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ABSTRACT

Nowadays, consumers, worldwide, are becoming increasingly aware of the relationship between food or food constituents and health. Sugar alcohol or polyols, which are being used as sucrose substitutes, are found in small quantities in nature, mainly in fruits and vegetables. The major aim of this research is to produce sugar alcohol from *meranti* wood sawdust using three important phases of the processes; pretreatment (pre-delignification, first treatment and second treatment), hydrolysis and fermentation. The objective of this research is to recover cellulose content and parallel with lignin degradation. Besides, enzymatic hydrolysis of cellulose recovery from pretreatment process to produce glucose with high yield also was being done. Fermentation of glucose production from cellulose to produce sugar alcohol is the major objective of this research. A series of preliminary studies were being performed prior to the investigation of the lignin degradation based on *meranti* wood sawdust to enhance enzymatic hydrolysis and also fermentation process to produce sugar alcohol. Furthermore, the characterization of *meranti* wood sawdust, glucose and sugar alcohol also respectively being discussed and that characterization was being obtained from Fourier Transform Infrared (FTIR), Ultraviolet Visible Spectroscopy (UV-VIS) and Scanning Electron Microscopic (SEM). After PAA treatment, the degree of delignification was increased by 77.5% from total of lignin (after alkaline) and the lignin content was decreased to 5.1%. This result indicated PAA was a very selective delignification agent and this chemical treatment will proceed with second stage of treatment using dilute sulfuric acid as an agent to remove hemicelluloses and lignin. Apparently, the optimal value of 86.0799% was obtained in the pretreatment stage. However, the optimal value of glucose production was 1028.35mg/L after RSM being applied.

ABSTRAK

Dewasa ini, semua barang-barang keperluan bukan sahaja di negara kita malah diseluruh dunia semakin hari semakin meningkat sejajar dengan permintaan masyarakat samada bahan makanan, perubatan malah kesihatan sekalipun. Gula alkohol ataupun dikenali sebagai polyol yang mana digunakan sebagai alternatif daripada sukrosa, telah ditemui dengan kunatiti yang sedikit dalam tumbuhan semulajadi ataupun alam sekitar khususnya pada buah-buahan dan juga sayur-sayuran. Sasaran yang paling besar dalam kajian ini adalah untuk menghasilkan gula alkohol daripada habuk kayu meranti menggunakan tiga kaedah iaitu pra-rawatan habuk kayu, proses penurunan selulosa kepada glukosa dan akhir sekali proses penapaian menggunakan bakteria. Sejajar dengan keperluan kesihatan masyarakat, mereka mula mengambil langkah berhati-hati dalam memilih keperluan nutrien yang terbaik. Polyol mula digunakan sebagai bahan alternatif dan digunakan sebagai bahan utama dalam menggantikan gula khususnya dalam bidang pergigian. Objektif kajian ini adalah untuk melindungi kandungan selulosa sekaligus menyahkan kandungan lignin dalam habuk kayu meranti. Selain itu, proses penurunan selulosa kepada glukosa juga dijalankan. Dan akhir sekali proses penapaian glukosa dijalankan untuk menghasilkan gula alkohol dan ini adalan tujuan utama kajian ini dijalankan. Sebelum itu, beberapa siri pra-rawatan dijalankan untuk mengenalpasti kaedah yang terbaik bagi menyahkan kandungan lignin dalam habuk kayu bagi menggalakkan proses hidrolisis menggunakan enzim dan juga sekaligus menghasilkan gula alkohol dalam proses penapaian. Selain itu, kaedah FTIR, UV-VIS dan SEM digunakan bagi mengkaji sifat-sifat habuk kayu meranti. Dengan menggunakan kaedah RSM, keadaan optimum bagi rawatan mengggunakan PAA tercapai iaitu 77.5% daripada seluruh kandungan lignin. Selain itu, dengan menggunakan kaedah RSM juga, nilai optimum bagi penghasilan glukosa daripada proses hidrolisis menggunakan enzim ialah 86.0799%.

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LIST OF ABBREVIATIONS

ABBREVIATIONS	MEANINGS	PAGE
e.g.	example	4
HPLC	High performance liquid chromatography	8
DNS	Dinitrosalicylic Acid	8
SEM	Scanning electron Microscope	8
FTIR	Fourier Transform Infra red	8
MSW	Material Sewage Waste	11
CO2	Carbon dioxide	20
DNA	Deoxyribonucleic acid	42
DW	Dry weight	54
L:S	Liquid:Solid	59
OFAT	One factor at a time	64
DOE	Design of experimental	64
ANOVA	Analysis of variance	75
PAA	Peracetic acid	90
PRT	Pretreatment reaction time	97
IU	International Unit	119

CHAPTER 1

INTRODUCTION

1.1 GENERAL BACKGROUND

Since the early 1960s, climate change and air quality have become major concern and are often controversial issue in many countries among groups from the governments to various scientific communities (Klass, 1998). Everywhere even in symposiums, in world discussions or in research groups; their discussions are all about on how to decrease the causes of climate change and also to decrease the abundant of waste in the world.

Wood is the most ancient and widely used fuel in the world. In 1976, about 390 million tons of wood fuels were being used in Asia for cooking, heating, generating power and manufacturing of charcoals (Anon, 1978; Ali and Hoi, 1990). It still remains the predominant source of fuel in Malaysia. In Malaysia, wood only accounts for about 10% of the energy consumption due to the country's substantial reserve of oil and natural gas. Peninsular Malaysia is currently harvesting approximately 6000 – 7000 hectares of timber annually or extracting about 9 - 11 million m^3 of logs. This represents only about 60 - 65% of the total harvested tree biomass and the remaining 35 - 40% is being left behind in the field to rot or wastefully burnt. When the logs arrive at the mills, they undergo further processing activities. In general, the average recovery rate of logs by Malaysian mills is less than 50%. The rest of the residues, in the form of sawdust, off cuts, slabs, shavings and bark, are being discarded. This means that about 65-75% of the

total tree biomass harvested in the country will end up as residues (Jalaluddin et al., 1984; Ali and Hoi, 1990).

Due to the abundance of the wood residues especially sawdust in Malaysian mills, it is imperative to innovate the waste into wealth. Probably the greatest potential for developing wood residues is as a valuable source for production of chemicals in the food or pharmaceutical industries would be pay attention. This is because, wood residues have no other potential commercial value and no research has yet been done to improve that waste which would give us benefits except it being useful in chicken and also in vegetable farms.

The other side, tooth decay, diabetes or obesity is one of the most critical problems in the world especially in our country, Malaysia. Malaysia has been experiencing a rapid phase of industrialization and urbanization in recent decades and has often been recognized as a role model for developing economies. Statistics available from several ministries for the last two decades suggest that as the populations achieve affluence, their intake of energy, fats and sugars increase, as being reflected in the rising and now substantial size of the food importation bills. The 'westernization' of global eating habits has also brought about an increase in the number of fast food outlets in Malaysia during the last decade (Ismail, 2002; Ismail et al., 2002). Due to this problem, researchers need to produce some product than can be replaced the role of the sugar to decrease the tooth decay, diabetes, obesity or cardiovascular diseases which are caused primarily from our foods consumptions.

1.2 RESEARCH BACKGROUND

Nowadays, the current rapid developments of the global economy and increase in population and living standards have posed great pressure on energy resources and the environment especially in Malaysia. Research into the development of renewable and

sustainable fuels has been a major endeavor owing to the shortage of petrochemical fuel resources and climate change. In recent years, the interest in biomass energy has increased considerably worldwide. Nowadays, forestry lignocellulosic residues have potential as cheap as materials to produce valuable foods and supplements. Lignocellulosic biomass also being recognized as potential sustainable source for production of power, biofuels and variety of commodity chemicals which would potentially add economic value to biomass. Biomass, especially woody biomass and energy crops, is already an important energy carrier contributing substantially to cover energy demands in many parts of the world (Zhou et al., 2011).

Sugar alcohol is a class of polyols in which sugar's carbonyl (aldehyde or ketone) is reduced to the corresponding primary or secondary hydroxyl group. They have characteristics similar to sugar and are used to improve the nutritional profile of food products owing to health promoting properties such as lower caloric content, noncariogenicity, and low glycemic index and insulin response. Other auspicious qualities as food additives include high enthalpies of solution and lack of reactive carbonyls. Sugar alcohols additionally find many applications in pharmaceuticals, chemical production, oral and personal care, and animal nutrition. They are found naturally in fruits and vegetables and are produced by microorganisms, serving as carbohydrate reserves, storage of reducing power, translocatory compound and osmoprotection.

The production of sugar alcohol involves an extensive process and varies depending on the type of biomass. The process mainly encompasses the upstream and downstream operations which involve pretreatment of the biomass, saccharification, fermentation and product recovery. The biomass pretreatment is a crucial process because it is essential for the fermentable sugars to be released and made available for the fermentation process. However, since the pretreatment process contributes significantly to the cost of production of sugar alcohol, the most economical pretreatment protocol must be selected in order to make sugar alcohol more economically attractive. An

efficient biomass pretreatment procedure must be energy efficient, cost effective, and simple to apply and must not degrade the fermentable sugars (Rabelo et al, 2009; Harun and Danquah, 2011). Traditional industrial production of most sugar alcohols is accomplished by hydrogenating sugars over nickel catalyst under high temperature and high pressure condition. Biosynthetic routes offer the potential for safer, environmentally friendly production with enhanced product specificity. Enzyme based process for the production of sugar alcohols via sugar reduction have been investigated but are not within the scope of the review.

1.3 POTENTIAL OF SUGAR ALCOHOL AS ALTERNATIVE OF SUGAR

Sugar provides quick energy and a concentrated source of calories. This benefit has its downside. However, because consuming too many calories, regardless of the source, contributes to obesity and meanwhile, sugar is a major contributor to tooth decay. Sugar alcohols are sometimes used as a substitute for sucrose. Mannitol, sorbitol, and maltitol occur naturally in fruits. Sugar alcohols add bulk and texture to food such as chewing gum and hard candies. Because they are metabolized by the body more slowly than sucrose, they are useful in foods for people following special diets, such as a diabetic diet (Anderson and Young, 2010).

Consumers say they regularly consume low calorie, sugar free foods and beverages to stay in better overall health or simply because they taste good. Many of these products contain ingredients called sugar alcohols frequently being referred to as polyols. A polyol (or sugar alcohol) is not a sugar, nor an alcohol. Polyols are a group of low digestible carbohydrates derived from the hydrogenation of their sugar or syrup source (e.g., lactitol from lactose). These unique sweeteners taste like sugar but have special advantages.

In addition to their clean sweet taste and unique functional properties, polyols offer important health benefits. For example, they are reduced in calories and do not cause sudden increases in blood sugar levels. Importantly, polyols are not readily converted to acids by bacteria in the mouth and, therefore, do not promote tooth decay. Since most polyols are not as sweet as sugar they are often used in combination with approved low-calorie sweeteners such as acesulfame potassium, aspartame, neotame, saccharin or sucralose. Scientific research supports the fact that these low calorie sweeteners, like polyols, do not promote tooth decay.

