperature during the microwave pre-treatment. The average overshot temperature was found to be around 6 °C higher than the designed temperature for all the runs except run 1 and 17. In run 1 and 17 the designed temperature was never reached and therefore the maximum temperature reached by the microwave before the pre-treatment finished was reported. It can be noticed from Table 5-2 that for the short time and high-temperature pre-treatment, the microwave power was at the maximum designed power. On the other hand, for a long time pre-treatment, the power average was lower than the designed power. A similar trend in the variation between the designed and the actual microwave pre-treatment conditions was reported by (Janker-Obermeier et al., 2012).

Table 5-2. The setting and the actual values for the microwave pre-treatment factors.

Run	Time (min)		Temperature (°C)		Power (W)		Distilled water volume (mL)
	Design	Reaching target	Design	Overshot	Design	Average power	
1	5	---	200	150	200	200	20
2	62.5	1.0	125	128	900	190	30
3	120	0.8	50	54	200	60	20
4	120	1.4	200	203	200	96	40
5	120	1.2	200	206	900	220	20
6	120	0.5	50	53	900	35	20
7	62.5	0.5	50	54	550	70	30
8	62.5	3.0	200	206	550	100	30
9	62.5	1.1	125	129	550	73	30
10	5	1.5	200	208	900	190	40
11	5	1.3	200	207	900	230	20
12	62.5	1.3	125	130	550	90	30
13	120	1.9	200	206	900	120	40
14	62.5	1.2	125	130	550	77	30
15	120	1.8	125	128	550	86	30
16	62.5	3.3	125	128	200	70	30
17	5	---	200	140	200	200	40
18	5	1.1	50	57	200	81	40
19	62.5	1.1	125	133	550	35	20
20	62.5	1.3	125	132	550	65	30
21	5	0.8	50	53	900	58	20
22	62.5	1.3	125	132	550	63	30
23	120	0.5	50	54	900	60	40
24	120	1.3	50	57	200	25	40
25	120	1.0	200	202	200	101	20
26	5	0.8	50	53	900	52	40
27	5	1.2	50	51	200	51	20
28	62.5	1.4	125	130	550	67	30
29	62.5	1.7	125	131	550	70	40
30	5	1.3	125	132	550	60	30

5.3 Hemicellulose Extracted and Total Reduced Sugar Yield after Enzymatic Hydrolysis

The liquid fraction collected after applying the microwave pre-treatment was analysed to determine the hemicellulose extraction (removal) from the WS according to the National Renewable Energy Laboratory (NREL) standard procedure (Sluiter et al., 2008a). The water insoluble solid (WIS) was also analysed using the NREL standard procedure (Sluiter et al., 2008c). The WIS was subjected to EH using Cellic CTec2 enzyme at pH 5.8. The extracted hemicellulose in the liquid fraction, sugars remained in the pre-treated WIS and the total reduced sugar after EH (72 hrs) concentrations are giving in Table 5-3. The results in Table 5-3 were calculated based on using 1 g of the WS in 50 mL of the solution. From Table 5-3, it can be noticed that the extracted hemicellulose concentration has a proportional relationship with microwave pre-treatment conditions. It was found that the highest hemicellulose extraction of (5.62 g L^{-1}) was recorded in run 5 where the WS was pre-treated at 200 ºC for 120 min and with microwave power of 900 W. On the other hand, the lowest hemicellulose extraction of (1.02 g L^{-1}) was monitored in run 27 where the WS was pre-treated at 50 ºC for 120 min with microwave power of 200 W. As for the total reduced sugar concentration after EH, Table 5-3 showed that the highest yield was established from run 9, 12, 14, 19, 20, 22 and 28 with an average concentration of 9.93 g L^{-1}. It was concluded that increasing the microwave pre-treatment time and temperature will increase the hemicellulose extraction and subsequently the total reduced sugar yield after EH. After analysing the liquid fraction after the microwave pre-treatment and WIS, it was found that the main extracted saccharides sugars were xylose and glucose from hemicellulose and cellulose, respectively. Therefore, xylose and glucose are used to refer to hemicellulose and cellulose, respectively.

Table 5-3. The extracted hemicellulose in the liquid fraction, the total sugars remaining in the WIS after microwave pre-treatment and the total reduced sugar after EH concentrations.

Run number	The extracted hemicellulose in the liquid fraction (g L^{-1})	Total sugars remaining after the microwave pre-treatment (g L^{-1})	Total reduced sugar concentration after EH (g L^{-1})
1	1.2	13.6	9.9
2	4.6	10.3	8.7
3	1.2	13.5	9.5
4	4.9	10.0	8.7
5	5.6	9.2	8.6
6	1.8	13.0	9.3
7	3.3	11.5	8.2
8	5.0	9.9	8.8
9	2.9	12.0	9.9
10	3.4	11.5	8.3
11	3.5	11.3	8.5
12	3.0	11.8	9.9
13	5.6	9.4	8.5
14	2.9	12.0	9.9
15	4.6	10.3	8.4
16	2.8	12.1	9.9
17	1.3	13.7	9.6
18	1.1	13.7	9.7
19	2.9	12.0	10.0
20	2.9	12.0	9.9
21	2.2	12.6	8.9
22	3.0	11.9	9.9
23	2.0	12.9	9.1
24	1.2	13.7	9.7
25	5.0	9.9	8.7
26	2.0	12.9	9.1
27	1.0	13.9	9.8
28	2.9	12.1	9.9
29	3.8	11.1	8.7
30	3.3	11.6	8.2

To have a better view and understanding of the results in Table 5-3, hemicellulose recovery yield in the liquid fraction was calculated based on the initial hemicellulose available in the raw WS. Total reduced sugar yield after EH was also calculated based on the sugars available in the WIS. Finally, the overall sugar recovery yield (a summation of the extracted sugars in the liquid fraction and the sugars produced after

EH) based on the sugars contained in the raw WS were calculated and the results are reported in Table 5-4.

Table 5-4. The hemicellulose recovery in the liquid fraction after the microwave pre-treatment, the total reduced sugar yield after EH and the Overall sugar recovery yields.

Run	Hemicellulose recovery (%)	Total reduced sugar yield liberated after EH (%)	Overall sugars recovered yield (%)
1	18.6	72.1	72.4
2	70.7	84.6	87.6
3	18.9	72.1	72.3
4	76.6	87.4	89.9
5	87.1	92.6	93.4
6	27.6	71.0	72.7
7	50.5	70.8	75.2
8	77.8	88.9	90.8
9	45.3	82.7	84.7
10	52.1	71.8	76.5
11	54.0	75.0	78.9
12	46.8	84.3	85.2
13	86.2	90.6	92.8
14	44.7	82.6	84.5
15	71.6	82.3	86.1
16	43.7	81.9	83.8
17	19.2	70.6	71.8
18	17.7	70.5	71.4
19	44.7	82.7	84.6
20	44.3	82.6	84.4
21	34.7	70.6	73.4
22	46.2	83.6	85.1
23	31.3	70.3	73.2
24	18.3	70.5	71.6
25	77.2	88.3	90.4
26	30.7	70.4	73.1
27	15.8	70.7	71.4
28	44.0	82.1	84.2
29	58.0	77.7	81.7
30	50.9	70.6	75.6

Table 5-4 showed that the maximum and minimum hemicellulose recovery in the liquid fraction was obtained from run 5 and 27 with a yield of 87.12% and 15.8%, respectively.

In a study carried by Janker-Obermeier et al (2012), the WS was pre-treated with microwave at 60–140 °C for 10–60 min and with different NaOH concentration (2 – 5 wt %). It was concluded that using NaOH (4 – 5 wt %) can remove 73% of hemicellulose in the liquid fraction (Janker-Obermeier et al., 2012). Adding NaOH decreased the microwave pre-treatment temperature and time in Janker-Obermeier et al (2012) study compared to the microwave pre-treatment temperature and time used in the current study. However, the hemicellulose recovery yield with the present of the NaOH was 73% whilst the hemicellulose recovery yield in the current study from run 13 was 86.2% without using NaOH.

The maximum total reduced sugar yield after EH in Table 5-4 of (92.6%) and (90.6%) was obtained from the WIS in run 5 and 13, respectively. In which the WS was pre-treated at 200 ºC for 120 min with microwave power 900 W and distilled water volume 20 mL and 40 mL, respectively. On the other hand, all the samples pre-treated for 5 min and at 50 ºC gave the lowest sugars yield after EH. It was concluded that increasing the hemicellulose recovery in the liquid fraction will essentially increase the total reduced sugar yield after EH. Overall sugar recovery yield varies from 71.4% to 90.4%. The highest overall sugar recovery yield where it exceeding the 90 % was recorded from runs 5, 7, 13 and 25 with a yield of 93.4%, 90.8%, 92.8% and 90.4%, respectively.

Hu and Wen (2008) pre-treated switchgrass with microwave-assisted alkali at a range of temperature, time, solid content and alkali loading. The reported optimum microwave pre-treatment conditions for switchgrass were 190 °C, 30 min, alkali loading 0.1 g g^{-1} and solid content 50 g L^{-1}. At these optimum conditions, 99% of potential sugars available in the switchgrass was recovered in the liquid fraction and after EH (Hu and Wen, 2008). The reported sugars recovery of 99% from switchgrass

was slightly higher than the overall sugar recovery yield of 93.4% reported in Table 5-4 (run 5). Nevertheless, the overall sugar recovery yield of 93.4% in the current study was obtained from WS pre-treated with distilled water only.

In a study carried out by Aguilar-Reynosa et al (2017), the corn residues (stover and cob) was pre-treated with microwave and conduction-convection heating for comparison. The corn residues were immersed in water (10% solid loading) and pre-treated with microwave at temperature ranged in 160 °C to 200 °C for a time period of 10 to 50 min. It was reported that the microwave pre-treatment at 200 °C for 30 min gave the highest sugars conversion up to 95.1% (Aguilar-Reynosa et al., 2017). The difference between corn residue used by Aguilar-Reynosa et al (2017) and WS used in the current study might be one of the reasons explaining the difference in the sugars yield of 95.1% in 30 min and 93.4% in 120 min, respectively.

In another study carried by Kim and Han (2012), the rice straw was pre-treated at temperature ranged in (60 – 100 °C), time (30 – 90 min) and NaOH concentration. Glucose yield after EH of 85.49% was obtained from the rice straw pre-treated at 100 °C for 60 min with 4.0% of NaOH (Kim and Han, 2012). The average total reduced sugar yield after EH in Table 5-4 for WS samples pre-treated at 125°C for 62.5 min were approximately 83% which is similar to the yield reported by Kim and Han (2012) of 85.49%. Nevertheless, the yield reported in the current study was achieved by pre-treating the WS using distilled water only during the microwave pre-treatment. It was concluded that increasing the microwave pre-treatment temperature and time will improve hemicellulose extraction and subsequently the total reduced yield after EH.

5.4 DoE Model Analysis of the Microwave Pre-treatment

DoE was applied to evaluate the microwave pre-treatment factors (A, B, C and D) effects and interactions on the overall sugar recovery yield. The design matrix of the factors A, B, C and D and the response (overall sugar recovery yield) are presented in Table 5-5.

Table 5-5. Microwave pre-treatment factors (conditions) matrix and the Overall sugar recovery yield (response).

Run	Factor 1	Factor 2	Factor 3	Factor 4	Response
	A: Time (min)	B: Temperature (°C)	C: Distilled water (mL)	D: Power (W)	Overall sugar recovery yield (%)
1	5.0	200	20	200	72.4
2	62.5	125	30	900	87.6
3	120.0	50	20	200	72.3
4	120.0	200	40	200	89.9
5	120.0	200	20	900	93.4
6	120.0	50	20	900	72.7
7	62.5	50	30	550	75.2
8	62.5	200	30	550	90.8
9	62.5	125	30	550	84.7
10	5.0	200	40	900	76.5
11	5.0	200	20	900	78.9
12	62.5	125	30	550	85.2
13	120.0	200	40	900	92.8
14	62.5	125	30	550	84.5
15	120.0	125	30	550	86.1
16	62.5	125	30	200	83.8
17	5.0	200	40	200	71.8
18	5.0	50	40	200	71.4
19	62.5	125	20	550	84.6
20	62.5	125	30	550	84.4
21	5.0	50	20	900	73.4
22	62.5	125	30	550	85.1
23	120.0	50	40	900	73.2
24	120.0	50	40	200	71.6
25	120.0	200	20	200	90.4
26	5.0	50	40	900	73.1
27	5.0	50	20	200	71.4
28	62.5	125	30	550	84.2
29	62.5	125	40	550	81.7
30	5.0	125	30	550	75.6

5.4.1 Analysis of Variance (ANOVA)

The CCD design obtained for the microwave pre-treatment factors and the response were analysis and found to be significant terms for the quadratic power. The microwave pre-treatment design equation in terms of coded factors are:

Total sugars yield = 84.80 + 4.33*A + 5.70*B - 0.4167*C + 1.48D + 4.15*AB + 0.125*AC-0.4375*AD - 0.2250*BC + 0.7125*BD - 0.0625*CD - 4.06*A^2 - 1.91*B^2 - 1.76*C^2 + 0.7851*D^2 (5.1)

The analysis of variance (ANOVA) was used to analyse the microwave pre-treatment model and the results are illustrated in Table 5-6.