In some people, over consumption of polyols containing foods may cause gastrointestinal symptoms, including laxative effects, similar to reactions to beans, cabbage and certain high-fiber foods. Such symptoms are dependent upon an individual's sensitivity and the other foods eaten along with the polyol containing product. Any gastrointestinal symptoms (such as a feeling of fullness) from consuming foods with polyols, if they occur at all, are usually mild and temporary. Most people will adapt to polyols after a few days, the same way they do to high fiber foods. Food manufacturers are advised to inform consumers of these possible effects through product labeling (Anderson and Young, 2010).

1.4 STATEMENT OF THE PROBLEM

Nowadays, consumers, worldwide, are becoming increasingly aware of the relationship between food or food constituents and health. Functional products, i.e. foods or beverages offering specific health benefits beyond basic nutrition, are increasingly valued. Within functional foods, probiotics, with prebiotics, represent one of the fastest growing sectors. Probiotics foods are fermented formulates containing sufficient numbers of selected live microorganisms to beneficially modify the intestinal microbiota of the host (Fuller, 1989; Havenaar and Huis in't Veld, 1992).

Sugar alcohol or polyols, which are being used as sucrose substitutes, are found in small quantities in nature, mainly in fruits and vegetables. Polyols are interesting properties, such as anticariogenicity, a strongly negative heat solution, and being slowly or incompletely metabolized. Polyols improve confectionaries with their cooling effect on the mouth and are recommended to patients with diabetes, heart disease, or cancer of the gastrointestinal tract (Suryadi et al., 1999).

Obesity is a growing problem not only in Western countries but also in Eastern countries. Therefore, special diets and dietary ingredients for body weight control are of major interest to the food industry. Belonging to the family of low calorie sugar, polyols such as mannitol and sorbitol are non metabolized sugar alcohols that can replace sucrose or lactose in food products, with a nearly equivalent sweetness and taste (Salminen, 1993).

In the higher education institutions, many scientists have worked with new plant foods that have the potential to be effective natural remedies and as well as food supplement and health care. As a result, they come up with new products in the form of drinks, juice, herbal tea, dietary supplements or even pills. In Malaysia, many plant foods that have the potential to be effective natural remedies have been abandoned due to lack of information about its nutritional values. However in this case, we try to use waste from our forest industry to make it valuable soon.

Parallel to that problem, environmental pollution also is a worldwide threat to public health has given rise to a new massive industry for environmental restoration. Biological degradation, for both economic and ecological reasons, has become an increasingly popular alternative for the treatment of agricultural, industrial, organic as well toxic waste (Milala et al., 2009). For this research, we use the waste from forestry sawdust was being used to make it valuable. This research is therefore aimed to determine the content of cellulose in wood sawdust and to hydrolyze the cellulose to produce glucose and then to produce the sugar alcohol from fermentation of glucose. The

scope of this study is also to compare the highest cellulose content among hardwood sawdust, softwood sawdust and industry sawdust.

1.5 OBJECTIVE OF THE RESEARCH

The major aim of this research is to produce sugar alcohol from *meranti* wood sawdust using three important phases of the processes; pretreatment (pre-delignification, first treatment and second treatment), hydrolysis and fermentation. To achieve the objectives, the following stages have been carried out:

1) Studied the recovering of cellulose from pretreatment process using pre-delignification treatment, first stage of pretreatment and second stage of pretreatment,

2) Optimized the removal of lignin in raw materials (*meranti* wood sawdust) using design of expert software version 6.0.8,

3) Optimized the production of glucose from enzymatic hydrolysis of cellulose. The cellulose fibers were produced from treatment of *meranti* wood sawdust. In this phase, design of expert software version 6.0.8 also being used, and

4) Produced sugar alcohol (sorbitol especially) from fermentation of glucose that was being produced from hydrolysis process and also commercial glucose.

1.6 SCOPE OF THE RESEARCH

The study involved the pretreatment process, enzymatic hydrolysis process and fermentation process. Details of the scopes of research are as below:

- Determination of chemical compound in untreated *meranti* wood sawdust,
- Determination of diluted sulfuric acid concentration, time and temperature in second stage of pretreatment,
- Determination of lignin degradation using Kappa Number equation,
- Optimization of lignin degradation in second stage of pretreatment using Design of Experimental software (version 6.0.8),
- Kinetic study of cellulase and β-Glucosidase enzyme,
- Optimization of glucose production in enzymatic hydrolysis process,
- Analysis of the glucose production using DNS method,
- Investigation of cultivation period, pH, and temperature of fermentation process,
- Analysis of the amount of sugar alcohol being ferment using HPLC (column: Rezex RCM-Monosaccharide Ca+2, detector: RI, size: 300 X 7.8 mm, mobile phase: H_2O, flowrate: 0.6mL/min, temp: 75^0C), and
- Characterization of *meranti* wood sawdust using SEM method and FTIR method.

1.7 RATIONALE OF THE RESEARCH

As the general public becomes more health conscious, they have also become more aware of the significance of good nutrition. The polyols are being used to sweeten a number of sugar free products, the most important of which in terms of caries control in the United States in chewing gum. The polyols are most frequently being used in chewing gum are sorbitol, a hexatol derived from glucose, and xylitol, a pentatol that occurs widely in nature. It has been well known for years that polyols do not promote caries because polyols are being metabolized either slowly or not at all in dental plaque.

The most commonly used polyol in the United States is sorbitol, which is the standard sweetener in several sugar free chewing gums and over the counter medicines. Sorbitol is 60 percent as sweet as sucrose and is much less expensive than xylitol. Sorbitol is less effective than xylitol in controlling caries, but its lower cost makes it appealing to food manufacturers. In terms of cariogenesis, sorbitol has an advantage over sugars because, in small amounts, it does not lower the pH of plaque to a point where enamel demineralization occurs.

The use of biological means has greater advantages over the use of chemicals for degradation because biotechnological synthesized products are less toxic and environmentally friend (Liu et al., 1998). With current consumers demanding for low calorie, sugar free products, as well as the increased availability of sorbitol and innovations in food technology, additional good tasting sugar free and reduce calorie products are being expected to be available. These products may assist consumers in maintaining good oral health, maintaining or reducing weight and reducing glycemic load.

CHAPTER 2

LITERATURE REVIEW

This chapter reviews the materials properties including biomass residues and *meranti* wood sawdust as well as materials that could be applied in this research to produce cellulose fibers and then continues with other process to produce other valuable chemicals such as glucose and sugar alcohol. Due to the current issues in relation to environmental requirement and the loss of life property, the alternative of the sugar or sweeteners need to be explored to convert the waste from our world to become valuable things.

2.1 BIOMASS RESIDUES

When energy experts talk about biomass, they mean organic matter that could be used to produce energy to heat homes, power automobiles, or produce electricity. Wood, for example, is a kind of biomass burned in fireplaces and campfires to produce heat and to cook food. The term biomass refers to plant materials and animals wastes being used for energy, especially tree and grass crops, and forestry, agricultural, and urban wastes. It is the oldest source of renewable energy known to human, being used since our ancestors learned the secret of fire (Garza, 2007).

Biomass sources are therefore diverse. Biomass has always been a major source of energy for mankind, and accounts for about 14% of the world's total energy supply

(Bhattacarya et al., 2005). The current rapid developments of global economy and the increase in population and living standards have posed great pressure on energy resources and the environment especially in Malaysia. Research into the development of renewable and sustainable fuels has been a major endeavor owing to the shortage of petrochemical fuel resources and climate change. In recent years, the interest in biomass energy has increased considerably worldwide. There are several reasons for this; biomass is widely available and it has the potential to produce modern energy carriers such as electricity and liquid transport fuels that are clean, convenient and easily used in the present energy supply system. Biomass energy can also be produced in a carbon neutral way and can contribute to (local) socioeconomic development. The present contribution of modern biomass to the primary energy consumption is estimated at 6 or 7 $EJyr^{-1}$. Combined with traditional biomass, its share in the total primary energy supply is 9 – 13% (Hoogwijk et al., 2009).

The sustainable biomass resource available for energy use could be divided into five categories: agricultural residues, forest residues, and biomass production on surplus degraded land, organic wastes, and others (Table 2.1). Biomass production on surplus degraded land is the biomass that can be produced on deforested or agriculturally degraded or marginal land that is still suitable for grassland, or reforestation use. Organic wastes are the biomass released after human material use. These include MSW, sludge from sewage disposal plants, waste wood and so on. The dominant product is MSW, which consists of combustible and incombustible components.MSW usually can be harmlessly disposed in three main methods: landfill, compost, and incineration. The combustible organic components such as waste rubber tyre, waste plastic and waste paper, could be directly combusted in MSW incinerators or blended with other fuel such as coal. Other biomass resources include biomass from animal manure or directly on used as a feedstock for material end used options like pulp and paper, or for petrochemical industry, or small quantity biomass energy crops, e.g., short rotation crops, fast growing energy trees, and some kinds of grass species, etc (Zhou et al., 2011).

Table 2.1: The biomass resource categories distinguished to assess the theoretically available potential of biomass for energy use

Category	Description
Agricultural Residues	The residues released together with food production and processing
Forest Residues	The residues released together with food production and processing
Biomass production on surplus degraded land	The biomass that can be produced on deforested or agriculturally degraded or marginal land that is still suitable for grassland, or reforestation use
Organic wastes	Biomass released after human's materials use, e.g., MSW, sludge from sewage disposal plant, waste wood, etc.
Others, e.g., animal manure, bio materials, small quantity biomass energy crops	Biomass from animal manure or directly on used as a feedstock for material end-use options like pulp and paper, or fro petrochemical industry, or small quantity biomass energy crops, etc.

Source: (Liao et al., 2004; Zhou et al., 2011)

The idea of generating energy from forest biomass has a long history; humans have been using wood heat for millennia and are still doing so today. Over the years more sophisticated technologies have been developed, and today woody biomass could also be converted to bioelectricity, biofuel (liquid energy), and biogas. The popularity of biomass in industrialized countries waned for a period in the face of competition from fossil fuels, but is experiencing a global resurgence as more carbon neutral and locally beneficial sources of energy are sought (Demirbas, 2009; Stidham, 2011).

Traditionally, all biomass fuels were being used more or less in the same geographical region in which they were being produced (Hillring et al., 2000; Parikka, 2004). In recent years, this pattern has been changed, especially in Northern Europe, by large scale use of recycled wood, forest and wood residues and densified biofuels for district heating (Vesterines et al., 2001; Parikka 2004). Solid biofuels like wood residues (e.g. industrial byproducts: bark and sawdust, recycled wood), upgraded biofuels (e.g. wood pellets, briquettes) and wood chips are today traded, e.g. in Europe and North America. In several countries there is growing interest in international biomass trade because it could provide biomass fuels at lower prices (Hillring et al., 2000; Parikka, 2004).

Biomass, especially woody biomass and energy crops, is already an important energy carrier contributing substantially to cover energy demands in many parts of the world. Biomass is an abundant and renewable energy source with low net CO_2 emission (Zhou et al., 2011). Biofuel from biomass becomes a sink for GHG because biomass is low in carbon and carbon is fixed during the biomass growth. This energy carrier has the potential to contribute even more to provide energy to substitute the use of fossil fuel energy, especially in industrialized countries as well as in developing countries. However, the exploitation of this potential is only advisable if there are promising economic and/or environmental effects. An important aspect is the assessment of the potentials and appraisal of resources in the setting of targets and limits for their practical utilization. There is also an ongoing discussion on the acceptability and availability of renewable energy. International biofuel trade is going to be an important factor in the future. These facts are beneficial when considering production of densified biofuels based on biomass (Parikka, 2004).

Biomass has a large energy potential. A comparison between the available potential with the current use shows that, on a worldwide level, about two fifths of the existing biomass energy potential is used. In most areas of the world the current biomass use is clearly below the available potential. Only for Asia does the current use exceed the available potential, i.e. non-sustainable biomass use. Therefore, increased biomass use,

e.g. for upgrading is possible in most countries. A possible alternative is to cover the future demand for renewable energy, by increased utilization of forest residues and residues from the wood processing industry, e.g. for production of densified biofuels (Parikka, 2004).

Experts and decision makers widely agree that alleviation of climate change is mankind's greatest threat and challenge for the 21st century and beyond (Yong et al., 2007). Biomass fuels and residues can be converted to more valuable energy forms via a number of processes including thermal, biological, and mechanical or physical processes (Bridgwater, 2011). Biomass has gained increased attention in the past decade because it not only provides an effective option for the provision of energy services from a technical point of view but is also based on resources that can be utilized on a sustainable basis all around the globe (Yong et al., 2007).

At present, biomass residues from the forestry or agricultural sector are mostly used to produce modern biomass energy carriers at low costs. However, biomass originating from energy crops has a much larger potential than biomass from residue flows (Berndes et al., 2003; Hoogwijk, 2009). But specially cultivated biomass for energy purposes currently results in high fuel and electricity costs in most cases; particularly where land and labor costs are high. Therefore, insight in potential cost and supply developments of energy crops and biomass energy carriers is important. Cost and supply curves of biomass energy have been studied at a regional or national level but no research has been conducted at global scale. This would be interesting for both energy modelers and engineering approaches for policy assessments (Junginjer, 2001; Hoogwijk, 2009).

The wood residues potential is especially important in countries where forests cover a considerable part of the whole land area. It is obvious that the potential from wood residues is in the range of about one fourth of the overall potential of all investigated biomass streams (Parikka, 2004). The use of biomass differs significantly

throughout the world. Residues produced at industrial processing sites, like bark and sawdust in sawmills, are currently the largest commercially used biomass source.

2.2 WOOD SAWDUST

Biomass, whether as sugar crops, starch crops, or cellulosic materials, provides a unique resource for sustainable production of organic fuels and chemicals that are now primarily being made from petroleum. Furthermore, cellulosic materials including agricultural (e.g. corn stover) and forestry (e.g. sawdust) residues and herbaceous (e.g. switch grass) and woody (e.g. poplar trees) crops can be sufficiently abundant to provide a major resource for making commodity products (Charle, et al., 2005).

Wood sawdust is among the most abundant and widely distributed biomass resources. However, due to its complex multi component structure it is difficult to use it directly as a chemical feedstock. Normally it is first being separated into its main components, cellulose, lignin, and hemicelluloses, which are then further processed, while transformations of unseparated wood are less common (Andrej et al., 2009).

Sawdust is composed of fine particles of wood. This material is being produced from cutting with a saw, hence its name. It has a variety of practical uses, including serving as mulch, or as an alternative to clay cat litter, or as a fuel, or for the manufacture of particleboard. Until the advent of refrigeration, it was often being used in ice houses to keep ice frozen during the summer. Historically, it has been treated as a byproduct of manufacturing industries and can easily be understood to be more of a hazard, especially in terms of its flammability. It has also been used in artistic displays and as scatter. It is also sometimes used in bars in order to soak up spills, allowing the spill to be easily swept out the door.

It is estimated that there are 3870 (10^6) ha of forest worldwide or 30% of the earth's land area, of which about 95% are natural forests and 5% are plantations (FAO, 2001; Parikka, 2004). Tropical and subtropical forests comprise 56% of the world's forests, while temperate and boreal forests account for 44% (Parikka, 2004). The average area of forest and wooded land per inhabitant varies regionally (Table 2.2).The area varies between 6.6 ha in Oceania, 0.2 ha in Asia, and 1.4 ha in Europe (3.4 ha in the Nordic countries). This fact indicates that the potential contribution of wood to the energy supply also varies from country to country. There are also large regional differences in accessibility to forests. Therefore, the possibilities for production of densified biofuels vary widely between regions. The total above ground wood volume (m^3) and woody biomass (tonnes) in forest has been estimated in 166 countries, representing 99% of the world's forest area (FAO, 2001; Parikka, 2004). The world's total aboveground biomass in forests is 420 (109) tones (Table 2.2), of which more than 40% is being located in South America and about 27% is in Brazil alone. The worldwide average above ground woody biomass is 109 tonnes/ha.

Table 2.2: Forest resources, above ground biomass volume and biomass (m^3 and tonne)

	Forest Area (ha) (10^9)	Volume (m^3/ha)	Volume (m^3) (10^9)	Woody Biomass (tonne/ha)	Woody Biomass (tonne) (10^9)
Africa	649	72	46	109	70
Asia	547	63	34	82	44
Europe	1039	112	116	59	61
North and Central America	549	123	67	95	52
Oceania	197	55	10	64	12
South America	885	125	110	203	179
World	3869	100	386	109	421

Source: (FAO, 2001; Parikka, 2004)

Biomass currently represents approximately 14% of world's final energy consumption (IEA, 1998; Parikka 2004). About 25% of the usage is in industrialized countries, where a significant level of investment in environmental protection has been made to meet emissions standards, especially air emissions (Overend 2002; Parikka 2004). The other 75% of primary energy use of biomass is in heat production for developing country household energy needs and in process heat production for biomass-based industries through the use of their generated residues (Overend 2002; Parikka 2004).

The residues being generated from the forest products industry could be divided into two categories:

(1) Logging residues, generated from logging operations, e.g. from final felling and
(2) Industrial byproducts, generated by the forest industries during processing of timber, plywood, particleboard, pulpwood, etc. (FAO, 1993; Parikka, 2004). Sources and types of residues are shown in Table 2.3.

Table 2.3: Sources and types of residue

Source or residues	Type of residues
Forest operations	Branches, needles, leaves, stumps, roots, low-grade and decayed wood, slashing and sawdust
Sawmilling and planning	Bark, sawdust, trimmings, split wood, planer shavings
Plywood production	Bark, core, sawdust, veneer clippings and waste, panel trim, sander dust
Particle board production	Bark, screening fines, panel trim, sawdust, sander dust

Source: (FAO, 1993)

Nowadays, wood is one of the alternative and renewable resources. Wood is an essential material for man. It is a material source for energy and constructional works. Wood industries such as pulp and paper plants and sawmills generate large quantities of biomass residues every year (Perez et al., 2006). Wood sawdust, byproduct of wood processing, pollutes environmental even though this sawdust are materials suited for biodegradation. Wastes and their disposal become enough substances of environmental concern worldwide especially when these wastes are biodegradable to useful goods and services (Shide et al., 2004). Even though wood is a renewable resource, conservation is essential to forest conservation and environmental protection.

Wood consists of an orderly arrangement of cells with cell walls being composed of varying amounts of cellulose, hemicelluloses, and lignin. The great diversity of woody plants is reflected in their varied morphology and chemical composition (Carmen et al., 2009). Typically, two general groups, hardwoods (angiosperms) and softwood (gymnosperms) can be easily distinguished. Hardwoods have pores or vessel elements that occur among fiber and parenchyma cells. It is well known that the cellulose content ranges from 40 to 50 wt% and the lignin content is comprised between 15 and 25 wt%, while that of the hemicelluloses varies from 15 to 25 wt%. Softwoods are composed of overlapping tracheids, connected by bordered pit apertures, and parenchyma cells and, in some cases, resin canals. Greater concentration of lignin, about 5 to 10% more than in hardwoods, are found in softwoods, and about the same amount of cellulose 40 to 50 %. Less hemicelluloses maybe found in softwood than hardwoods. The chemical composition of softwood is also different from hardwoods with different type of lignin (primarily guaiacyl propane units), hemicelluloses (mannose is the most common constituent) and wood extractives (different terpens, fatty acids, etc). Differences in composition are also common between temperate and tropical hardwoods. Woods such as teak, mahogany, and ebony have greater concentration of lignin and wood extractives than many temperate hardwoods such as maple, birch and aspen (Blanchette et al., 1997).

2.3 LIGNOCELLULOSIC BIOMASS MATERIALS

Lignocellulosic biomass is the most abundant material in the world. Its sources range from trees to agricultural residues. Long ago, these materials were being used as firewood, building materials and animal food. Nowadays, lignocellulosic materials are not just used in their old ways but their applications have expanded into the fiber level as in pulp and paper products. In some cases, the use of lignocellulosic is proceeding to the level of the chemical component itself. For example, cellulose, which is a major chemical constituent of lignocellulosic material, can be used for fibers in the textile industry; while lignin is used as an adhesive component in the composite industry. The chemical components of lignocellulosic can be divided into four major components. They are cellulose, hemicellulose, lignin and extractives. Lignocellulisic materials predominantly contain a mixture of carbohydrate polymers (cellulose and hemicelluloses), lignin, extractives, and ashes. The terms "holocellulose" is often being used to describe the total carbohydrate contained in a plant or microbial cell. Holocellulose is therefore comprised of cellulose and hemicelluloses in lignocellulosic materials (Taherzadeh and Karimi, 2007). Generally, the first three components have high molecular weights and contribute much mass, while the latter component is of small molecular size, and it is available in little quantity. Figure 2.1 shows the illusion of lignocellulosic fibers.