Table 5-6. Variance analysis of the RSM (ANOVA) for the microwave pre-treatment.

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	1551.97	14	110.85	93.61	< 0.0001
A-Time	337.13	1	337.13	284.68	< 0.0001
B-Temperature	584.82	1	584.82	493.82	< 0.0001
C-Water	3.13	1	3.13	2.64	0.1251
D-Power	39.31	1	39.31	33.19	< 0.0001
AB	275.56	1	275.56	232.68	< 0.0001
AC	0.25	1	0.25	0.21	0.6525
AD	3.06	1	3.06	2.59	0.1287
BC	0.81	1	0.81	0.68	0.4212
BD	8.12	1	8.12	6.86	0.0194
CD	0.06	1	0.0625	0.05	0.8214
A^2	42.81	1	42.81	36.15	< 0.0001
B^2	9.50	1	9.50	8.02	0.0126
C^2	8.07	1	8.07	6.81	0.0197
D^2	1.60	1	1.60	1.35	0.2637
Residual	17.76	15	1.18	---	< 0.0001
Lack of Fit	16.98	10	1.70	10.77	0.0086

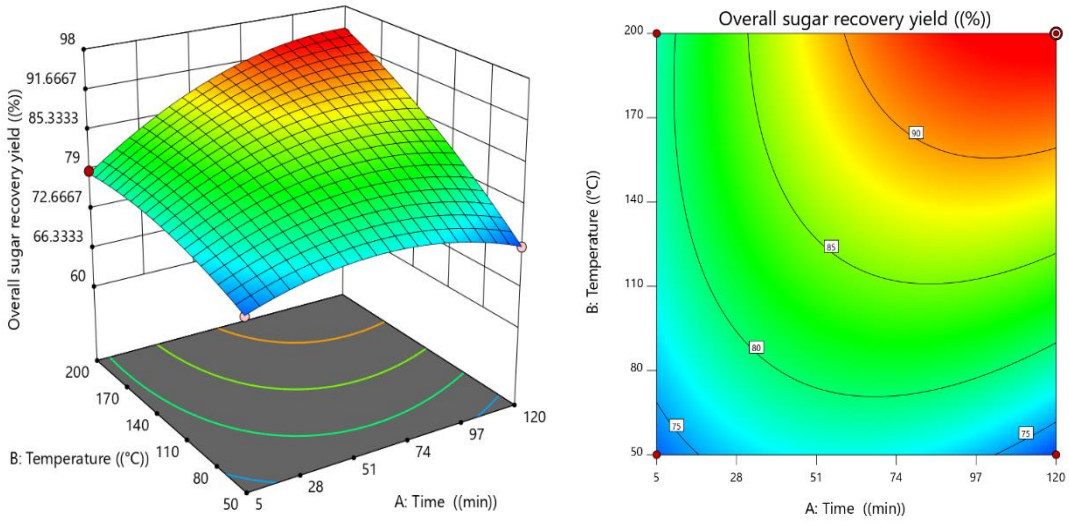
The F-value represent the ratio of the individual term mean square to the residual mean square. It can be seen from Table 5-6 that the model F-value was 93.61 which indicates that the design model is significant. Furthermore, the F-value for A, B, C and D factors indicates that factor B (temperature) and factor A (time) has the highest effect on the designed model with a value of 493.82 and 284.68, respectively.

Moreover, the Prob > F value (p-value) represent the probability of F-statistics value. The P-value is used to identify the significance of the coefficients and to understand the pattern of the interactions between the variables. In the case of the p-value is less than 0.05 which imply that the model terms are significates. A higher p-value (0.1 and higher) indicate that the model terms have no significant influence on the response. The results in Table 5-6 showed that factors A, B and D have a significant effect on the response (overall sugar recovery yield). Moreover, BD, A^2, B^2 and C^2 p-values were also less than 0.05. Meanwhile, factor C (distilled water volume) has no significant effect on the response. When the model was analysed with ANOVA, the adjusted and predicted R^2 values were found to be 0.9781 and 0.9471, respectively. The adjusted R^2 value means that 97.81% of the overall sugar recovery yield can be defined by the selected model. On the other hand, the predicted R^2 of 0.9471 is with an agreement with the adjusted R^2 since the difference between them is less than 0.2.

The DoE provides a 3-D surface response and a project contour plots which describes the process parameters effect and their interaction on the response(s). From the 3-D and the project contour graphs, the interaction level and effect force of the factors on the response can be identified. In case the fitted response surface in the 3-D graph was plane and the project contour lines are straights or parallels then there is no (or minor) interaction between the system factors. On the other hand, disfigured response surface

in the 3-D and elliptic shape of the contour graphs indicate that the two factors under study have high interaction and influence on the response (Liu and Chiou, 2005).

Figure 5-1 (a) and (b) show the effect of factor A (microwave pre-treatment time) and factor B (microwave pre-treatment temperature) and their interaction on the response (overall sugar recovery yield) in a 3-D response surface and project contour plots corresponding to design Equation 5-1. The maximum overall sugar recovery yield of 93.4% in Table 5-4 was achieved by conducting the microwave pre-treatment at 200 °C for 120 min. The increase in the overall sugar recovery yield has a proportional relationship with the microwave pre-treatment temperature and time. A remarkable increase in the overall sugar recovery yield of 90% can be achieved by setting the microwave pre-treatment temperature and time on 200 °C and approximately 60 min, respectively, as shown in Figure 5-1 (a) and (b). These results in Figure 5-1 (a) and (b) indicates that the interaction effect of the microwave pre-treatment temperature and time has a significant influence on the response, as confirmed by the low p-value of < 0.0001 for the AB term in Table 5-6.



(a) (b)

Figure 5-1. Effect of the microwave pre-treatment time (hrs) and temperature (°C) and their interaction on the overall sugars recovery: (a) three dimensional surface response plot and (b) project contour plot.

5.5 Optimization

An overall sugar recovery yield of 93.4% was obtained from run 5 in Table 5-4, however, the pre-treatment time was relatively long (120 min) and therefore the needs for optimization was essential. DoE provides different approaches for optimization including minimizing, maximize or search in the range of the selected targets. The microwave pre-treatment was optimized by selecting the following targets: factor A (pre-treatment time) to be minimized, the response (overall sugar recovery yield) to be maximized and factors B, C, and D to be in the range. The optimization numerical results are listed in Table 5-7. Table 5-7 gives a high response (overall sugars recovery) yield prediction ranged between 86.2% – 88.4%.

The maximum overall sugars yield obtained for run 5 in Table 5-5 was (92.3%) which was reached after pre-treating the WS for 120 min. After applying optimization, the maximum overall sugars yield decreased to 88.4% which was achieved by pre-treating the WS for only 42.8 min at the same temperature (200 ºC). Although the overall sugar recover yield of 93.4% in Table 5-5 before optimization was higher, however, the pre-treatment time after the optimization decreased significantly from 120 min to only 42.8 min, respectively.

Ma et al (2009) reported that the optimum microwave pre-treatment conditions of rice straw are as follow: 680 W, 75 g L^{-1} (substrate concentration) and 24 min. At these optimum conditions, the total saccharification was found to be 30.3% (Ma et al., 2009). The optimum overall sugar recovery yield of 88.4% in Table 5-7 demonstrate an improvement in the yield comparing to Ma et al (2009) results.

Table 5-7. The optimum microwave pre-treatment conditions suggested by the DoE software and the predicted overall sugar recovery.

No.	Time (min)	Temp. (°C)	Distilled water (mL)	Power (W)	Overall sugar recovery yield (%)
1	38.6	200	27.8	900	87.6
2	38.4	200	27.9	900	87.6
3	39.5	200	27.5	900	87.8
4	38.8	200	27.5	900	87.6
5	38.3	199	27.8	900	87.5
6	36.5	200	28.0	900	87.2
7	40.5	200	27.3	900	88.0
8	40.5	200	27.0	900	88.0
9	35.8	200	28.3	900	87.0
10	37.8	200	26.7	900	87.4
11	38.9	200	27.8	897	87.6
12	38.5	200	27.9	896	87.5
13	37.3	200	26.3	900	87.3
14	38.1	200	28.0	896	87.4
15	37.9	200	26.4	900	87.4
16	36.7	200	26.2	900	87.2
17	36.9	195	27.5	900	87.2
18	42.8	200	26.9	900	88.4
19	38.1	200	24.7	900	87.3
20	38.8	200	31.1	900	87.4
21	35.6	200	24.7	900	87.8
22	39.1	200	24.1	900	87.4
23	40.9	187	28.0	900	87.8
24	38.8	200	32.4	900	87.3
25	36.4	179	29.0	900	86.8
26	44.1	200	23.5	900	86.3
27	42.3	200	37.6	900	86.6
28	40.5	200	26.6	724	86.2

Figure 5-2 illustrate the microwave pre-treatment factors: A (time), B (temperature), C (distilled water volume) and D (power) values at which the response (overall sugar recovery yield) was at the optimum value of 88.4%.

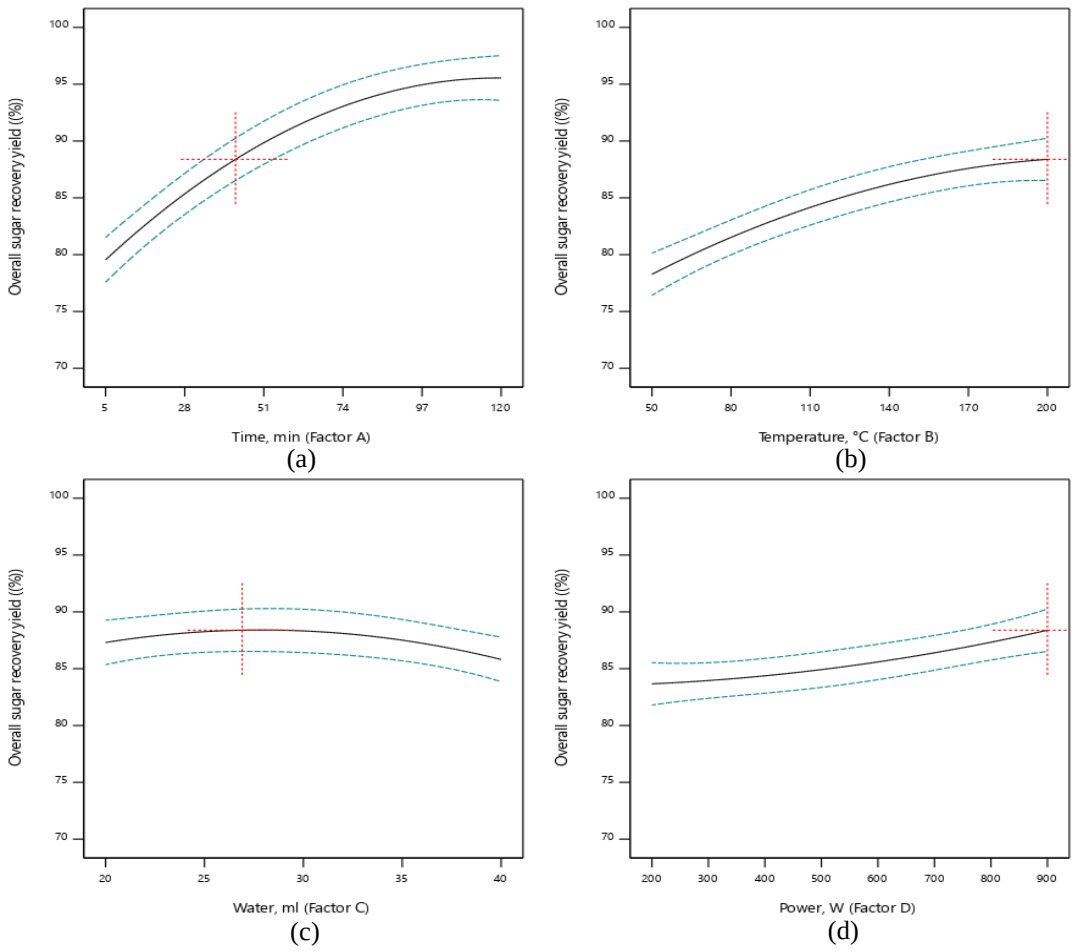


Figure 5-2. The microwave pre-treatment factors: (a) time (factor A), (b) temperature (factor B), (c) water volume (factor C) and (d) power (factor D) and the optimum response (overall sugar recovery yield).