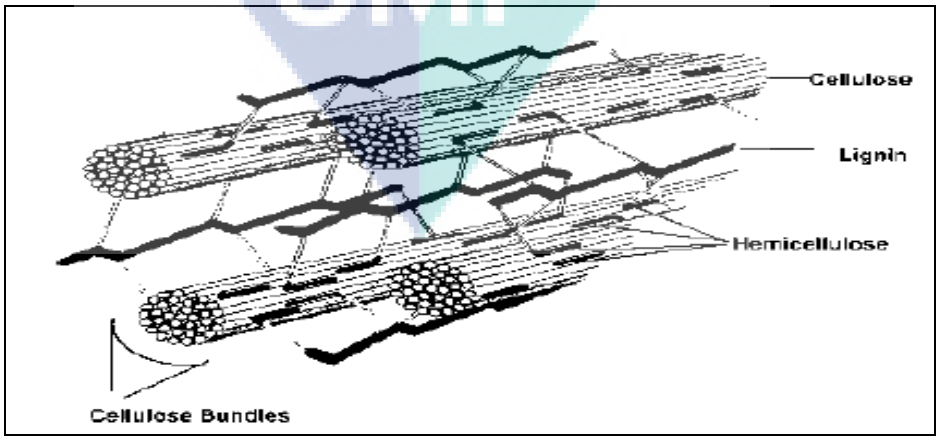
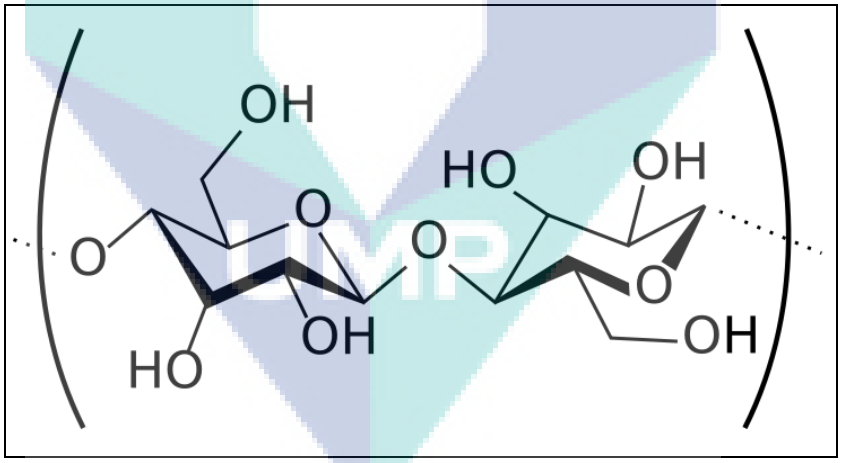


Figure 2.1: Lignocellulose (Lignin, Hemicellulose, Cellulose) (Hammel, 1997)

2.3.1 CELLULOSE

Many biomass materials containing higher cellulose, such as agricultural residues, forest waste, are currently being used as resources to produce chemicals and biofuel. Cellulose is abundantly available and has a potential use as an alternative to fossil resource for production of chemicals and fuels which decrease the generation of CO_2 as compared to the utilization of fossil fuels (Wang et. al., 2011). Cellulose is an unbranched linear polymer. The length of a cellulose molecule (polymer) is being determined by the number of glucan units in the polymer, referred to as the degree of polymerization. The degree of polymerization of cellulose depends on the type of plants and typically is being estimated to be from 2000 to 27000 glucan units (Moroshi, 1991; Sjöström, 1993; Taherzadeh and Karimi, 2007). Figure 2.2 shows the structure of the cellulose.



Source: Wang et al., 2011

Figure 2.2: Structure of cellulose

Cellulose is being composed of D-glucoses monomers joined together by β-1, 4-glycosidic bonds could be primarily converted into glucose. Cellulose is the dominant polymer in the biosphere. It is an optically anisotropic system being made up of poly-

(1→4)-β-D-glucose chains (Atalla et al., 1997). The numerous polar groups make cellulose molecules predestined for building up hydrogen bonds within the molecule and between the different molecules. In the generally accepted structure of cellulose I, intra molecular hydrogen bonds of type's 3-OH-O-5 and 2-OH--O-6 are present for both sides of the chain. As a result of hydroxyl groups showing different polarities, cellulose has different crystalline structure, ranging from cellulose I (native cellulose) to cellulose IV (O'Sullivan, 1997).

By forming intra molecular and intermolecular hydrogen bonds between OH groups within the same cellulose chain and the surrounding cellulose chains, the chains tend to arrange in parallel and form a crystalline supermolecular structure. Then, bundles of linear cellulose chains (in the longitudinal direction) form a microfibril which is oriented in the cell wall structure. The amounts of the carbohydrate polymers and lignin depend on the type of material (Table 2.4).

Table 2.4: Chemical composition of some typical cellulose-containing materials

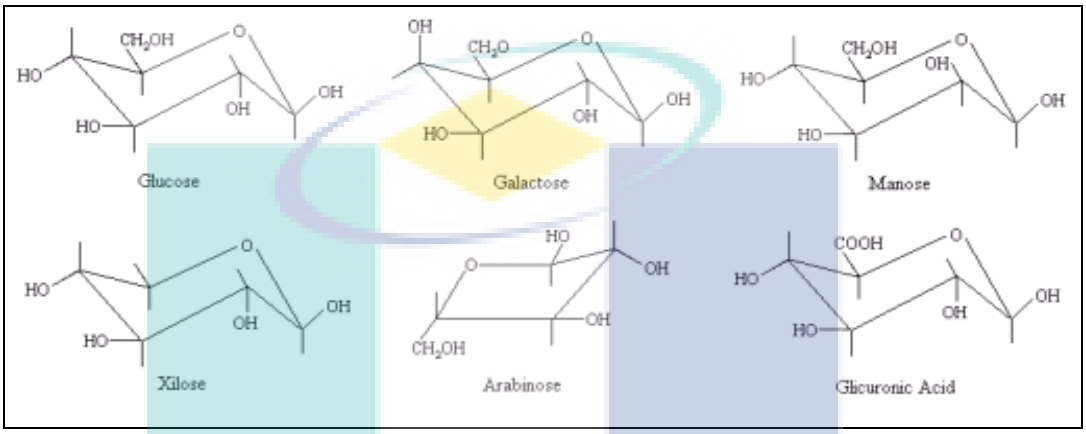
Source	Composition (%)			
	Cellulose	Hemicellulose	Lignin	Extractive
Hardwood	43-47	25-35	16-24	2-8
Softwood	40-44	25-29	25-31	1-5
Bagasse	40	30	20	10
Coir	32-43	10-20	43-49	4
Corn cobs	45	35	15	4
Corn stalks	35	25	35	5
Cotton	96	2	1	0.4
Flax (retted)	71	21	2	6
Flax (unretted)	63	12	3	13
Hemp	70	22	6	2
Henequen	78	4-8	13	4
Istle	73	4-8	17	2
Jute	71	14	13	4
Kenaf	36	21	18	2
Ramie	76	17	1	6
Sisal	73	14	11	2
Sunn	80	10	6	3
Wheat straw	30	50	15	5

Adapted from (Hon, 1996)

2.3.2 HEMICELLULOSE

Hemicelluloses belong to a group of heterogeneous polysaccharides. The amount of hemicelluloses is usually between 11% and 37% of the lignocellulosic dry weight. Hemicelluloses are relatively easily hydrolyzed by acids compared to their monomer

components consisting of xylose, mannose, glucose, galactose, arabinose, and small amounts of rhamnose, glucuronic acid, methyl glucuronic acid, and galacturonic acid (Morohoshi, 1991; Sjöström 1993; Taherzadeh and Karimi, 2007). Figure 2.3 shows the hemicelluloses monomer components.



Source: Taherzadeh and Karimi, 2007

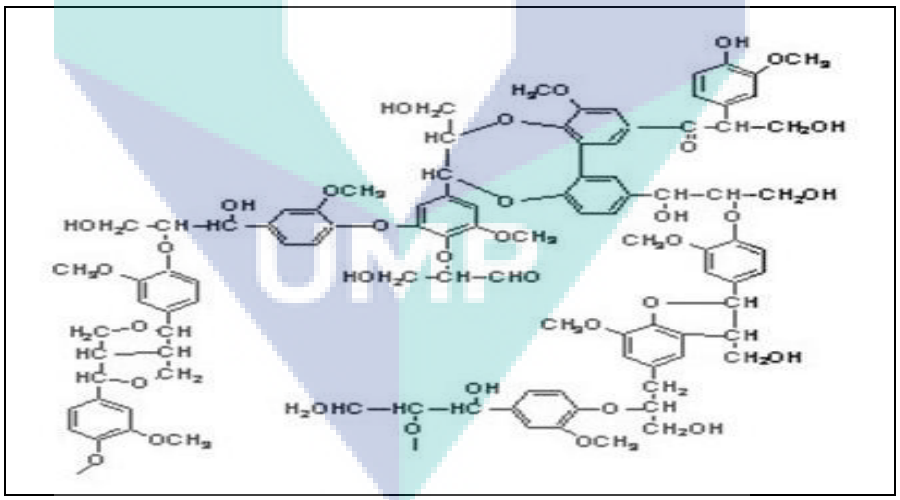
Figure 2.3: Monomer components of hemicelluloses

Unlike cellulose, hemicelluloses consist of different monosacharide units. In addition, the polymer chains of hemicellulose have short branches and are amorphous. Because of the amorphous morphology, hemicellulose are partially soluble or swellable in water. The backbone of the chains of hemicellulose can be a homopolymer (generally consisting of single sugar repeat unit) or a heteropolymer (mixture of different sugars. Among the most important sugar of the hemicelluloses component is xylose. In hardwood xylan, the backbone chain consists of xylose units which are linked by β-(1, 4)-glycosidic bonds and branched by β-(1, 2)-glycosidic bonds with 4-O methyl glucuronic acid groups. For softwood xylan, the acetyl groups are fewer in the backbone chain. However, softwood xylan has additional branches consisting of arabinofuranose units linked by β-(1, 3)-glycosidic bonds to the backbone. Among the carbohydrates components, starch is the only structure that has linear and branched chains. The linear chain is known as amylose. Their anhydroglucose units are linked by β-(1, 4)-glycosidic

bonds. In the case of branched chains, which is known as amylopectin, the backbone is like amylose but it also has β-(1, 6)-glycosidic bonds at the branch position.

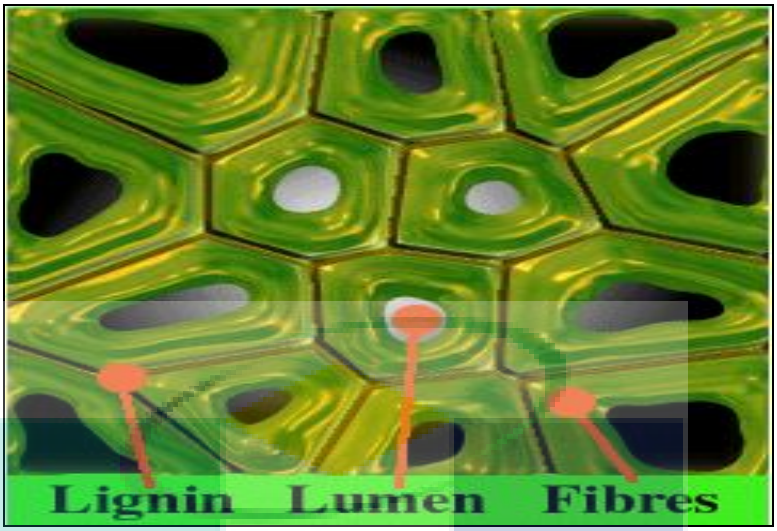
2.3.3 LIGNIN

Lignin is a very complex molecule, cross linked polymer that forms a large molecular structure. Lignin gives mechanical strength to wood by gluing the fibers together (reinforcing agent) between the cell walls. Lignin also serves as a disposal mechanism for metabolic waste. The monomeric building units of lignin are shown in Figure 2.4. The guaiacyl unit is dominant in the softwoods. In contrast, syringyl units are dominant in hardwoods. Figure 2.4 shows the structure of the lignin compound and figure 2.5 shows the cross section of the lignin impregnated wood.



Source: Taherzadeh and Karimi, 2007

Figure 2.4: Lignin structure



Source: Taherzadeh and Karimi, 2007

Figure 2.5: Cross section; lignin impregnated wood

Besides, lignin is a three dimensional, highly branched poly-phenolic polymer, most commonly being derived from wood and an integral part of the secondary cell wall of plants that presents a three dimensional structure. Lignin fills the spaces in the cell wall between cellulose, hemicellulose and pectin components, conferring mechanical strength to the cell wall. Lignin accounts for 23–33% of softwood mass, for 16–25% of hardwood mass and for 26–33% of plant biomass. In plants, lignin is second in natural abundance, cellulose being the first. Lignin macromolecule has a high surface area: $180 m^2 g^{-1}$ and molecular weight from 2000 to 15,000 $gmol^{-1}$.

There are many different types of lignin in different types of lignocellulosic materials, the lignin from grasses, softwoods and hardwoods differ in the methoxyl substitution and the degree of carbon–carbon linkage between phenyl groups. Lignin is covalently linked with xylans in hardwoods and with galacto-glucomannans in softwoods. Lignin also comprises a variety of functional groups such as aliphatic and phenolic hydroxyl groups (9–11%), methoxyl groups (13–26%) and carbonyl groups.

Although the principal structural elements in lignin have been largely clarified, many aspects of its chemistry remain unclear. Chemical bonds have been reported between lignin and hemicelluloses and even cellulose. Lignins are extremely resistant to chemical and enzymatic degradation (Palmqvist and Hahn-Hägerdal, 2000; Taherzadeh, 1999; Taherzadeh and Karimi, 2007).

2.3.5 EXTRACTIVES

Extractives are wood compounds that are soluble in neutral organic solvents or water. The extractives usually represent a minor fraction (between 1-5%) of lignocellulosic materials. They contain a large number of both lipophilic and hydrophilic constituents. The extractives can be classified in four groups: (a) terpenoids and steroids, (b) fats and waxes, (c) phenolic constituents, and (d) inorganic components (Sjöström, 1993; Taherzadeh, 1999; Taherzadeh and Karimi, 2007).

Extractives are the organic substances which have low molecular weight and are soluble in neutral solvents. Resin (combination of the following components: terpenes, lignans and other aromatics), fats, waxes, fatty acids and alcohols, terpentines, tannins and flavonoids are categorized as extractives. They only represent between 4-10 % of the total weight of dry wood, and the contents of extractives vary among wood species, geographical site and season. The extractives can be found mostly in resin canal and ray parenchyma cells and small amount in middle lamella and cell walls of tracheids. Some extractives are toxic and this is an advantage for the wood to resist attack by fungi and termites. Tannins are the main component of the red oak extractives. Natural tannins can be subdivided into hydrolyzable and condensed tannins. The hydrolyzable tannins are being classified as gallotannins (yielding gallic acid after hydrolysis) and ellagitannins (yielding ellagic acid after hydrolysis). The condensed tannins are widely used in leather industries as chemical treatments. The main structures in the condensed tannins are catechins type.

2.4 PRETREATMENT OF THE BIOMASS

The production of bioethanol involves an extensive process and varies depending on the type of biomass. The process mainly encompasses the upstream and downstream operations which involve pretreatment of the biomass, saccharification, fermentation and product recovery. The biomass pretreatment is a crucial process because it is essential for the fermentable sugars to be released and made available for the fermentation process. However, since the pretreatment process contributes significantly to the cost of production of bioethanol, the most economical pretreatment protocol must be selected in order to make bioethanol more economically attractive. An efficient biomass pretreatment procedure must be energy efficient, cost effective, and simple to apply and must not degrade the fermentable sugars (Rabelo et. al., 2009; Harun and Danquah, 2011). Pretreatment of the cellulose whether chemical treatment, physical treatment or biological treatment is typically required to increase the accessible area of cellulose for a reasonable rate of enzymatic hydrolysis.

2.4.1 PHYSICAL PRETREATMENT

Biomass could be pretreated in three different ways; physical, biological and chemical. Physical pretreatment refers to the reduction of physical size of biomass feedstock to increase enzyme accessible surface areas. The physical pretreatment process normally uses mechanical comminuting (such as chipping, milling and grinding) and pyrolysis, where the biomass is exposed to high temperatures. Although this procedure is quite simple, the high energy consumption associated with it makes it not preferable to be implemented in a commercial scale production.

Nowadays, physical pretreatment, i.e., biomass size reduction, has been overlooked. For example, significantly size reduced materials are being used in chemical pretreatments, but no information about the size reduction process was provided. In fact,

physical pretreatment or size reduction was even not included in process cost analyses in some key literature (Galbe and Zacchi, 2007; Lynd et al., 2008; Zhu and Pan, 2010). This is most likely because most research studied agricultural biomass, and energy consumption in the size reduction of agricultural biomass was insignificant. Unfortunately, size reduction has also been ignored in woody biomass studies.

2.4.2 CHEMICAL PRETREATMENT

Chemical pretreatment as a method for the conversion of biomass into useful chemicals has been the topic of much research since the late 1800s (Avellar et al., 1998). The chemical pretreatment process has been successfully proven for various biomass: corn (Chen et al, 2009; Harun and Danquah, 2011), switchgrass (Van et al., 2006; Harun and Danquah, 2011), sugar cane (Dawson et al., 2008; Harun and Danquah, 2011) and straw (Abenifar et al., 2009; Harun and Danquah, 2011). Chemical pretreatment exactly refers to the process of using chemicals to remove or modify key chemical components that interfere with biomass cellulose saccharification, mainly hemicellulose and lignin. The chemicals commonly applied in the pretreatment process are either acid (hydrochloric acid and sulfuric acid) or alkaline (lime and sodium hydroxide), which are everyday industrial chemicals carrying minimal toxicity in their applied concentrations. The acid pretreatment is more preferable as it provides higher efficiency in converting cellulosic materials. During acid pretreatment process, various parameters significantly influence the total amount of fermentable sugars being released. These include process time, temperature, amount of substrate loading and acid concentration.

Several types of chemical pretreatment for lignocellulosic biomass conversion have been propose before, including uncatalysed steam explosion, liquid hot water, pH controlled hot water, flow through liquid hot water, dilute acid, flow through acid and ammonia pretreatments as cost effective alternatives compared to traditional comminution and chemical treatments involving strong acids or bases or other highly

polluting solvents. To avoid some typical drawbacks of the commonly applied pretreatments, such as considerable production of polluting and not easily disposable byproducts, not satisfactory delignification of the starting material, partial oxidation of cellulose, thermal degradation of sugars, and considerable thermal energy consumption, some mild acid or alkaline or oxidative pretreatments have been proposed and successfully applied on different wastes (Spigno et. al., 2008).

Dilute acid pretreatment is the most studied process for agriculture biomass (Wyman et al., 2005; Zhu and Pan, 2010). The application of dilute acid pretreatment to woody biomass can achieve some level of success in that it can provide satisfactory cellulose conversion with certain hardwood species.

2.4.3 BIOLOGICAL PRETREATMENT

Biological pretreatment techniques of lignocellulosic materials have not been developed as intensively as physical and chemical methods (Fan et al. 1982; Lee et al., 1983; Moo-young et al., 1985; Tong and Hamzah 1989; Adaskaveg et al., 1990; Tong et al., 1993). If the capacity of microorganisms is to be utilised more fully, a better understanding of microbial lignin degradation is necessary (Crawford 1981; Tong et al., 1993). The most promising organisms for biological pretreatment of lignocellulose are the white-rot fungi (Hatakka 1983; Tong et al., 1993). It is possible to use these microorganisms to degrade the lignin component in lignocellulosic waste materials to make the cellulose and hemicellulose components more accessible for further biotechnological use (Tong et al., 1993).

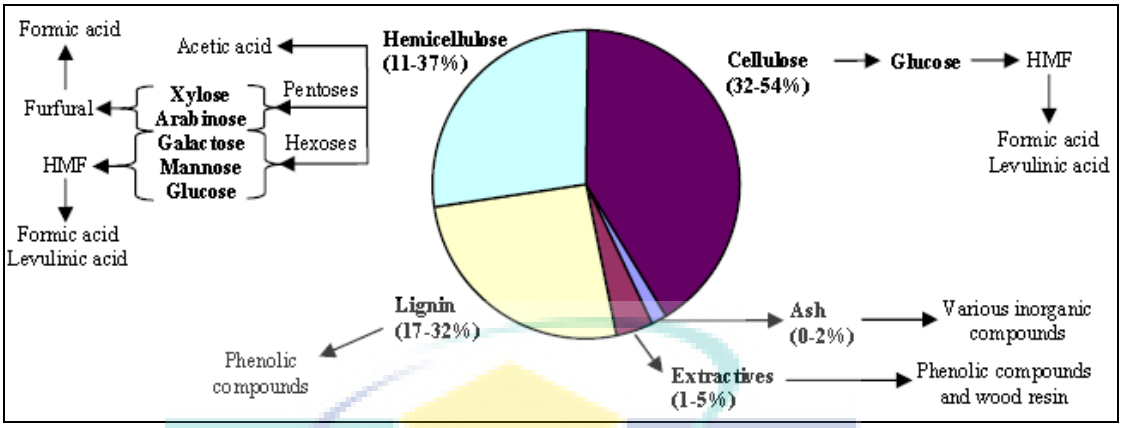
2.5 HYDROLYSIS

The carbohydrate polymers in the lignocellulosic materials need to be converted to simple sugars before the fermentation process, through a process called hydrolysis. However, several products can result from hydrolysis (Figure 2.6). There are several possible methods to hydrolyze lignocelluloses. The most commonly applied methods could be classified in two groups: chemical hydrolysis and enzymatic hydrolysis. Cellulose and hemicelluloses can be converted to ethanol, while lignin remains as a byproduct (Taherzadeh and Karimi, 2007).

$$Cellulose \xrightarrow{Hydrolysis} Glucose \xrightarrow{Fermentation} Ethanol \quad (2.1)$$

$$Hemicellulose \xrightarrow{Hydrolysis} Pentoses\ \&\ Hexoses \xrightarrow{Fermentation} Ethanol \quad (2.2)$$

In addition, there are some other hydrolysis methods in which no chemicals or enzymes are applied. For instance, lignocelluloses may be hydrolyzed by gamma ray or electron beam irradiation, or microwave irradiation. However, these processes are far from being commercially applied (Taherzadeh, 1999; Taherzadeh and Karimi, 2007).



Source: Taherzadeh and Karimi, 2007

Figure 2.6: Composition of lignocellulosic materials and their potential hydrolysis products

Hydrolysis involves cleaving the polymers of cellulose and hemicelluloses into their monomers. Complete hydrolysis of cellulose results in glucose, whereas the hemicellulose gives rise to several pentoses and hexoses. While softwood hemicellulose is mainly composed of mannose, the dominant sugar in hemicellulose being derived from hardwood and crop residues is usually xylose (Karimi et al., 2006; Taherzadeh et al., 1997; Taherzadeh and Karimi, 2007). The hydrolysis could be carried out chemically or enzymatically.