Figure 5-2 (b) and (d) show that the optimum response (overall sugar recovery yield) of 88.4% was achieved by applying the microwave pre-treatment at maximum temperature and power of 200 °C and 900 W, respectively. Moreover, the optimum distilled water volume in Figure 5-2 (c) was found to be approximately 27 mL. As for microwave pre-treatment time, it can be seen from Figure 5-2 (a), the microwave pre-treatment time effect on the response (overall sugar recovery yield) is significant. It was concluded that, if, the microwave pre-treatment power and temperature were fixed at their maximum values, then the microwave pre-treatment time will be the dominant variable affecting the overall sugar recovery yield. Therefore, a second set of the

microwave pre-treatment experiments were carried out at 200 °C and 900 W for pre-treatment time ranged between 10 to 35 min. The distilled water volume was fixed at 27 mL based on the optimum volume suggested by DoE in Table 5-7. Furthermore, the WS was also pre-treated at the optimum conditions suggested by DoE (200 °C, 27 mL 900 W and 42.8 min) to measure the accuracy between prediction and experimental overall sugar recovery yield. Similar to Table 5-2, the designed and actual pre-treatment conditions for the second set experiments are reported in Table 5-8.

Table 5-8. The microwave pre-treatment conditions for the second set of experiments.

Run	Time (min)		Temperature (°C)		Power (W)		Distilled water volume (mL)
	Design	Reaching Target	Design	Overshot	Design	Average power	
1	10	1:30	200	180	900	86	27
2	15	1:42	200	208	900	120	27
3	20	1:30	200	207	900	130	27
4	25	1:30	200	206	900	118	27
5	30	1:28	200	205	900	120	27
6	35	1:26	200	206	900	104	27
7	42.8	1:27	200	206	900	91	27

All the experiments in Table 5-8 were performed three times and the average values were reported. The overall sugar recovery yield obtained after applying the pre-treatment conditions in Table 5-8 are presented in Figure 5-3. As can be seen from Figure 5-3, the overall sugar recovery yield has a proportional relationship with the microwave pre-treatment time. Furthermore, a yield of (88.6 ± 2.1%) was obtained from the WS pre-treated for 42.8 min which indicates a high precision of the optimizing model since the predicted overall sugars yield in Table 5-7 was 88.4%. Although the WS pre-treated for 10 min gave the lowest overall sugar recovery yield

of 75.4 ± 2.8%. Nevertheless, pre-treatment time was 4 times less than the WS pre-treated for 42.8 which gave a yield of 88.6 ± 2.1%.

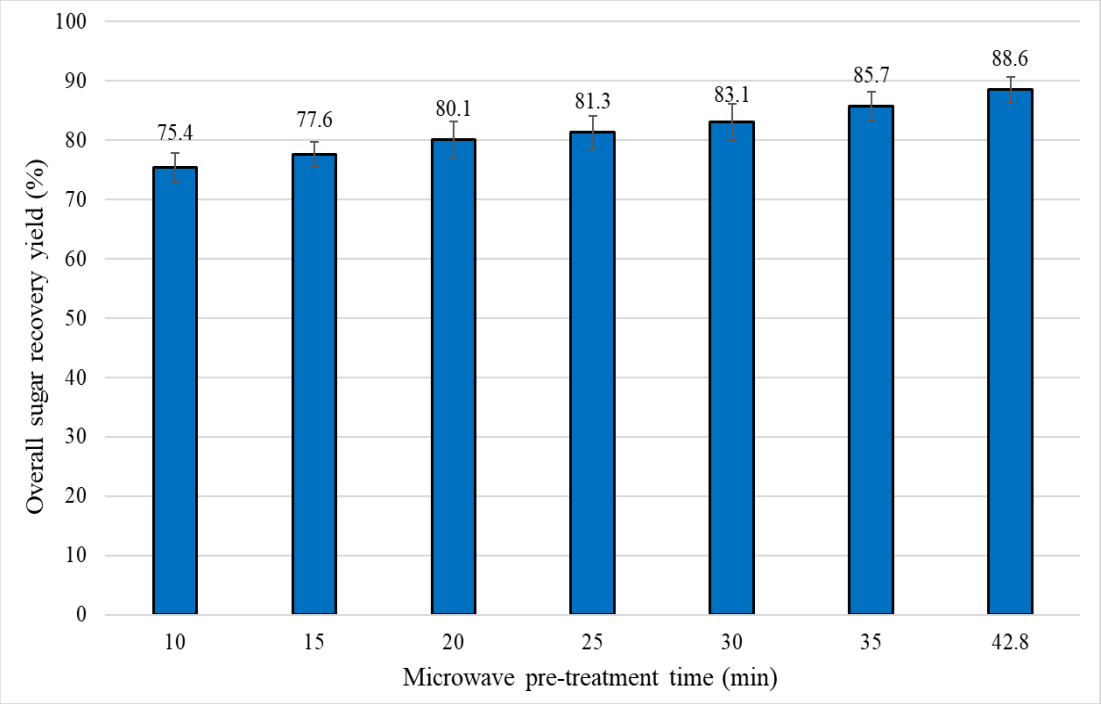


Figure 5-3. Overall sugar recovery yield from the WS pre-treated by the microwave at 200 ºC, 900 W for different time periods.

5.6 Fermentation

The EH solution was filtered and the pH was adjusted to 6.0 before adding the activated *Saccharomyces Cerevisiae* yeast (see Chapter 2). The fermentation process was performed on a few samples including the run 5 sample in Table 5-4 (pre-treated for 120 min) which gave the highest overall sugar recovery yield, the optimum sample in Table 5-7 (pre-treated for 42.8 min) and the sample pre-treated for 10 min in Table 5-8.

Before applying the fermentation process, the solution after EH was analysed to determine the hexose sugar. It was found that the majority of the hexose sugars was glucose with a negligible proportion of galactose. Since the three samples were pre-treated at the same temperature and power for a different time. Therefore, the pre-treatment time will be used to refer to the run 5 sample, optimum sample and the

sample pre-treated for 10 min in Table 5-8. The hexose sugars after EH, ethanol concentration after fermentation and the ethanol yield (based on the theoretical yield if all the available C6 sugars after EH were fermented) for sample 120 min, 42.8 min and 10 min are presented in Table 5-9.

Table 5-9. Hexose sugars concentration after EH, ethanol concentrations after fermentation and the ethanol yield for the microwave pre-treatment samples.

Sample No.	Microwave pre-treatment time (min)	Hexose sugars concentration liberated after EH (g L^{-1})	Ethanol concentration (g L^{-1})	Ethanol yield (%)
1	120 min	8.54 ± 0.3	3.31 ± 0.1	75.9 ± 2.1
2	42.8 min	10.19 ± 0.2	4.23 ± 0.1	81.3 ± 3.3
3	10 min	10.26 ± 0.4	4.41 ± 0.2	84.1 ± 3.1

The results in Table 5-9 showed that a satisfying ethanol yield was reached by all the samples. The highest ethanol yield of (84.1 ± 3.1%) was obtained from sample 3 whilst the lowest ethanol yield of (75.9 ± 2.1%) was recorded from sample 1. The relatively low ethanol yield in sample 1 can be explained by the possibility of forming complex sugars during the microwave pre-treatment since the WS was pre-treated for a long time (120 min). Zhu et al (2006) pre-treated WS with microwave-assisted alkali (NaOH) and the ethanol yield was 69.3% (Zhu et al., 2006). In another study conducted by Zhang et al (2013), WS was pre-treated with alkali (NaOH). By using DoE, the predicted and actual ethanol yield was found to be 69.49% and 70.76%, respectively (Zhang et al., 2013). The ethanol yield obtained from sample 1, 2 and 3 in Table 5-9 presented an improvement in the ethanol yield compared to Zhu et al (2006) and Zhang et al (2013). Aguilar-Reynosa et al (2017) reported an ethanol yield of 92% from corn residues pre-treated with the microwave. Fermentation was

performed by using simultaneous saccharification and fermentation (SSF) method (Aguilar-Reynosa et al., 2017). The reported ethanol yield was higher than the ethanol yield obtained from sample 1, 2 and 3 in Table 5-9. The difference in the ethanol yield might be a result of using corn residues while the ethanol yield in Table 5-9 was acquired from pre-treated WS.

5.7 Summary

To summarize, the microwave pre-treatment time, temperature, power and distilled water volume effect on the overall sugar recovery yield was investigated with means of DoE. The hemicellulose recovery in the liquid fraction, total reduced sugar yield after EH and the overall sugar recovery yield were measured at different microwave pre-treatment conditions. The results in Table 5-4 showed that the maximum hemicellulose recovery, total reduced sugar and overall sugar recovery yield were 87.1%, 92.6% and 93.4%, respectively. The overall sugar recovery yield varies between 71.4% and 93.4%. The results in Table 5-5 showed that the increasing in the microwave pre-treatment power, temperature and time will results in increasing the overall sugar recovery yield. By analysing the microwave pre-treatment condition in Table 5-6, it was found the temperature has the highest effect on the overall sugar recovery yield followed. The microwave pre-treatment conditions were optimized with the aim of reducing the pre-treatment time and increase the overall sugar yield recovery. The optimum microwave pre-treatment conditions were found to be 42.8 min, 200 °C, 900 W and 27 mL. At these optimum conditions, the overall sugar recovery yield of 88.4 % was achieved.

Chapter Six: Disk Refiner Pre-treatment

6.1 Introduction

WS was pre-treated using atmospheric disk refiner (ADP) and pressurized disk refiner (PDR) in Bangor University as a collaboration project with Hull University and Vivergo Fuels Ltd. The WS used in this chapter was generously supplied from East of England (harvest winter 2015).

6.2 Wheat Straw Composition Analysis

The composition analysis results for the raw WS (harvest in winter 2015) used in this are presented in Table 6-1.

Table 6-1: Composition analysis for WS used by Bangor University for atmospheric and pressurized disk refiner.

Component	(wt/wt %)
Cellulose	37 ± 3
Hemicellulose	24 ± 3
Lignin	23 ± 5
Ash	8 ± 3
Others	8 ± 3

6.3 Atmospheric Disk Refiner

6.3.1 Introduction

The atmospheric disk refiner (ADR) pre-treatment procedure is described in detail earlier (see Chapter 2). In summary, the WS was pre-processed with chopper or hammer mill. The pre-processed WS was suspended in water at 55 °C for 60 min before it was fed to the rotating plates. Then the ADR pre-treatment was carried at a consistency of 2 – 4% (i.e. 1 kg WS suspended in 50 L of water) to facilitate the wet

WS loading into the disk refiner. Towards the end of the refining process, the refined fibre was dewatered using a Vincent CP4 screw press. ADR pre-treatment method was conducted to evaluate the influence of pre-processing types, plate gaps for the disk refiner and the number of passes in order to achieve the maximum yield of WS. Samples codes, type of the pre-process, number of passes in the ADR and refiner plate gaps are listed in Table 6-1.

Table 6-2. Experimental parameters and the samples codes for the ADR pre-treatment conducted at Bangor University.

Sample Code	Pre-processing method prior to refining	Refining method for the (ADR)	Refiner plate gap
VWS1	Forage chopper (12 mm cutter length)	Single pass through the refiner	0.5 mm
VWS2	Forage chopper (12 mm cutter length)	Two passes through the refiner	0.5 mm
VWS3	Hammer mill (12 mm screen)	single pass through the refiner	0.5 mm
VWS4	Hammer mill (12 mm screen)	Two passes through the refiner	0.5mm
VSW5	Forage chopper (12 mm cutter length)	Single pass through the refiner	1.0 mm
VWS6	Forage chopper (12 mm cutter length)	Two passes through the refiner	1.0 mm
VWS10	Forage chopper (12 mm cutter length)	Single pass- using sharpened refiner plates	0.5 mm
VWS11	Forage chopper (12 mm cutter length)	Single pass- using sharpened refiner plates	0 mm

6.3.2 Composition Analysis of the Wheat Straw after Applying Atmospheric Disk Refiner Pre-treatment

The ADR samples were analysed according to the National Renewable Energy Laboratory (NREL) standard protocol (Sluiter et al., 2008a) to identify cellulose and hemicellulose composition. It was found that the margin of difference between cellulose and hemicellulose weight in all ADR samples were very small which can be neglected. The average weight of cellulose and hemicellulose was found to be 360 ± 10 mg and 220 ± 9 mg in 1 g of all ADR samples, respectively.

Unfortunately, the liquid fraction after the ADR process was not analysed to determine the hemicellulose extraction due to technical limitations at the time the project was carried out. However, the carbohydrates compositions for the raw WS and ADR samples were analysed and the extracted hemicellulose in the liquid fraction was calculated using the material balance equation.

6.3.3 Enzymatic Hydrolysis

The content of moisture for the pre-treated samples was analysed (Sluiter et al., 2008b) and found to be 25 – 30%. The samples were dried before applying EH. The total reduced sugars concentration during EH for all the samples were measured and the results are drawn in Figure 6-2.

Figure 6-2 shows the total reduced sugar concentration increasing rapidly for the majority of the samples until 50 hrs. The observation of a plateau increases until it reaches the final concentration at 72 hrs. The EH was carried beyond 72 hrs for another 24 hrs (results not shown). The total reduced yield showed no changing after 72 hrs. Therefore, 72 hrs was deemed more than sufficient to achieve the maximum sugar yield.