Hydrolysis literally means reaction with water. It is a chemical process in which a molecule is being cleaved into two parts by the addition of a molecule of water. One fragment of the parent molecule gains a hydrogen ion (H^+) from the additional water molecule. The other group collects the remaining hydroxyl group (OH^-). To illustrate this process, some examples from real life and actual living systems are being discussed here. The most common hydrolysis occurs when a salt of a weak acid or weak base (or both) is being dissolved in water. Water auto ionizes into negative hydroxyl ions and hydrogen ions. The salt breaks down into positive and negative ions. For example, sodium acetate dissociates in water into sodium and acetate ions. Sodium ions react very little with hydroxyl ions whereas acetate ions combine with hydrogen ions to produce

neutral acetic acid, and the net result is being a relative excess of hydroxyl ions, causing a basic solution.

However, under normal conditions, only a few reactions between water and organic compounds occur. Generally, strong acids or bases must be added in order to achieve hydrolysis where water has no effect. The acid or base is being considered a catalyst. They are meant to speed up the reaction, but are being recovered at the end of it. Acid base catalyzed hydrolyses are very common; one example is the hydrolysis of amides or esters. Their hydrolysis occurs when the nucleophile (a nucleus seeking agent, e.g., water or hydroxyl ion) attacks the carbon of the carbonyl group of the ester or amide. In an aqueous base, hydroxyl ions are better nucleophiles than dipoles such as water.

In acid, the carbonyl group becomes protonated, and this leads to a much easier nucleophilic attack. The products for both hydrolyse are compounds with carboxylic acid groups. Perhaps the oldest example of ester hydrolysis is the process called saponification. It is the hydrolysis of a triglyceride (fat) with an aqueous base such as sodium hydroxide (NaOH). During the process, glycerol, also commercially named glycerin, is being formed, and the fatty acids react with the base, converting them to salts. These salts are called soaps, commonly used in households.

Moreover, hydrolysis is an important process in plants and animals, the most significant example being energy metabolism and storage. All living cells require a continual supply of energy for two main purposes: for the biosynthesis of small and macromolecules, and for the active transport of ions and molecules across cell membranes. The energy derived from the oxidation of nutrients is not used directly but, by means of a complex and long sequence of reactions, it is channeled into a special energy storage molecule, adenosine triphosphate (ATP).

The ATP molecule contains pyrophosphate linkages (bonds formed when two phosphate units are combined together) that release energy when needed. ATP can be hydrolyzed in two ways: the removal of terminal phosphate to form adenosine diphosphate (ADP) and inorganic phosphate, or the removal of a terminal diphosphate to yield adenosine monophosphate (AMP) and pyrophosphate.

The latter is usually cleaved further to yield two phosphates.This results in biosynthesis reactions, which do not occur alone, that, can be driven in the direction of synthesis when the phosphate bonds are hydrolyzed. In addition, in living systems, most biochemical reactions, including ATP hydrolysis, take place during the catalysis of enzymes. The catalytic action of enzymes allows the hydrolysis of proteins, fats, oils, and carbohydrates. As an example, one may consider proteases, enzymes that aid digestion by hydrolyzing peptide bonds in proteins.

They catalyze the hydrolysis of interior peptide bonds in peptide chains, as opposed to exopeptidases, another class of enzymes, which catalyze the hydrolysis of terminal peptide bonds, liberating one free amino acid at a time. However, proteases do not catalyze the hydrolysis of all kinds of proteins. Their action is stereo-selective, only proteins with a certain tertiary structure will be targeted. The reason is that some kind of orienting force is being needed to place the amide group in the proper position for catalysis. The necessary contacts between an enzyme and its substrates (proteins) are created because the enzyme folds in such a way as to form a crevice into which the substrate fits; the crevice also contains the catalytic groups. Therefore, proteins that do not fit into the crevice will not be hydrolyzed. This specificity preserves the integrity of other proteins such as hormones, and therefore the biological system continues to function normally.

2.5.1 **ACID HYDROLYSIS**

Chemical hydrolysis involves exposure of lignocellulosic materials to a chemical for a period of time at a specific temperature, and results in sugar monomers from cellulose and hemicellulose polymers. Acids are predominantly applied in chemical hydrolyses. Sulfuric acid is the most investigated acid (Harris et al., 1945; Taherzadeh and Karimi, 2007), although other acids such as hydrochloric acid (Hashem and Rashad, 1993; Taherzadeh and Karimi, 2007) have been used. Acid hydrolyses can be divided into two groups: (a) concentrated acid hydrolysis and (b) dilute acid hydrolysis. A comparison between concentrated and diluted acid hydrolysis methods is presented in Table 2.5.

Table 2.5: Comparison between concentrated and diluted acid hydrolysis methods

Hydrolysis method	Advantages	Disadvantages
Concentrated acid process	- Operated at low temperature - High sugar yield	- High acid consumption - Equipment corrosion - High energy consumption for acid recovery longer reaction time (e.g. 2-6 hours
Diluted acid process	- Low acid consumption - Short residence time	- Operated at high temperature - Low sugar yield - Equipment corrosion - Formation of undesirable byproducts

Hydrolysis of lignocellulosic by concentrated sulfuric acid or hydrochloric acids is a relatively old process. Barconnot in 1819 first discovered that cellulose can be converted to fermentable sugar by concentrated acid (Sherrar and Kressman, 1945;

Taherzadeh and Karimi, 2007). Concentrated single stage hydrolysis with sulfuric acid, and concentrated hydrolysis by liquid or vapor phase with hydrochloric acid were being used. Concentrated acid processes are generally reported to give higher sugar yield (e.g. 90% of theoretical glucose yield) and consequently higher ethanol yield, compared to diluted acid processes. Furthermore, the concentrated acid processes can operate at low temperature (e.g. 40^0C), which is a clear advantage compared to dilute acid processes.

However, the concentration of acid is very high and dilution and heating of the concentrated acid during the hydrolysis process make it extremely corrosive. Therefore, the process requires either expensive alloys or specialized non metallic constructions, such as ceramic or carbon-brick lining. The acid recovery is an energy demanding process. In addition, when sulfuric acid is used, the neutralization process produces large amounts of gypsum. Furthermore, the environmental impact strongly limits the application of hydrochloric acid. The high investment and maintenance costs have greatly reduced the potential commercial interest of this process (Jones and Semrau, 1984; Katzen et al., 1945; Taherzadeh and Karimi, 2007). Despite the disadvantages, the concentrated acid process is still of interest.

Among the chemical hydrolysis methods, dilute acid hydrolysis is probably the mst commonly being applied. It is a method that could be used either as a pretreatment preceding enzymatic hydrolysis, or as the actual method of hydrolyzing lignocelluloses to sugar (Qureshi and Manderson, 1995; Taherzadeh and Karimi, 2007). The first established diluted acid hydrolysis process was probably the Scholler process (Faith, 1945; Taherzadeh and Karimi, 2007). This was a batch process, in which the wood material was kept in 0.5% sulfuric acid at 11-12 bar for approximately 45 minutes. Nowadays, most of dilute acid hydrolysis processes are performed in a batch mode with a retention time of a few minutes (Karimi et al., 2006; Taherzadeh and Karimi, 2007).

One of the major problems with hydrolyzates being produced by acid hydrolysis is the poor fermentability being caused by the presence of inhibitors in the hydrolyzates.

Furthermore, the acidic hydrolysis results in serious corrosion hazard and the use of acid is wasteful and energy inefficient, which requires separation, recycling and treatment of the waste sulfuric acid.

2.5.2 **Enzymatic Hydrolysis**

Enzymatic hydrolysis is a catalytic decomposition of a chemical compound by reaction with water, such as the conversion of cellulosic materials into fermentable sugars by the addition of specific enzymes. This process is being used to convert starch and cellulose in plant stalks, leaves, wood fiber, and other biomass into glucose by the addition of enzymes, e.g. *cellulase* enzyme. It is also used for the breakdown of proteins into amino acids by the addition of proteases.

2.5.3 **Alkaline Hydrolysis**

Alkaline Hydrolysis is water based chemical resolving process using strong alkali in water at temperatures of up to 18^0C (35^0F) to rapidly yet sympathetically reduce the body to ash. It is essentially a highly accelerated version of natural decomposition chemistry. Hydrolysis as the name suggests is the process of forcing water molecules between the chemical bonds holding large tissue molecules such as fats, DNA and proteins together. This process breaks the tissue down to its original small molecular building blocks. This is a natural process found in body decomposition after death (Prakash, 2007.

2.6 **Enzyme**

Enzymes are the biological catalysts and are the basis of metabolism that keeps a cell or organism alive. They are able to accelerate the rate of chemical reactions in a living cell. Like catalysts, they are not used up in the reaction, but unlike catalysis they are produced by living cell only. These are the enzymes which run myriads of biochemical reactions under normal cellular conditions. For hydrolyzing starch in the food or example, high temperature or acidity was required. In the alimentary canal however, it is digested to glucose under relatively normal conditions of temperature and pH by the enzymes. Similarly the enzymes are able to hydrolyze other macromolecules such as the protein and lipid also under normal physiological conditions (Prakash, 2007).

Enzymes occur in all living cells but not all enzymes are found in all the cells. The enzymes catalyze a wide variety of biochemical reactions many of which are being localized in specific organs or are peculiar to certain species of plant or animal life. Thus, for example, pepsin is produced only in the cells of gastric mucosa and trypsin only in the pancreas. In the plant world lipases are not generally distributed but are found chiefly in plants that produce oilseeds. A few of the enzymes are present in most forms of life. For example, catalases and peroxidases are widely being distributed in all higher plants and animal. The amount of enzymes maybe different in different tissues.

In the past, enzymes were being named in a haphazard manner as and when they were discovered. The ways in which enzymes are or have been named are listed below:

1) The first enzymes studied have been named for their color, their localization within the body or after the person who discovered them. However, this nomenclature had not been agreeable to many.

2) Later, many enzymes have been named by adding the suffix ‘-ase’ to the name of the substrate, for example, urease catalyzes the hydrolysis of arginine to

ornithine and urea and so on. However, this nomenclature has not been always practicable.

3) Further, many other enzymes have been given chemically uninformative names, e.g. pepsin, trypsin and catalase. With the increasing number of newly discovered enzymes, this kind of nomenclature has not been accepted; this system has its limitations.

4) Finally, a systematic classification of enzymes has been adopted on the recommendation of an International Enzyme Commission as listed in the 1973 edition of Enzyme Nomenclature with a few exceptions. According to this, enzymes has been classified into six major classes and sets of subclasses based on the nature and type of reactions catalyzed (Prakash, 2007).

2.6.1 **Cellulase Enzyme**

Cellulase enzyme refers to a class of enzymes produced chiefly by fungi, bacteria, and protozoans that catalyze the cellulolysis (or hydrolysis) of cellulose. However, there are also cellulases produced by other types of organisms such as plants and animals. Several different kinds of cellulase enzymes are known, which differ structurally and mechanistically (Chapin, et al., 2002). Cellulase enzymes are relatively costly enzymes, and a significant reduction in cost will be important for their commercial use in biorefineries. Cellulase enzyme based strategies that will make the biorefinery processing more economical include; increasing commercial enzyme volumetric productivity, producing enzymes using cheaper substrates, producing enzyme preparations with greater stability for specific processes, and producing cellulases with higher specific activity on solid substrates.

Currently, most commercial cellulase enzymes (including β- glucosidase) are produced by *Trichoderma* species and *Aspergillus* species (Cherry and Fidantsef, 2003). Cellulase enzymes are used in the textile industry for cotton softening and denim finishing, in the detergent market for color care, cleaning, and anti deposition, in the food industry for mashing, and in the pulp and paper industries for deinking drainage improvement, and fiber modification (Cherry and Fidantsef, 2003). The cellulase enzyme market is expected to expand dramatically when cellulase enzymes are used to hydrolyze pretreated cellulosic materials to sugars, which can be fermented to commodities such as bioethanol and biobased products on a large scale (Cherry and Fidantsef, 2003).

As access of cellulose to the cellulose network is a key issue for hydrolysis, it is not surprising that success of hydrolysis depends on the quality of pretreatment. While one important factor contributing to the complex nature of cellulose hydrolysis is the presence of lignin, pretreatment even is believed to influence the extent of cellulose hydrolysis in the absence of lignin. As stated above, the degree of crystalline should influence the rate of hydrolysis. Also, unless the ratio of cellulose and *β*-glucosidase is optimized, inhibition of cellulose by cellobiose causes inefficient hydrolysis.

Cellulase is an enzyme which breaks down cellulose to beta-glucose. It is produced mainly by symbiotic bacteria in the ruminating chambers of herbivores. At least two steps in cellulose degradation by microorganisms begin with the preparatory prehydrolytic first step involving an enzyme (C1) which swells and/or hydrates anhydroglucose chains. The second step uses hydrolytic enzymes (Cx) and beta glucosidase (cellobiase). *Trichoderma reesei* has an extensively studied cellulase enzyme complex. This complex converts crystalline, amorphous, and chemically derived celluloses quantitatively to glucose. Cellulase derived from *Trichoderma longbrachiatum* is comprised of an enzyme complex consisting of cellulase, a glucosidase, cellobiohydrolase and a glucanase. This complex converts cellulose to beta-dextrins and ultimately to D-glucose. Cellulase is used as a digestive aid, particularly in animals, and for the management of flatulence.

Cellulose is an indigestible plant polysaccharide. It is the principal constituent of the cell wall of plants. Cellulase has cellulolytic activity, meaning that it hydrolyzes cellulose. Cellulase hydrolyzes the beta-D-1, 4-glycosidic bonds of cellulose. Cellulase enzymes show activity during the ripening of some fruits, where their effects on cell walls results in softening of the fruit. The vital characteristics of this cellulase complex are:

1) The system is multienzymatic;

2) At least three enzyme components are both physically and chemically distinct; and

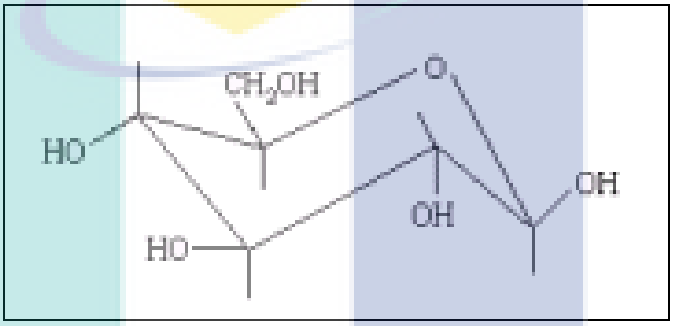
3) All three components play essential roles in the hydrolysis of cellulose to glucose.

The assay for cellulase activity uses a method which determines the effect of cellulase on microcrystalline cellulose with respect to glucose formation. Released glucose is determined in a hexokinase/glucose-6-phosphate dehydrogenase system at 340 nm.

Cellulase is an enzyme that breaks down cellulose, the carbohydrate that is the main part of the cell walls of plants. Cellulose is nondigestible by humans because we to not produce the enzyme cellulase. Cellulase is produced by grazing animals such as cows (with the aid of the beneficial bacteria that reside in the animal's digestive tract), and is the reason why they can get nutrition from plants such as grasses. The human body does not produce cellulase, however, it is available for supplementation since it can help us break down plant material better, thereby getting the most from the plants that we eat.

2.7 Glucose

Glucose is the main type of sugar in the blood and is the major source of energy for the body's cells. Glucose comes from the foods we eat or the body can make it from other substances. Glucose is carried to the cells through the bloodstream. Several hormones, including insulin, control glucose levels in the blood. Figure 2.7 below shows the structure of the glucose.



Source: Valyasevi and Rolle, 2000

Figure 2.7: Structure of glucose compound

2.8 Kinetic Study of Enzyme

Enzyme kinetics is the study of the chemical reactions that are being catalyzed by enzymes. In enzyme kinetics the reaction rate is measured and the effects of varying the conditions of the reaction are being investigated. Studying an enzyme's kinetics in this way can reveal the catalytic mechanism of this enzyme, its role in metabolism, how its activity is controlled, and how a drug or a poison might inhibit the enzyme. Enzymes are usually protein molecules that manipulate other molecules. These target molecules bind to an enzyme's active site and are transformed into products through a series of steps known as the enzymatic mechanism. These mechanisms can be divided into single substrate and multiple substrate mechanisms. Kinetic studies on enzymes that only bind

one substrate, aim to measure the affinity with which the enzyme binds this substrate and the turnover rate.

When enzymes bind multiple substrates, enzyme kinetics can also show the sequence in which these substrates bind and the sequence in which products are released. Examples of enzymes that bind a single substrate and release multiple products are proteases, which cleave one protein substrate into two polypeptide products. Others join two substrates together, such as DNA polymerase linking a nucleotide to DNA. Although these mechanisms are often a complex series of steps, there is typically one rate determining step that determines the overall kinetics. This rate determining step may be a chemical reaction or a conformational change of the enzyme or substrates, such as those involved in the release of products from the enzyme.

2.9 **Fermentation**

Fermentation is one of the oldest and most economical methods of producing and preserving foods. Natural fermentation has already been used for ages to increase the shelf life of various food materials. This process has resulted in a number of traditional food products such as the dairy products cheese, butter, buttermilk and yoghurt; fermented meat, plants and fruits such as sausages, silage, sauerkraut, olives and grapes; and finally fermented cereal products such as bread and beer (Caplice and Fitzgerald, 1999). Indigenous fermented foods were developed through traditional or village art methodologies, which were preserved over the years, in order to maintain the uniqueness and identity of these foods. Fermented foods are essential components of the diet in a number of developing countries, and are consumed either as main dishes or as condiments. They are prepared from both plant and animal materials, using processes in which microorganisms play an active role in the physical, nutritional and organoleptic modification of the starting material. Due to the lack of scientific and technological know

how, fermented foods are generally evaluated on the basis of qualitative attributes such as odor and flavor (Valyasevi and Rolle, 2000).

Biotechnological innovations have greatly assisted in industrializing the production of certain indigenous fermented foods. Indonesian Tempe and Oriental soy sauce are well known examples of indigenous fermented foods that have been industrialized and marketed globally. Nevertheless, the majority of fermented foods are produced using traditional methodologies at both the cottage and small scale levels in developing countries. In Africa, most of food fermentations are carried out on a household level, and are still conducted as spontaneous processes. Spontaneous fermentations typically result from the competitive activities of a variety of autochthonous and contaminating microorganisms.

Those best adapted to the conditions during the fermentation process will eventually dominate. Initiation of a spontaneous process takes a relatively long time, with a high risk for failure. Failure of fermentation processes can result in spoilage and/or the survival of pathogens, thereby creating unexpected health risks in food products. Thus, from both a hygiene and safety point of view, the use of starter cultures is recommended, as it would lead to a rapid acidification of the product and thus inhibit the growth of spoilage and pathogenic bacteria, and to a product with consistent quality.

2.10 **Lactic Acid Bacteria**

There are numerous application areas for the use of lactic acid bacteria (LAB) both in industry and human health, including preservation of foods (Stiles, 1996). The use of starter cultures for the production of fermented food is becoming necessary to guarantee safety and standardize properties. Lactic acid bacteria (LAB) are generally being recognized as safe microorganisms that have been used in the processing of fermented food for centuries (Essid et al., 2009).

Lactic acid bacteria (LAB) represent a group of microorganisms that are functionally related by their ability to produce lactic acid during fermentation. Acidification due to lactic acid production and other enzymatic processes contribute to the flavor, texture, and keeping qualities of fermented food and feed products. Understanding the mechanisms involved in the growth performance of an appropriate LAB strain to be used for food and feed fermentations is of crucial importance to obtaining high-quality fermented product.

2.10.1 **Lactobacillus Plantarum**

Lactobacillus plantarum is a facultative heterofermentative LAB encountered in a large number of environmental niches, from dairy and meat products to a variety of vegetable and plant fermentation. It is also one of the *Lactobacillus* species present in the human intestinal tract. Due to its high acid tolerance, *Lactobacillus plantarum* has been used as the starter culture of food and feedstuffs, but it has also been used as a probiotic and as a delivery vehicle for therapeutic compounds due to its ability to survive and persist in the gastrointestinal tract. Therefore, studies of the adaptation of *Lactobacillus plantarum* to different environmental niches are of great interest due to the extensive use of this bacterium in food and feed fermentations.

In the context of polyol production, *Lactobacillus plantarum* possesses some relevant characteristics. It is a food-grade microorganism belonging to the group of lactic acid bacteria. *Lactobacillus plantarum* is a normal member of the human intestinal microbiota and can also be isolated from the oral cavity (Pretzer, et. al., 2005). This study was being performed with *Lactobacillus plantarum* NCIMB8826 grown in MRS broth.

Lactobacilli are normal inhabitants of the gastrointestinal tract (GIT) of many mammalian hosts. Their administration as probiotics in functional foods is currently a

frequent practice, mainly because of their benefits to host health. It is therefore of interest to study the impact of administration of exogenous strains of Lactobacillus normally used as probiotics upon endogenous microbial populations (Susana et al., 2008).

Lactobacillus plantarum is a versatile and widespread microorganism found in environments ranging from vegetable, dairy and meat fermentations to the human gastrointestinal (GI) tract (Kleerebezem et al., 2003). *Lactobacillus plantarum* is a facultative heterofermentative LAB, metabolically very flexible and versatile, encountered in many environmental niches, and with broad applications, e.g. as a starter culture in vegetable (Salovaara, 2004) and meat (Ammor and Mayo, 2007) fermentations, as probiotic for humans (Goossens et al., 2005) and animals (Demecková et al., 2002), and lately as a delivery vehicle for therapeutic compounds (Pavan et al., 2000). *Lactobacillus plantarum* has been much investigated due to its wide adaptation capacity and its numerous applications. Research has been mainly focused on the bacterial responses to stress factors such as heat shock (De Angelis et al., 2004), the presence of high concentrations of lactic acid (Pieterse et al., 2005).