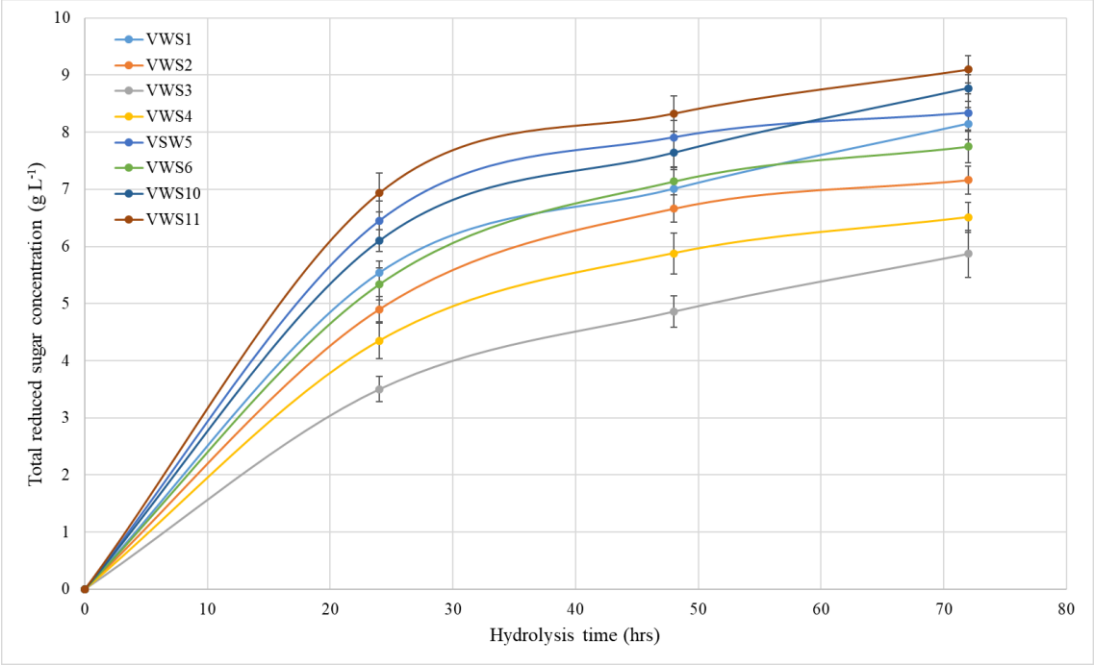


Figure 6-1. Total reduced sugar concentration during EH for the ADR samples.

The total reduced sugar yield percentage was calculated based on the sugars available in the ADR samples. The results are presented in Figure 6-3

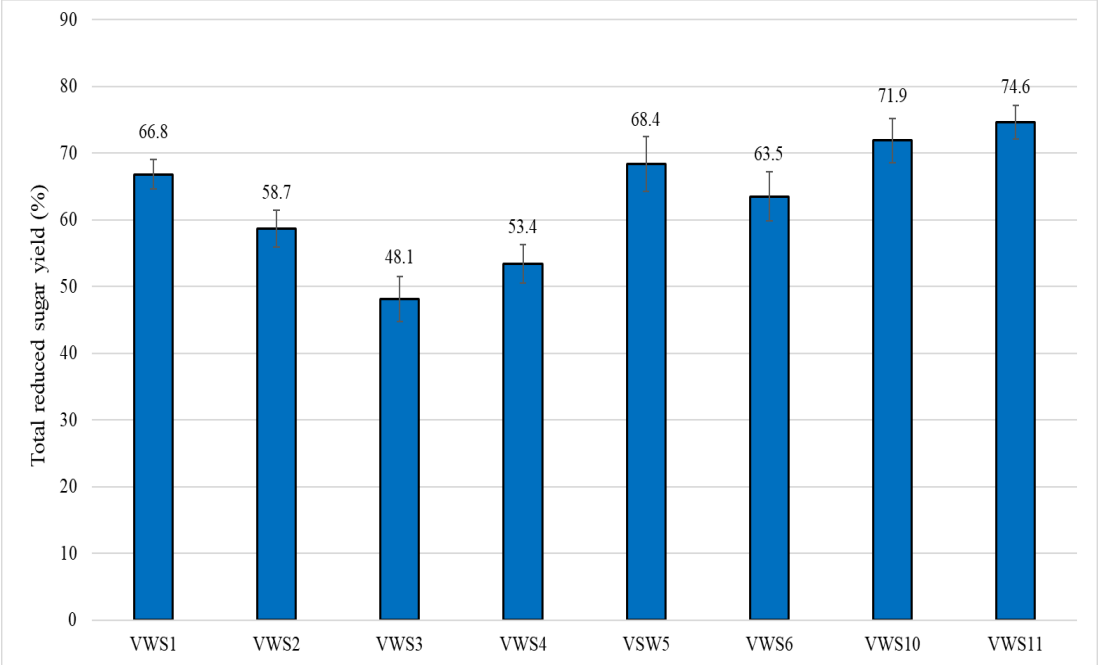


Figure 6-2. Total reduced sugar yield at the end of the EH for the ADR samples.

As can be seen from Figure 6-3, the highest total reduced sugar yield was obtained from sample VWS11 and VWS10 with a total reduced sugar yield of 74.6 ± 2.5% and 71.9 ± 3.3%, respectively. The highest yield recorded from VWS11 and VWS10

samples might be a result of using sharpened plats with low gap 0 and 0.5 mm. Furthermore, it is noticeable from Figure 6-3 that VWS5 and VWS1 gave a relatively high reduced sugar 68.4 ± 4.1% and 66.8 ± 2.2%, respectively. The lowest reduced sugars yield were obtained from the hammer milled single and two pass samples. VWS3 gave the lowest yield (48.1 ± 3.4%). This can be attributed to the loss of fine particle size from the hammer milled pre-process that causes an increase in the larger particle size percentage in the pre-processed sample. As a result, enzymes accessibility to cellulose and hemicellulose during EH will decrease and as a result lower total reduced sugar yield.

In a study carried by da Silva et al (2010), sugarcane bagasse and straw were pre-treated with Wet Disk Milling (WDM) using two nonporous ceramic disks. It was reported that the maximum glucose and xylose yield for the sugarcane straw was 68.0% and 44.9%, respectively (da Silva et al., 2010). In the current study, higher glucose and xylose were obtained for sample VWS11 with 81.1 ± 3.5% and 51.8 ± 3.2%, respectively. Both glucose and xylose yield in the current study was higher than the yield reported by da Silva et al (2010). Although da Silva et al (2010) used sugarcane straw with a lower particle size of < 2 mm, the sugars yield was still lower than the sugars yield obtained in the current study from the WS with a particle size of 12 mm.

Hideno et al (2009) pre-treated rice straw with ball milling, hot-compressed water pre-treatment and WDM. The glucose and xylose yield percentages were 89.4% and 54.3%, respectively (Hideno et al., 2009). The yield was slightly higher than the glucose (81.1 ± 3.5) and xylose (51.8 ± 3.2) yield obtained from VWS11 in the current stay. The difference in the sugars yield might be attributed to the usage of rice straw with a particle size of < 2 mm in Hideno et al (2009) compared to WS with a particle

size of < 12 mm which was used in the current study. In the current study, ADP pre-treatment on the WS with 12 mm length gave a higher glucose and xylose yield compared to Hideno et al (2009) where the WS length was < 2 mm.

6.4 Pressurized Disk Refiner

6.4.1 Introduction

PDR pre-treatment procedure includes chopping the WS with forage chopped and feeding it to an Andritz Sprout-Bauer 12 inch (30.5 cm) pressurised refiner. The chopped WS passed through a modular screw device (MSD) where the plug was formed at the desired pressure. The plug was then passed through several units until it reaches the refining section with one rotation plate. After refining, the sample was passed to the blowing line where a sudden pressure decrease similar to SE was applied. The pre-treated WS was filtered, dewatered and frozen until further using.

The aim of this section is to evaluate the pressure effects and the refiner plate configurations on the WS by monitoring the total reduced sugar yield after EH. Two types of plates were fitted in the disk refiner, Andritz D2-503 plates and Andritz D2-516. Plates types are shown in Figure 6-4. PDR pre-treatment conditions as well as the samples codes are illustrated in Table 6-2.

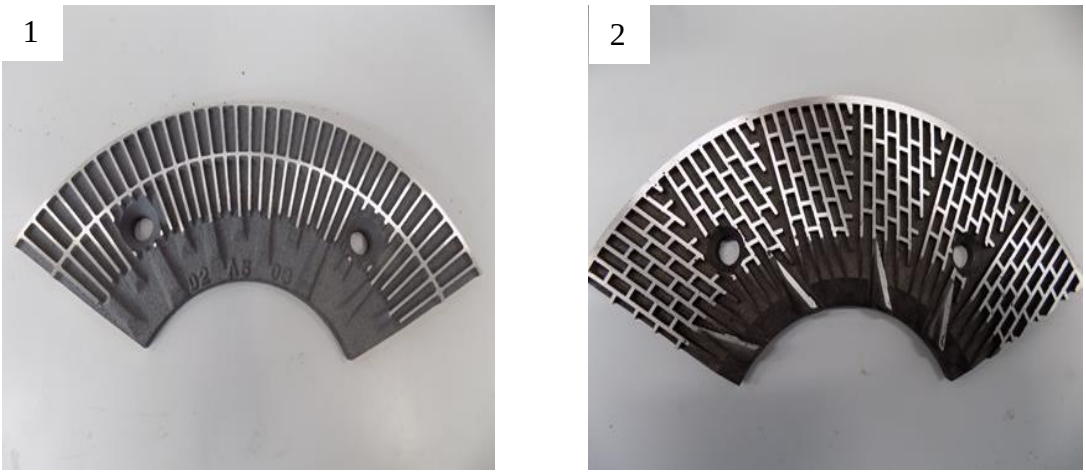


Figure 6-3. Refiner plates used during the PDR pre-treatment in Bangor University: (1) Andritz D2-503 plates and (2) Andritz D2-516 plates (right)

Table 6-3. Experimental parameters for the pilot scale PDR pre-treatment conducted at Bangor University (including the samples codes).

Sample code	PDR plate type	Pressure (bar)	Refiner plate gap (µm)	Length of refining time at each pressure (min)
VWS12-4	Andritz refiner plates, high intensity, D2-503	4	4	15
VWS12-6		6		
VWS12-8		7		
VWS12-10		10		
VWS13-4	Re-sharpened Andritz refiner plates, low intensity, D2-516	4		
VWS13-6		6		
VWS13-8		7		
VWS13-10		10		

6.4.2 Composition Analysis of the Wheat Straw after Applying Pressurized Disk Refiner Pre-treatment

The refined samples were analysed using NREL standard protocol (Sluiter et al., 2008a) to characterize cellulose, hemicellulose and lignin percentages. The results are shown in Figure 6-5.

It can be seen in Figure 6-5 that hemicellulose and lignin percentage decreases with increasing refining pressure. The extraction of the hemicellulose and lignin in the liquid fraction will expose cellulose to enzymes during EH which will improve the sugar yield significantly.

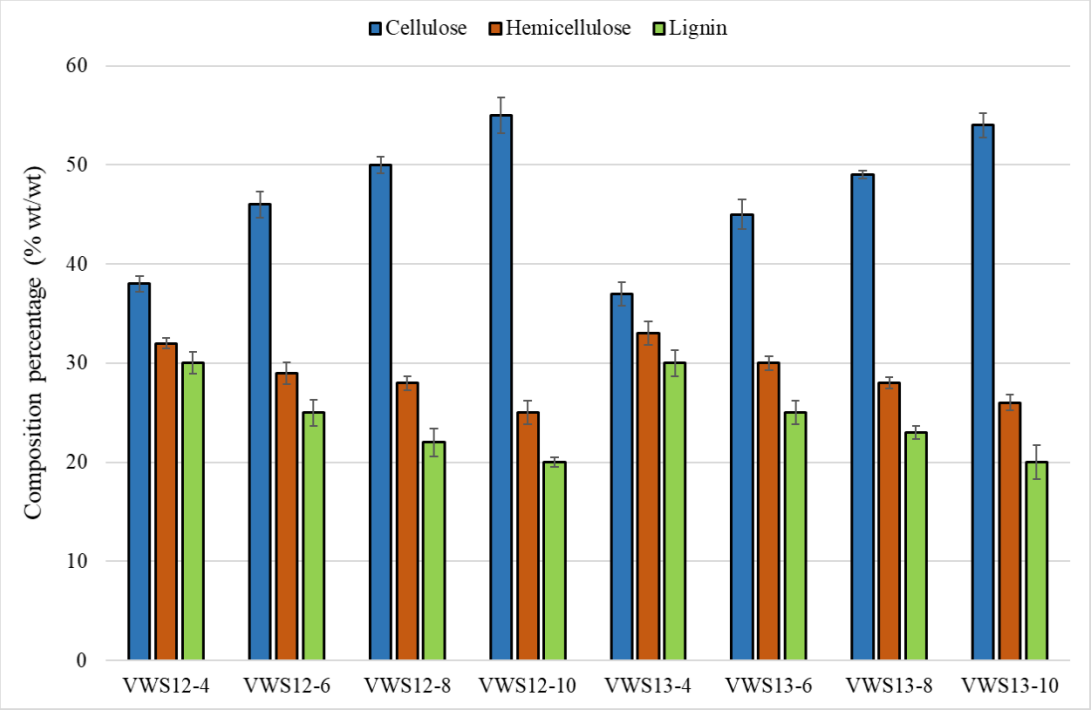


Figure 6-4. WS solid analysis after pressurized disk refiner pre-treatment at different pressures and plates intensity.

In general, the refined WS fibre composition was similar for both high and low intensity plates (VWS12 and VWS13). It can be seen from Figure 6-5 that, the cellulose percentage has a proportional relationship with the refining pressure for the high and low intensity plates. On the other hand, increasing the disk refining pressure degrease the hemicellulose and lignin percentage. Decreasing the hemicellulose might be a results of the hemicellulose dissolving and hydrolysis at high pressure with the present of water. Furthermore, the WS compositions after PDR at different pressure for the low high and low intensity plates showed a great similarity. Therefore, it can be concluded that the refining intensity (plate's type) has insignificant effect on the final fibre composition and hence can be neglected.

6.4.3 Enzymatic Hydrolysis

All the refined WS samples were dried and subjected to EH at similar condition described previously. The EH was carried by using Cellic CTec2 enzyme with 15

FPU/g DM dosage @ pH 5.8. The total reduced sugar yield at the end of EH (72 hrs) for all the refined samples are indicated in Figure 6-6.

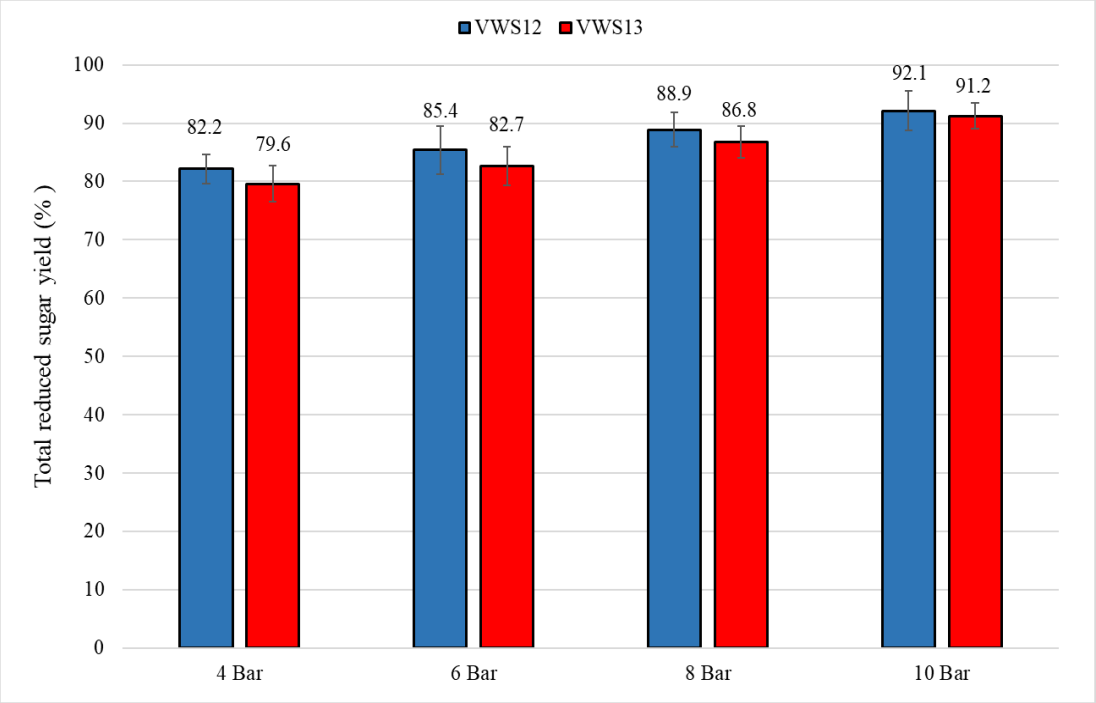


Figure 6-5. Total reduced sugar yield after EH for the VWS12 and VWS13 samples.

As predicted, removing hemicellulose and lignin with increasing the refining pressure facilitate enzymes accessibility to cellulose during EH. Increasing refining pressure led to the increase of the total reduced sugar yield after EH.

A study carried out by Fang et al (2011) on the pressure effects during disk refining pre-treatment of the WS showed to the achievement of a total reduced sugar yield of 93.3% after EH when the WS was pre-treated at 15 bar with 6 min residence time. Moreover, they reported that the total reduced sugar yield decreased to 88.7% when the residence time was 4 min. The present study provides a similar total reduced sugar yield to Fang et al (2011) from sample VWS12-10. In the current study, the total reduced sugar yield of 92.1 ± 2.2% was established by using less pressure (10 bar) and with shorter residence time (1 min) compared to (Fang et al., 2011).

Additional EH experiment was performed by using Cellic CTec2 enzymes at pH 4.8 on the VWS12-(4, 6, 8, 10) and VWS13-(4, 6, 8, 10) samples. The objective is to verify the finding in Chapter 4. The total reduced sugar yield for all the samples @ pH 4.8 and pH 5.8 are listed in Table 6-3. The results in Table 6-3 confirmed the finding and conclusion in Chapter 4 related to the carrying out the EH at a pH of 5.8 -6.0 rather than pH of 4.8.

Table 6-4. The total reduced sugar yield after the EH process @ pH 4.8 and pH 5.8 for the pressurized disk refining WS samples using Cellic CTec2 enzyme.

Sample	Total reduced sugar yield @ pH 4.8 (%)	Total reduced sugar yield @ pH 5.8 (%)
VWS12-4	69.2 ± 2.1	82.2 ± 2.5
VWS12-6	72.2 ± 2.8	85.4 ± 4.1
VWS12-8	72.9 ± 3.2	88.9 ± 2.9
VWS12-10	74.7 ± 3.3	92.1 ± 3.4
VWS13-4	70.1 ± 3.7	79.6 ± 3.1
VWS13-6	70.5 ± 3.5	82.7 ± 3.3
VWS13-8	72.6 ± 2.6	86.8 ± 2.7
VWS13-10	74.5 ± 2.4	91.2 ± 2.2

6.5 Fermentation

Samples VWS11 and VWS3 (which gave the highest and lowest total reduced sugar yield in the ADR pre-treatment), as well as VWS12 and VWS13 at 4, 6, 8 and 10 bar samples were fermented using *S. Cerevisiae* yeast. The total reduced sugar after EH was analysed and the hexose sugar (mainly glucose) was identified since the yeast has the ability to ferment C6 sugar only. Furthermore, the theoretical ethanol

concentration (if all the glucose released after EH was fermented) was calculated based on 1 mole of glucose produces 2 moles of ethanol. Glucose concentration after EH, theoretical ethanol concentration, actual ethanol concentration and ethanol yield percentage are presented in Table 6-4.

Table 6-5. The hexose sugars concentrations after EH, theoretical ethanol concentrations, actual ethanol concentrations and the ethanol yield percentage for VWS11, VWS3 and all the PDR samples.

Sample	Hexose sugars concentration after EH ($g\ L^{-1}$)	Theoretical ethanol concentration ($g\ L^{-1}$)	Actual ethanol concentration ($g\ L^{-1}$)	Ethanol yield percentage (%)
VWS11	5.50 ± 0.4	2.81 ± 0.1	2.49 ± 0.2	88.7 ± 3
VWS3	3.66 ± 0.3	1.87 ± 0.1	1.74 ± 0.2	93.2 ± 4
VWS12-4	6.10 ± 0.3	3.12 ± 0.1	2.76 ± 0.3	88.5 ± 5
VWS12-6	5.76 ± 0.2	2.95 ± 0.4	2.57 ± 0.1	87.4 ± 5
VWS12-8	5.96 ± 0.2	3.05 ± 0.3	2.63 ± 0.2	86.6 ± 2
VWS12-10	6.26 ± 0.5	3.20 ± 0.2	2.71 ± 0.3	84.9 ± 3
VWS13-4	5.88 ± 0.3	3.01 ± 0.4	2.64 ± 0.3	88.1 ± 4
VWS13-6	6.00 ± 0.1	3.07 ± 0.3	2.69 ± 0.4	87.3 ± 3
VWS13-8	5.95 ± 0.2	3.04 ± 0.3	2.62 ± 0.3	86.1 ± 2
VWS13-10	6.14 ± 0.3	3.14 ± 0.3	2.67 ± 0.4	85.2 ± 4

The data shown in Table 6-4 demonstrates that the difference in ethanol yield percentage among PDR samples was relatively low and all the samples showed great similarity in ethanol yield. The ethanol yield percentage indicates that almost all the available hexose sugars after EH were fermented for all the samples.

The two ADR samples VWS11 and VWS3 in Table 6-4 gave an ethanol yield of 88.7 ± 3% and 93.2 ± 4%, respectively. A similar ethanol yield of 91.8% was reported by da Silva et al (2010) when *S. Cerevisiae* was used on the sugarcane straw. Furthermore, da Silva et al (2010) reported a lower ethanol yield (78%) when C6/C5

modified fermenting strain yeast was used on the sugarcane straw (da Silva et al., 2010).

Although VWS3 sample gave the lowest hexose sugars concentration after EH compared to the other samples in Table 6-4. Nevertheless, VWS3 reached the highest hexose sugars conversion to ethanol with a yield of 93.2 ± 4%. On the other hand, VWS12-10 and VWS13-10 samples which produced the highest hexose sugars yield after EH gave the lowest ethanol yield of 84.9 ± 3% and 85.2 ± 4%, respectively. The decreasing in the fermented hexose sugars could be explained by the pre-treating pressure. Subjecting WS to high-pressure pre-treatment might cause a forming of the inhibitors and un-fermentable complex sugar increases which affect the ethanol yield (Fang et al., 2011).

6.6 Summary

In ADR pre-treatment, the WS samples pre-processed with forage chopper shows a higher sugar yield than the samples pre-processed with hammer mill. Furthermore, pre-processing the WS with single pass results in higher sugar yield than applying two passes for all the samples. Decreasing the gap between the plates in the refiner process increased the sugar yield. The highest total reduced sugar yield after EH (74.6 ± 2.5%) was obtained from sample VWS11.

As for PDR pre-treatment, it was concluded that increasing the refining pressure will increase the total reduced sugar yield after EH. Refining with elevated pressure will remove higher hemicellulose percentage from the WS and consequentially improve EH. The lowest sugar yield was obtained from VWS13-4 and VWS12-4 samples with a yield of 79.6 ± 3.1% and 82.2 ± 2.5%, respectively. Samples VWS13-10 and VWS12-10 which were refined at 10 bar using low and high intensity plates produced the highest sugar yield of 91.2 ± 2.2% and 92.1 ± 3.4%, respectively.

For all the samples refined at various pressures, there were no significant differences in the sugar yield after EH observed from refining with the low and the high intently plates. Both ADR and PDR samples showed a high conversion of hexose sugars to ethanol. The ethanol yields for the ADR and PDR samples varied from 85.2 ± 4% to 93.2 ± 4%. The lowest ethanol yield was obtained from VWS13-10 whilst the highest ethanol yield was obtained from VWS3.

Chapter Seven: Steam Explosion and Liquid Hot Water Pre-treatment

7.1 Introduction

Steam Explosion (SE) and Liquid Hot Water (LHW) pre-treatment experiments on WS were carried out at The Italian National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA) institution as part of collaboration work. The WS used for both SE and LHW pre-treatment was firstly chipped to a length of 2000 µm using a blender at the University of Hull and transported to ENEA.

SE and LHW pre-treatments were conducted using distilled H_2O (SE-H_2O and LHW-H_2O) and also in the presence of H_2SO_4 (3%, wt/wt), which are denoted as SE-H_2SO_4 and LHW-H_2SO_4, respectively.

SE-H_2O and SE-H_2SO_4 pre-treatment were carried out according to the method described in Chapter 2. In summary, 500 g of the chipped WS was soaked for 5 min in 500 mL of H_2O and 500 mL of the H_2SO_4 solution (3%, wt/wt) for the preparation of SE-H_2O and SE-H_2SO_4, respectively. The wet WS was then loaded to the SE vessel and the experiments were conducted at 224 °C for 10 min and at 200 °C for 5 min corresponding to severity log Ro 4.53 and 3.35, respectively.

As for LHW pre-treatment experiment, 5 g of the chipped WS was suspended in 100 mL of distilled water (LHW-H_2O) and 100 of the H_2SO_4 solution (3%, wt/wt) LHW-H_2SO_4. The LHW pre-treatment was conducted in (PARR-type) reactor and stirred at 400 rpm. Towards the end of the pre-treatment, the reactor was kept sealed to cool down to 50 °C before opening the reactor. After the pre-treatment, the sample slurry was then filtered and both liquid and the solid fraction were analysed.

The LHW-H_2O and LHW-H_2SO_4 were carried out at 204 °C and 164 °C with 30 min (holding time) corresponding to log Ro 4.54 and 3.36, respectively.

It was found that the reactor during LHW pre-treatment took a relatively long time to reach the desired pre-treatment temperature. Therefore, the heating and cooling as well as holding period temperature were recorded every 2 min. The temperature recording was initiated when the reactor temperature reached 100 °C and through the holding time (30 min) as well as during the cool-down stage until the reactor temperature dropped below 100 °C. The temperature readings with time are graphically shown in Figures 7-1 and 7-2 for the LHW-H_2O and LHW-H_2SO_4, respectively. The red line in Figures 7-1 and 7-2 represent the holding time (30 min) for LHW-H_2O and LHW-H_2SO_4 at 204 °C and 164 °C, respectively.

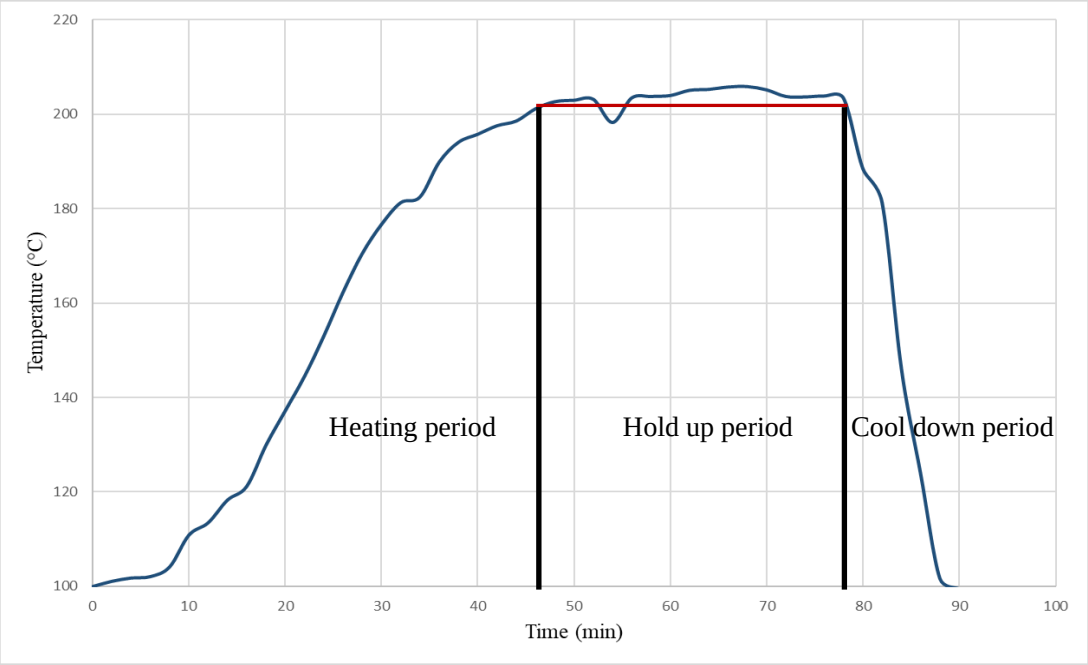


Figure 7-1. Temperature detection with time for LHW-H_2O pre-treatment.

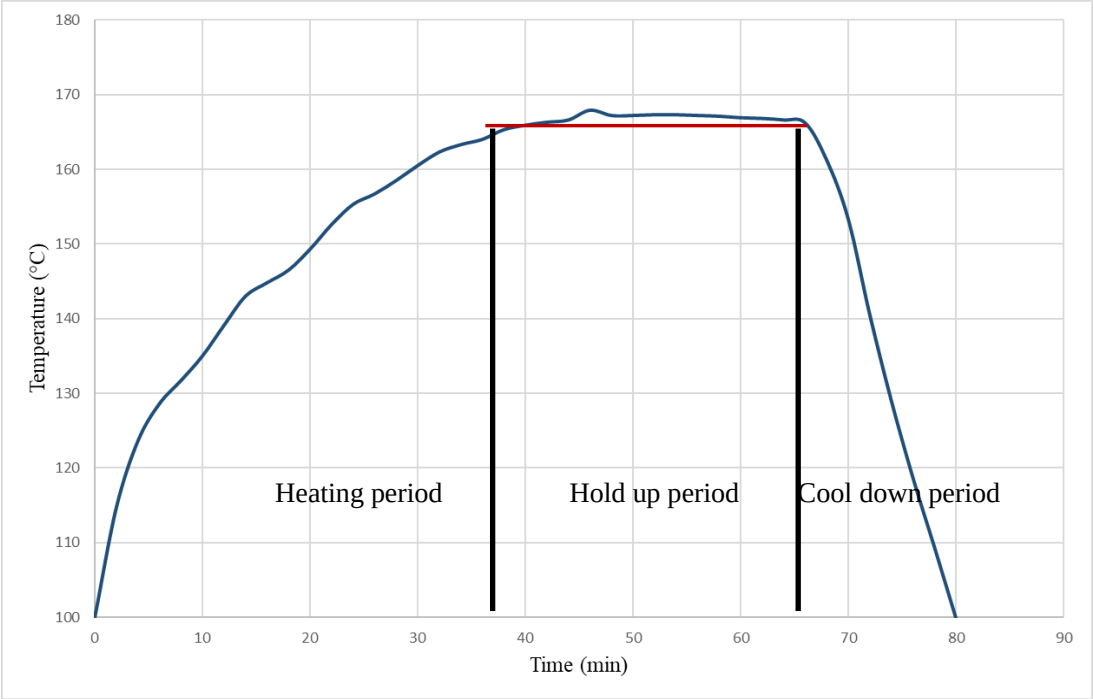


Figure 7-2. Temperature detection with time for LHW-H_2SO_4

It is noticeable from Figures 7-1 and 7-2, that the reactor consumed ~46 min and ~34 min to reach the set temperature for the LHW-H_2O and LHW-H_2SO_4, respectively. There was a slight fluctuation in temperature during the hold time for both samples due to the control system in the reactor. Both LHW-H_2O and LHW-H_2SO_4 consumed approximately 14 min to cool-down below 100 °C.

Since the reactor took a relatively long time to reach the pre-treatment temperatures. Therefore, the LHW pre-treatment period was considered from the time the reactor reached 100 °C until it was cooled down back to 100 °C. As a result, the average severity Log Ro was calculated for both LHW-H_2O LHW-H_2SO_4.

One of the project objectives was to perform SE and LHW at the same severity for comparison. Since the average severity for LHW was nominated as the LHW actual severity. Therefore the SE pre-treatment conditions and subsequently the severity was altered to match the LHW actual severity.

The designed and actual severity and pre-treatment conditions for LHW-H_2O, LHW-H_2SO_4, SE-H_2O and SE-H_2SO_4 are given in Table 7-1.

Table 7-1. The designed and the actual Log Ro as well as the pre-treatment conditions for the LHW-H_2O, LHW-H_2SO_4, SE-H_2O and SE-H_2SO_4 samples.

Pre-treatment type	**Temperature (°C)**	**Time (min)**	**Log Ro**	**Std-error**	**Note**
LHW-H_2O	204	30	4.54	---	Designed Log Ro
LHW-H_2O	191	94	4.66	± 0.084	Actual Log Ro
LHW-H_2SO_4	164	30	3.36	---	Designed Log Ro
LHW-H_2SO_4	159	78	3.62	± 0.183	Actual Log Ro
SE-H_2O	220	10	4.53	---	Designed Log Ro
SE-H_2O	224	10	4.65	0.084	Actual Log Ro
SE-H_2SO_4	190	5	3.35	---	Designed Log Ro
SE-H_2SO_4	200	5	3.64	0.205	Actual Log Ro

The designed severity was chosen based on previous experiments done by ENEA. It can be seen from Table 7-1 that the actual severity log Ro for LHW-H_2O and LHW-H_2SO_4 is slightly higher than the designed severity. The designed severity for LHW-H_2O and LHW-H_2SO_4 represent the severity during the 30 min holding time period only at 204 °C and 164 °C, respectively. Furthermore, the actual temperature for LHW-H_2O (191 °C) and LHW-H_2SO_4 (159 °C) represent the average temperature during the pre-treatment time 94 min and 78 min, respectively.

7.2 Sugars Recovery

7.2.1 Steam Explosion

After SE-H_2O and SE-H_2SO_4 pre-treatment experiment was established, the slurry was filtered and both liquid fraction and water insoluble solid (WIS) was analysed using the National Renewable Energy Laboratory (NREL) standard protocol (Sluiter et al., 2008a, Sluiter et al., 2008c). Glucose and galactose were considered as a cellulose whilst xylose and arabinose were considered hemicellulose. The results are displayed in Table 7-2.

Table 7-2. The composition analysis for the liquid fraction and the WIS after applying SE-H_2O and SE-H_2SO_4 pre-treatment.

Chemicals	SE-H_2O			SE-H_2SO_4		
	Liquid fraction (mg)	Solid fraction (mg)	Lost (mg)	Liquid fraction (mg)	Solid fraction (mg)	Lost (mg)
Cellulose	58 ± 2.8	380 ± 3.1	---	95 ± 2.5	346 ± 3.1	---
Hemicellulose	88 ± 3.1	103 ± 3.2	---	136 ± 2.2	74 ± 3.2	---
Lignin	15 ± 3	159 ± 4.4	---	59 ± 1.9	109 ± 4.4	---
Others	24 ± 2.8	22 ± 3.5	---	37 ± 2.6	16 ± 3.5	---
CH_2O_2	1.6 ± 0.2	---	---	0	---	---
CH_3COOH	1.4 ± 0.2	---	---	2.8 ± 0.2	---	---
HMF	1.7 ± 0.3	---	---	2.5 ± 0.2	---	---
$C_5H_4O_3$	4.3 ± 0.4	---	---	8.7 ± 0.3	---	---
Lost	---	---	142 ± 2.6	---	---	114 ± 2.9
Total	1000			1000		

The statistical calculation in Table 7-2 was based on 1000 mg of WS dry matter. For the SE-H_2O sample, the results showed that around 88 ± 3.1 mg (which represent about 27% of the total hemicellulose in the un-treated WS) was extracted in the liquid fraction. On the other hand, only 13% (58 ± 2.8 mg) of cellulose was extracted in the liquid fraction. Moreover, an insignificant proportion of lignin (15 mg) which is equivalent to 8% of the available lignin was soluble in the liquid fraction. In addition, applying SE-H_2O pre-treatment increased the cellulose content in the WIS from 43.6% to 57.2% compared to the un-treated WS. Alvira et al (2016) reported that, 58.6% of cellulose was recovered in the WIS after applying SE on WS at log Ro 3.944 (which is resembled 200 °C and 10 min). Moreover, Alvira et al (2016) reported that 9.9% and 9.5% of glucose and xylose was extracted in the liquid fraction, respectively. Although the cellulose content in the WIS was analogous, the extracted sugars in the liquid fraction reported in the current study were higher than the results reported (Alvira et al., 2016).

The addition of H_2SO_4 to the SE improved hemicellulose and lignin extraction significantly. Approximately 42% of the hemicellulose was extracted in the liquid fraction which is about 2.5 times higher than hemicellulose extracted from SE-H_2O. Moreover, lignin solubility increased approximately 4 times in SE-H_2SO_4 compared to SE-H_2O. The lignin removal from SE-H_2SO_4 was about 31.4% of the initial lignin in the un-treated WS.

The H_2SO_4 effect exceeded hemicellulose and lignin to include cellulose extraction in the liquid fraction. The results in Table 7-2 showed that about 21.8% of cellulose was extracted in the liquid fraction. Nevertheless, cellulose percentage increased in the WIS from 43.6% to 63.5% compared to un-treated WS. Furthermore, it can be also

noticed that the inhibitors such as CH_2O_2, CH_3COOH, HMF and $C_5H_4O_3$ concentration increased in SE-H_2SO_4 sample.

Chen et al (2011) reported SE pre-treatment on rice straw was conducted at 180 °C for 2 min with the present of H_2SO_4 at different concentrations (1% to 15%). It was concluded that hemicellulose concentration in the WIS decline with increase in the acid concentration and levelled up at approximately 3% (wt/wt). Moreover, it was reported that using H_2SO_4 increases cellulose content in WIS from 34.5% to 49.3% (Chen et al., 2011a). A higher cellulose concentration in the WIS (63.5%) was obtained from SE-H_2SO_4 sample.

After SE pre-treatment, 1 g of WIS collected from SE-H_2O and SE-H_2SO_4 was subjected to EH by using Cellic CTec2 enzyme in sodium citrate buffer (pH 5.8).

After EH, it was found that cellulose produced predominantly glucose and a minor amount of galactose whilst hemicellulose produced mainly xylose and a minor amount of arabinose. Due to the low amount of galactose and arabinose released after EH, hexose sugars from cellulose and pentose sugars for hemicellulose will be referred to as glucose and xylose, respectively.

Cellulose (as hexose sugars), hemicellulose (as pentose sugars) yield after EH for both SE-H_2O and SE-H_2SO_4 samples are presented in Table 7-3.

At the end of EH, the aqueous solution was centrifuged at 4400 rpm for 10 min and filtrated with 2 µm filter paper. The solid residue was dried, weighted and reported as a solid remaining.

Table 7-3. The total sugars available in the WIS and the total sugars liberated after the EH process for the SE-H_2O and SE-H_2SO_4 samples.

Compositions	SE-H_2O		H_2SO_4	
	WIS (mg)	After EH (mg)	WIS (mg)	After EH (mg)
Cellulose (Glucose)	380 ± 3.1	314 ± 3.2	346 ± 3.1	318 ± 3.2
Hemicellulose (Xylose)	103 ± 3.2	89 ± 2.3	74 ± 3.2	66 ± 2.3
Lignin	159 ± 4.4	151 ± 3.1	109 ± 4.4	105 ± 3.1
Others	22 ± 3.5	---	16 ± 3.5	---
Solid reaming	---	110 ± 4.2	---	56 ± 4.2
Total	664	664	545	545

The solid residue was essentially containing lignin with non-hydrolysed cellulose and hemicellulose as well as undefined material.

As can be seen from Table 7-3, glucose liberated from WIS for SE-H_2O and H_2SO_4 was 314 ± 3.2 mg and 318 ± 3.2 mg, respectively. The xylose liberated from WIS was found to be 89 ± 2.3 mg and 66 ± 2.3 mg for SE-H_2O and H_2SO_4, respectively. The results in Table 7-3 indicates that the majority of the monosaccharide sugars in cellulose and hemicellulose were liberated after EH.

Similar work on acid effect during SE pre-treatment was carried out by Linde et al (2008). The WS was pre-soaked in H_2SO_4 for 60 min. The pre-soaked WS was dewatered by applying pressure filtration to obtain a dry matter with a moisture content of 30% (wt/wt) before conducting SE. They reported that the optimum SE pre-treatment conditions are 190 °C for 10 min and by using 0.2% H_2SO_4. At these conditions, the total glucose and xylose yield obtained from the pre-treatment liquid

fraction and from EH was 39.6 g and 21.6 g per 100 g DM, respectively (Linde et al., 2008)

A higher glucose yield of 42.3 g per 100 g DM was achieved from SE-H_2SO_4 sample. On the other hand, xylose yield (approximately 20.4 g per 100 g DM) was obtained from SE-H_2SO_4 sample which was slightly lower than to Linde et al (2008).

Glucose and xylose yield percentage after EH based on their availability in the WIS for SE-H_2O and SE-H_2SO_4 are graphically presented in Figure 7-3.

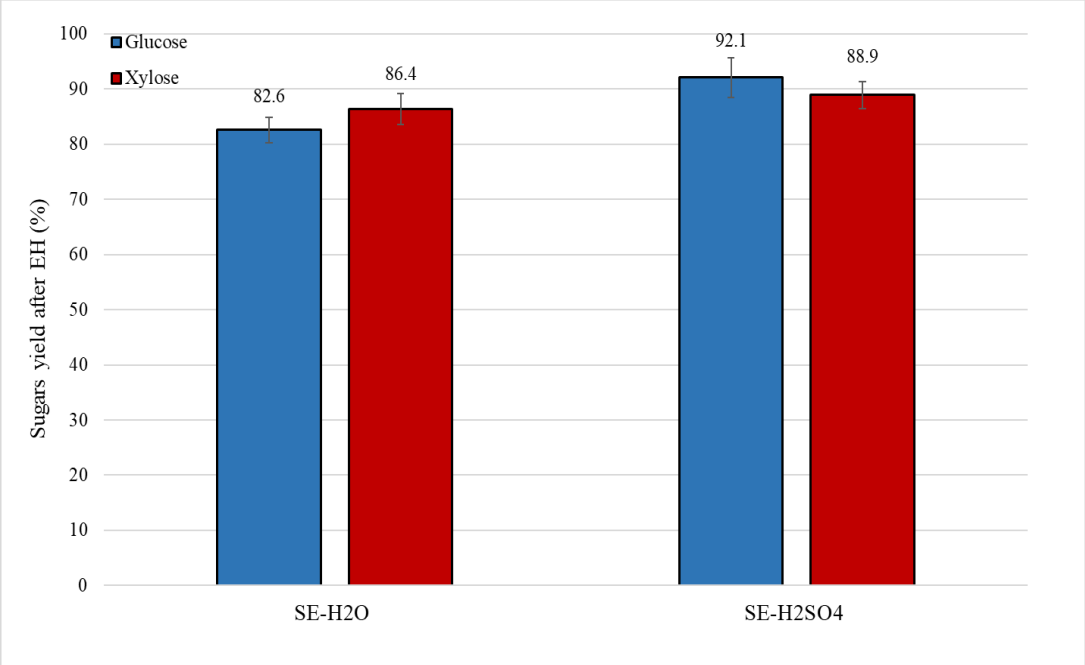


Figure 7-3. Cellulose and hemicellulose yield for the SE-H_2O and SE-H_2SO_4 samples after EH for the WIS.

The result showed that a high yield for glucose and xylose was accomplished after EH from both SE-H_2O and SE-H_2SO_4 samples. The glucose and xylose yield for SE-H_2O reached 82.6 ± 2.3% and 86.4 ± 2.8% based on the available cellulose and hemicellulose in the WIS, respectively. On the other hand, SE-H_2SO_4 gave glucose (92.1%) and xylose (88.9%) yield after EH, respectively. It can be seen from Figure 7-3 that the sugars yield liberated from SE-H_2SO_4 was higher the sugars yield liberated from SE-H_2O after EH.

SE-H_2SO_4 in the current study presented a higher glucose and xylose yield of 92.1% and 88.9% than the yield obtained by Alvira et al (2016) with 91.7% and 66.2%, respectively. The differences in glucose and xylose yield obtained from SE-H_2SO_4 and Alvira et al (2016) can be attributed to several variables including the difference in biomass, the difference in the severity factor and difference in pH value during EH.

A glucose and xylose yield of 102% and 96%, respectively were reported by (Linde et al., 2008). Although it is not very clear how the glucose exceeded 100%. However, the results indicate that the use of dilute H_2SO_4 prior to SE improves sugars yield after EH which is similar to the finding and conclusions of this study.

7.2.1 Liquid Hot Water

After LHW pre-treatment, the slurry was vacuum filtered with 2 µm filter paper. Both the liquid and the solid fraction was analysed using NREL standard procedure for LHW-H_2O and LHW-H_2SO_4 (Sluiter et al., 2008a, Sluiter et al., 2008c). The results are reported in Table 7-4. The results in Table 7-4 were based on 1000 mg of WS dry matter.

Table 7-4. The composition analysis of the liquid fraction and WIS after applying LHW-H_2O and LHW-H_2SO_4 pre-treatment.

Chemicals	LHW-H_2O			LHW-H_2SO_4		
	Liquid fraction (mg)	**Solid fraction (mg)**	**Lost (mg)**	**Liquid fraction (mg)**	**Solid fraction (mg)**	**Lost (mg)**
Cellulose	52 ± 3.3	370 ± 2.8	---	117 ± 3.1	302 ± 3.4	---
Hemicellulose	98 ± 4.2	184 ± 3.3	---	224 ± 2.4	80 ± 3.1	---
Lignin	25 ± 3.4	151 ± 5	---	91 ± 3.3	87 ± 3.8	---
Others	28 ± 3.7	21 ± 3.6	---	36 ± 1.9	15 ± 2.2	---
CH_2O_2	1.9 ± 0.3	---	---	0.64 ± 0.2	---	---
CH_3COOH	3.9 ± 0.1	---	---	5.06 ± 0.4	---	---
HMF	0.8 ± 0.3	---	---	4.8 ± 0.3	---	---
$C_5H_4O_3$	8.4 ± 0.2	---	---	14.1 ± 0.3	---	---
Lost	---	---	56 ± 2.9	---	---	23.4 ± 2.3
Total	218	726	56	492.6	484	23.4

For the LHW-H_2O, it can be seen that 98 ± 4.2 mg (approximately 30%) of the hemicellulose available in the un-treated WS was extracted in the liquid fraction. As for cellulose, 52 ± 3.3 mg (approximately 1%) was extracted in the liquid fraction whilst the majority of cellulose (approximately 84%) was counted in the slurry. Furthermore, approximately 13% of lignin was removed during the LHW-H_2O pre-treatment.

The addition of H_2SO_4 to the LHW pre-treatment enriched the hemicellulose removal to reach 224 ± 2.4 mg (~70%). On the other hand, 117 ± 3.1 mg (~27%) of cellulose was extracted in the liquid fraction in the LHW-H_2SO_4.

Based on the results in Table 7-4, the cellulose in the WIS for the LHW-H_2O and LHW-H_2SO_4 was found to be 50.9% and 62.4%, respectively.

Pérez et al (2008) investigated LHW pre-treatment time and temperature effect on WS. At the optimum pre-temperature conditions (188 °C and 40 min), the hemicellulose extraction in the liquid fraction was 43.6%. Moreover, the cellulose percentage in the WIS varies between 47% - 64% depending on the pre-treatment conditions (Pérez et al., 2008).

Nitsos et al (2013), reported the beech wood was pre-treated with LHW at a range of severity log Ro (1.9 – 4.69). Crystallization index, xylose extraction in the liquid fraction and enzymes digestion on cellulose were monitored to evaluate the severity effects. It was concluded that applying LHW pre-treatment at log Ro= 3.8 – 4.1 will results in maximum xylose extraction in the liquid fraction. Furthermore, extraction of hemicellulose (up to 100%) can be reached by using relatively high pre-treatment severity of 4.7 (Nitsos et al., 2013). Despite the difference in optimum severity between the LHW-H_2O and the optimum severity suggested by Nitsos et al (2013), a reasonable hemicellulose removal of (30%) was established from LHW-H_2O with low inhibitors forming during the pre-treatment. The difference in the pre-treated biomass has a major effect on the optimum severity value (Alvira et al., 2016).

In a similar work reported by Michelin and Teixeira (2016), WS among other biomass was pre-treated with LHW. An observation of hemicellulose extraction, cellulose crystallinity degree and EH yield were detected (Michelin and Teixeira, 2016a). They established 39.26% hemicellulose extraction at log Ro 4.13 which was slightly higher than the hemicellulose extraction obtained from LHW-H_2O of 30.4%.

Adding acid during LHW pre-treatment will improve hemicellulose extraction. However, the risk of inhibitors forming will increases as well (Yang and Wyman, 2004, Sun et al., 2016b). The concentration of HMF, CH_2O_2 and $C_5H_4O_2$ acid were

increased by 6, 1.3 and 1.7 times in LHW-H_2SO_4 comparing to LHW-H_2O, respectively.

The collected WIS from LHW-H_2O and LHW-H_2SO_4 was subjected to EH using Cellic CTec2 at pH 5.8 (see Chapter 2). The dominant monosaccharide sugars were found to be glucose and xylose. Therefore, glucose was counted for cellulose and xylose represent the hemicellulose. The composition analysis after EH for LHW-H_2O and LHW-H_2SO_4 are shown in Table 7-5.

Table 7-5. The composition analysis of the WIS and liquor solution after apply the EH process on the LHW-H_2O and LHW-H_2SO_4 samples.

Chemicals	**LHW-H2O**		**LHW-H2SO4**	
	WIS (mg)	**After EH (mg)**	**WIS (mg)**	**After EH (mg)**
Cellulose (Glucose)	370 ± 2.8	256 ± 3.1	302 ± 3.4	232 ± 3.9
Hemicellulose (Xylose)	184 ± 3.3	121 ± 2.9	80 ± 3.1	76 ± 2.5
Lignin	151 ± 5	145 ± 3.7	87 ± 3.8	84 ± 3.4
Others	21 ± 3.6	---	15 ± 2.2	---
Solid reaming	---	204 ± 3.7	---	92 ± 2.7
Total	726	726	484	484

Table 7-5 showed that 256 ± 3.1 mg (~69.2%) and 232 ± 3.9 mg (~76.8%) of glucose was liberated after EH of LHW-H_2O and LHW-H_2SO_4, respectively. on the other hand, a xylose yield of 121 ± 2.9 (~65.7%) mg and 76 ± 2.5 mg (~95%) was reached after EH of LHW-H_2O and LHW-H_2SO_4, respectively. The glucose and xylose yield was calculated based on their availability in the WIS.

LHW-H_2SO_4 presented a high conversion of the hemicellulose (available in WIS) to pentose monosaccharides sugars after EH compared to LHW-H_2O. On the other hand,

the cellulose conversion to hexose sugar was similar for both LHW-H_2O and LHW-H_2SO_4. The H_2SO_4 addition advantage relies on the EH process improvement and at the same time the reduction of the pre-treatment severity.

The obtained glucose yield from LHW-H_2O in the current study after EH was similar to glucose yield of (59.94%) which was reported by (Michelin and Teixeira, 2016a).

7.3 Fermentation

The EH solution was prepared for fermentation and fermented according to the method described in Chapter 2. As mentioned previously, *S. Cerevisiae* yeast has the ability to ferment hexose sugars only. Therefore, hexose sugars (mainly glucose) concentration after EH was determined for SE-H_2O, SE-H_2SO_4, LHW-H_2O and LHW-H_2SO_4. The ethanol yield was calculated based on the theoretical ethanol concentration (if all the available hexose sugars after EH was fermented). The total reduced sugar and hexose sugars concentrations after EH, ethanol concentration after fermentation and the ethanol yield are listed in Table 7-6.

Table 7-6. The total sugars and the hexose sugars concentrations after EH, the ethanol concentrations and yields for the SE-H_2O, SE-H_2SO_4, LHW-H_2O and LHW-H_2SO_4 samples.

Sample	Total reduced sugar concentration after EH (g L^{-1})	Hexose sugars concentration after EH (g L^{-1})	Ethanol concentration (g L^{-1})	Ethanol yield (%)
SE-H_2O	8.06 ± 0.4	6.28 ± 0.2	2.86 ± 0.3	89 ± 2.2
SE-H_2SO_4	7.92 ± 0.5	6.56 ± 0.4	2.85 ± 0.1	85 ± 3.4
LHW-H_2O	7.54 ± 0.3	5.12 ± 0.4	2.25 ± 0.5	86 ± 3.1
LHW-H_2SO_4	6.16 ± 0.2	4.64 ± 0.2	1.97 ± 0.1	83 ± 2.4

Table 7-6 summarized the ethanol yield for SE and LHW pre-treatment. The obtained results from all the samples showed a similarity in the ethanol yield in which the yield ranged between 83% - 89%.

A lower ethanol yield of 67% from WS pre-treated with acid SE was reported by (Linde et al., 2008). The ethanol yield (67%) was lower compared to ethanol yield obtained from SH-H_2SO_4 which was (85%). The difference in the ethanol yield might be a result of applying SSF method in Linde et al (2008) compared to SHF method used in the current study.

In another study carried by Lu et al (2012), reed was pre-treated with LHW. The WIS was then washed with H_2O until pH 7.0 was reached. Then the WIS was fermented using SSF and SHF fermentation methods. The reported ethanol yield was 85.5% and 99.5% from SSF and SHF methods, respectively (Lu et al., 2012). The washing step of the WIS could result in removing detached inhibitors formed during the pre-treatment process and therefore improves both EH and fermentation. However, the WIS washing step might also lead to losing in the pre-hydrolysate sugars from the pre-treatment process. Moreover, Lu et al (2012) managed to ferment the sugars present in liquid fraction after SE pre-treatment. Therefore, the reported ethanol yield of 99.5% represents the fermentation of the sugars presented in the liquid fraction and WIS. On the other hand, the ethanol yield from SE-H_2SO_4 of (85%) was achieved by fermenting the WIS only.

7.4 Summary

SE and LHW pre-treatment without and with H_2SO_4 improved EH process. The primary effect of SE-H_2O and SE-H_2SO_4 was on hemicellulose extraction. For the SE-H_2O, 27% of the hemicellulose available in the raw WS was extracted in the liquid fraction. On the other hand, the addition of H_2SO_4 (3% wt/wt) in the SE-H_2SO_4 results

in 42% hemicellulose extraction. Furthermore, cellulose extraction in liquid fraction for SE-H_2O and SE-H_2SO_4 was 13% and 21%, respectively.

As for the LHW pre-treatment, 30% of the hemicellulose was extracted in the liquid fraction from LHW-H_2O sample. Similar to SE, the introduction of H_2SO_4 in LHW-H_2SO_4 increased the hemicellulose extraction in the liquid fraction up to two times compared to LHW-H_2O. The removal of hemicellulose from the WS will exposure of cellulose and therefore facilitated enzymes accessible during EH. It was concluded that introducing H_2SO_4 to the SE and LHW pre-treatment will improve hemicellulose extraction and lowering the severity (Yang and Wyman, 2004). Moreover, dilute acid can release soluble sugars as monomers that recombinant organisms can readily ferment them to ethanol (Ingram and Doran, 1995). The LHW-H_2SO_4 and subsequently SE-H_2SO_4 severity was lower than LHW-H_2O and SE-H_2O to reduce the risk of sugar degradations due to the H_2SO_4 present (Saha et al., 2005).

The design severity for both LHW-H_2O and LHW-H_2SO_4 were 4.54 and 3.36, respectively. Due to the time-consuming during heating up period, the average severity was calculated from the time the LHW reactor reached 100 °C until cooled down to 100 °C again. The average (actual) severity was 4.66 and 3.62 for LHW-H_2O and LHW-H_2SO_4, respectively. For the purpose of compassion, the severity of SE-H_2O and SE-H_2SO_4 was adjusted to 4.65 and 3.64, respectively.

Glucose and xylose yield after EH for SE-H_2O and SE-H_2SO_4 were found to be 82.6% and 86.4%, 88.9% and 92.1%, respectively. On the other hand, LHW-H_2O and LHW-H_2SO_4 produced about 69% and 65%, 77% and 77% glucose and xylose, respectively. All the samples showed a similar ethanol yield ranged between 83% - 89% of the glucose available after EH.

In general, LHW and SE pre-treatment gave similar results. However, SE-H_2O and SE-H_2SO_4 pre-treatment time was 10 to 5 min, respectively. On the other hand, LHW-H_2O and LHW-H_2SO_4 holding time was 30 min omitting heating up and cooling down periods.

The overall sugar recovery yield including the extracted sugars in the liquid fraction and the sugars liberated after EH of the LHW-H_2O, LHW-H_2SO_4, SE-H_2O and SE-H_2SO_4 samples are presented in Figure 7-4.

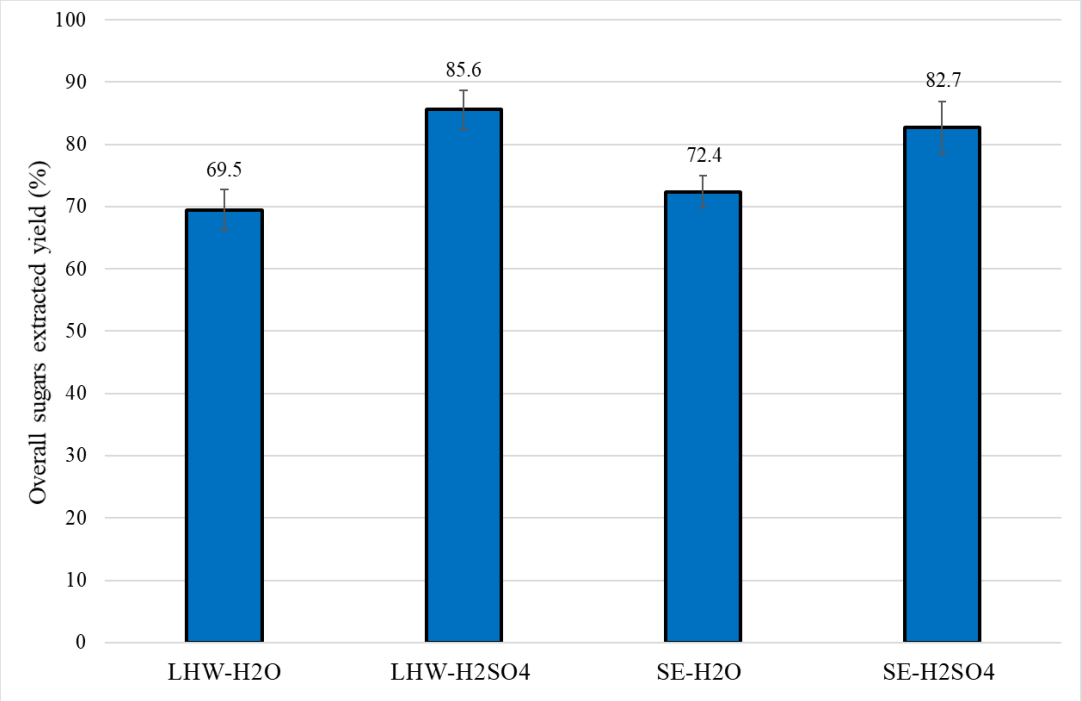


Figure 7-4. Overall sugar recover yield (sugars extracted in the liquid fraction and after EH) for the LHW-H_2O, LHW-H_2SO_4, SE-H_2O and SE-H_2SO_4 samples.

Chapter Eight: Conclusions and Future Work

8.1 Conclusions

Several pre-treatment methods including grinding, microwave, ADR, PDR, SE and LHW have been developed to pre-treat the WS. The pre-treatment effectiveness was determined by evaluating the total reduced sugar yield after EH, ethanol yield after fermentation and hemicellulose removal percentage (if applicable).

In grinding pre-treatment, WS was grounded to particle size range (> 2000 – < 250 µm) using ceramic disk grinder. The EH was carried out at pH 4.8 using three enzymes cocktails namely: Celluclast 1.5 L supplements with Novozymes 188 enzymes, Cellic CTec2 and endo-1, 4-β-Xylanase enzymes. The results demonstrate an increase in total reduced sugar yield after EH with the decrease of WS particle size. The high total reduced sugar yield of 34.0 ± 4.1%, 58.0 ± 5.5% and 35.3 ± 2.6% were obtained after applying EH on the grounded WS to a particle size of < 250 µm sample using Celluclast 1.5 L supplements with Novozymes 188 enzymes, Cellic CTec2 and endo-1, 4-β-Xylanase enzymes, respectively.

The pH effect on EH was evaluated by conducting EH using Celluclast 1.5L supplement with Novozymes 188, Cellic CTec2 and endo-1, 4-β-Xylanase enzymes at different pH values. The grounded WS < 250 µm was used in the pH investigation. The results demonstrate that conducting EH at pH 5.6 – 6.0 will improve the EH process and increases the total reduced sugar yield for all the enzymes. Furthermore, Cellic CTec2 gave the highest total reduced sugar yield compared to the other enzymes at pH 6.0. Therefore, it was chosen to perform EH for the other pre-treatment methods at pH 6.0.

In microwave pre-treatment process, extracted hemicellulose in liquid fraction after the pre-treatment was calculated alongside with total reduced sugar yield after EH.

The results showed that approximately 87.1% of hemicellulose was extracted when WS pre-treated at 200 °C, 900 W, 30 mL of water and for 120 min. Moreover, when the water insoluble solid (WIS) was subjected to EH, the sugar yield was found to be 92.6% (based on the sugars availability in WIS). At these conditions, Overall sugar recovery yield (in liquid fraction after pre-treatment and after EH) was found to be 93.4%. It was concluded that increasing the pre-treatment time at 200 °C will increase hemicellulose extraction and subsequently total reduced sugar yield after EH. To minimize the pre-treatment time, optimization was performed and the Overall sugar recovery yield of 88.4% was obtained when WS pre-treated for 42.8 min.

The total reduced sugar yield after EH increased with increasing PDR pre-treatment pressure. The maximum total reduced sugar yield of 92.1 ± 3.4% was obtained for the WS pre-treated at 10 bar. On the other hand, applying ADR at plate gap 0 mm (samples VWS11, Table 6-1) produced 74.6 ± 2.5% which was the highest sugar yield after EH established by ADR.

The addition of H_2SO_4 during SE and LHW showed a significant effect on hemicellulose extraction during the pre-treatment and the total reduced sugar yield after EH. SE and LHW pre-treatment without and with H_2SO_4 was carried at severity 4.65 and 3.64, respectively. Approximately 27% and 30% of hemicellulose extraction in the liquid fraction was established after applying SE and LHW without H_2SO_4. On the other hand, approximately 42% and 70% of hemicellulose were extracted after the addition of H_2SO_4 in SE and LHW pre-treatment, respectively. Furthermore, the total reduced sugar yield from SE-H_2O, SE-H_2SO_4, LHW-H_2O and LHW-H_2SO_4 after EH was found to be 72.4 ± 2.6%, 82.7 ± 4.2%, 69.5 ± 3.3% and 85.6 ± 3.1%, respectively. In general, all the samples used in the present work showed a high glucose conversion to ethanol yield by using *Saccharomyces Cerevisiae* yeast during fermentation.

8.2 Future Work

For future work, it is recommended to:

i. Measure energy consumption and cost during the pre-treatment process, EH and fermentation to establish economic analysis for the bio-ethanol production system.

ii. Study pH and temperature effect on the total reduced sugar concentration during EH to have a better understanding of their effects and interaction during EH.

iii. Perform SE and LHW with diverse acids, different concentration and at a range of severity.

iv. Prepare bio-char from EH solid residue and carried out the isothermal study. Moreover, investigate initial concentration and temperature influence on the MB removal rate.

v. Simulate bio-ethanol complete production process in Aspen Hysys in order to optimize each process separately and as a complete process.

vi. Perform microwave pre-treatment and EH at pilot scale and study the influence of upscaling EH on the total reduced sugar yield.

vii. Ferment hexose and pentose sugars by using C6/C5 modified yeast.

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