2.11 **Sugar Alcohol (Polyols)**

Polyols, also being called sugar alcohols, are low calorie bulking agents and sweeteners that are widely used throughout the food industry to reduce calories, act as a humectants and control water activity. They are also being used in cosmetic and pharmaceutical applications to provide viscosity as well as for their functional properties in industrial applications. Polyols are carbohydrates but they are not sugars, making them sugar free sweeteners. They are used cup for cup (volume for volume) in the same amount as sugar is being used, unlike low calorie sweeteners which are used in very small amounts.

Because the polyols taste good, people can improve the healthfulness of their diets without having to sacrifice the pleasure of eating sweet foods they enjoy. Scientists call polyols as "sugar alcohols" because part of sugar alcohol structure chemically resembles sugar and part is similar to alcohols. However, these sugar free sweeteners are neither sugars nor alcohols (http://www.caloriecontrol.org/sweeteners-and-lite/polyols, 11 June 2011).

Polyols provide fewer calories per gram than sugar. They provide significantly less than the traditional four calories per gram assigned to carbohydrates in general. The eight polyols currently available for use are erythritol, hydrogenated starch hydrolysates (including maltitol syrups), isomalt, lactitol, maltitol, mannitol, sorbitol and xylitol. These ingredients may be found in a wide range of products, including chewing gums, candies, ice cream, baked goods and fruit spreads. They are also used in toothpastes, mouthwashes, breath mints and pharmaceuticals such as cough syrups or drops and throat lozenges.

2.11.1 **Glucitol (Sorbitol)**

Sorbitol is a polyol with a sweet and refreshing flavor, and can be used as sweetener, moisturizer, texturizer and softener in the food industry. Other uses include vitamin C, sorbose, glycerol propylene, plastics and resin production. Sorbitol can be used in dietetic foods for diabetics because it is not insulin-dependent. World sorbitol production has been estimated to be more than 500.000 ton per year, traditionally from glucose catalytic hydrogenation using nickel as the catalyzer, at high pressures and temperatures. After the catalytic hydrogenation process, the sorbitol is collected and purified, making the process expensive. However, other methods can be used to obtain sorbitol, such as the biotechnological process by fermentation.

Sorbitol also known as glucitol, is a sugar alcohol that the body metabolizes slowly. It is obtained by reduction of glucose changing the aldehyde group to an additional hydroxyl group hence the name sugar alcohol. Sorbitol is used in sugar free mints and various cough syrups and is usually listed under the inactive ingredients. Sorbitol is a sugar substitute often used in diet foods, including diet drinks and ice cream and sugar free chewing gum. It also occurs naturally in many stone fruits and berries from trees of the genus Sorbus. Sorbitol is also referred to as a nutritive sweetener because it provides dietary energy: 2.6 kilocalories (11 kilojoules) per gram versus the average 4 kilocalories (17 kilojoules) for carbohydrates.

Sorbitol is identified as a potential key chemical intermediate from biomass resources. Complete reduction of sorbitol opens the way to alkanes such as hexane which can be used as a biofuel. Sorbitol itself provides much of the hydrogen required for the transformation. Even in the absence of dietary sorbitol, cells produce sorbitol naturally. When too much sorbitol is produced inside cells, it can cause damage. Diabetic retinopathy and neuropathy may be related to excess sorbitol in the cells of the eyes and nerves. The source of this sorbitol in diabetics is excess glucose, which goes through the sorbitol aldose reductase pathway.

The most commonly used polyol in the United States is sorbitol, which is the standard sweetener in several sugar-free chewing gums and over-the-counter medicines. Sorbitol is 60 percent as sweet as sucrose and is much less expensive than xylitol. Sorbitol is less effective than xylitol in controlling caries, but its lower cost makes it appealing to food manufacturers. In terms of cariogenesis, sorbitol has an advantage over sugars because, in small amounts, it does not lower the pH of plaque to a point where enamel demineralization occurs. Sorbitol, however, should be considered a low cariogenic sweetener rather than a noncariogenic one because consumption of larger amounts (more than two sticks of chewing gum per day) increases both the acid production in plaque and the number of sorbitol fermenting microorganisms. Sorbitol in a solution (such as in a soft drink) can be fermented, though slowly, by mutans

streptococci. Cariogenic microorganisms can “learn” to metabolize sorbitol when their sugar supply is restricted.

2.11.2 **Xylitol**

Xylitol is a sweetener similar to sucrose, which is found at low concentrations in fruits and vegetables. It has a broad range of applications in the food industry as well as in healthcare. The gastrointestinal effect of xylitol, derived from its slow absorption rate, is known. Despite a wide range of applications, the use of xylitol as an alternative sweetener is limited due to its high production costs. There are several technologies available for xylitol production, especially: extraction from vegetable and fruits, chemical synthesis and biotechnological procedures (Nahlik et al., 2003).

Xylitol is an alternative sweetener used in food, odontological, and pharmaceutical preparations (Carvalho et al., 2005), whose present chemical production is very expensive. Xylitol has recently become very attractive as a sugar substitute due to its high sweetening power and antic- ariogenic properties. This five carbon sugar alcohol is now produced by chemical synthesis through catalytic hydrogenation of xylose obtained from hemocellulosic hydrolysate.

However, this chemical process is expensive because xylose must be purified before the chemical reaction. Another way is the microbiological production of xylitol (Ikeuchi et. al., 1999).The first field trials for xylitol was conducted in the late 1960s and early 1970s in Turku, Finland. Xylitol can also be produced by microbiological methods with natural xylose utilizing yeasts, especially species of the genus *Candida*, such as C. *blankii, C. boidinii, C. guilfiermondii, C. petiicufosa, C. shehatae, C. tropicalis,* and C. *utilis, Saccharomyces bailii, S. rouxii,* and S. *uvarum, Schizosaccharomyces pombe,* and *Pachysolen tannophilus*. Use of yeasts, however, has the disadvantage of low xylitol productivity (Kim et al., 1996).

Recently it has been demonstrated that xylitol can prevent acute otitis media (AOM) (Uhari et. al., 1998; Prakash et al., 2011) in children. The value of xylitol market is currently $340 million with applications in mouthwashes, toothpastes and chewing gums as well as in foods for special dietary uses. Global xylitol consumption was 43,000 t in 2005, the US and Western Europe accounting for 30% and 37% of the total xylitol consumption respectively (Kadam et al., 2008; Prakash et al., 2011). Xylitol is produced from xylan-rich biomass by both chemical and biological methods (Winkelhausen and Kuzmanova, 1998; Prakash et al., 2011). The chemical process adapted so far is not ecofriendly and hydrogenation of xylose demands huge production costs in terms of temperature and pressure input as well as the formation of byproducts that require expensive separation and purification steps. In addition, xylose should be purified before it is hydrogenised, which further increases the capital investment and costs for xylitol production (Santos et al., 2005; Prakash et al., 2011).

2.11.3 **Mannitol**

Mannitol is the one of the sugar alcohol classified. Mannitol is the generic name for a Food and Drug Administration (FDA) approved drug used as an osmotic diuretic and a mild renal vasodilator. Orally, mannitol is used a sweetening agent in confections for people with diabetes and, in higher concentrations, as a laxative for children. Mannitol is typically administered intravenously, but can also be taken orally, depending on the purpose. Intravenously, it is used to treat excessive intracranial pressure, oliguria, and to expand openings in the blood brain barrier.

Chemically, mannitol is composed of carbon, hydrogen, and multiple hydroxyl groups (C_6H_8 $(OH)_6$) to make a sugar alcohol. It is prepared in a solution with water at concentrations or five, ten, fifteen, twenty, and twenty five percent. Mannitol is not permeable, meaning it will not pass through a cell membrane the way many other drugs will. Because mannitol is prone to making a solution acidic, the FDA has approved the adding of sodium bicarbonate to adjust the pH.

2.12 DESIGN EXPERIMENTAL USING RESPONSE SURFACE METHODOLOGY (RSM)

Optimization of pretreatment conditions is one of the most important stages in the development of an efficient and economic pretreatment method. The traditional 'one factor at a time approach' is time consuming and moreover the interactions between independent variables are not considered. Response surface methodology (RSM) is an effective optimization tool wherein many factors and their interactions affect the response can be identified with fewer experimental trials. RSM mainly includes central composite design, Box Behnken design, one factor design, D-optimal design, used-defined design and historical data design. RSM had been widely used in various fields ranging from food process operations including extrusion. Recently, RSM had been successfully applied to biomass pretreatment by many researchers (Karunanithy and Muthukumarappan, 2010).

Response surface methodology (RSM) is a statistical method that uses quantitative data from appropriate experiments to determine regression model equations and operating conditions. RSM is a collection of mathematical and statistical techniques for modeling and analysis of problems in which a response of interest is influenced by several variables. A standard RSM design called a central composite design (CCD) was applied in this work to study the variables of the lignin removal and also glucose yield. This method was suitable for fitting a quadratic surface and it helps to optimize the effective parameters with a minimum number of experiments, as well as to analyze the interaction between the parameters. Generally, CCD consists of a 2^n factorial runs with *2n* axial runs and n_c center runs (six replicates) (Hameed et. al., 2008).

Once the pretreatments were performed, the second order polynomial equation was used to describe the effects of independent variables in terms of linear, quadratic and interactions. The proposed model for the response, (Y_i) was:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_{ui} + \sum_{i=1}^{k} \beta_{ii} X_{ui} + \sum \sum_{1\langle j}^{k} \beta ij X_{ui} X_{uj} + \varepsilon \qquad (2.3)$$

Where Y_i is the predicted response; β_0 is the interception coefficient; β_i, β_{ii} and β_{ij} are coefficients of the linear, quadratic, and interaction terms; ε is the random error; and Xi is the independent variables studied. Design expert version 6.0.8 software was used for regression and graphical analysis of the data obtained. Statistical analysis of the model was performed to evaluate the analysis of variance.

CHAPTER 3

METHODOLOGY

3.1 RESEARCH DESIGN

This chapter describes the materials selection and techniques that were being implemented in the recovery of cellulose, glucose production and also fermentation techniques to produce sugar alcohol. There were three major process being involved in the production of sugar alcohol starting with *meranti* wood sawdust. The steps involved are being listed as below:

1) Pretreatment of wood sawdust to recover cellulose content and proportional with lignin degradation.
2) Enzymatic hydrolysis of cellulose recovery from pretreatment process to produce glucose with high yield.
3) Fermentation of glucose production from cellulose to produce sugar alcohol.

Section 3.2 describes the raw materials that were being used in this research and where the materials come from. In the preparation of the cellulose recovery process, the section 3.3 describes all the processes that were being used to remove lignin and also hemicelluloses in details. Section 3.3 also details up the experimental design of optimization of lignin degradation in second stages of pretreatment. In the 3.4 section, the enzymatic hydrolysis were being described in details and in this section, the design of experimental software also was used to optimize the glucose production. After that,

section 3.5 details about the fermentation process that run up to produce sugar alcohol. In the section 3.6, the details about response surface methodology was discussed. Lastly, in section 3.7 detailed about the instruments and techniques used for materials characterization test during the experimental process. The flow of the experimental procedures conducted in this study was being summarized in figure 3.1.

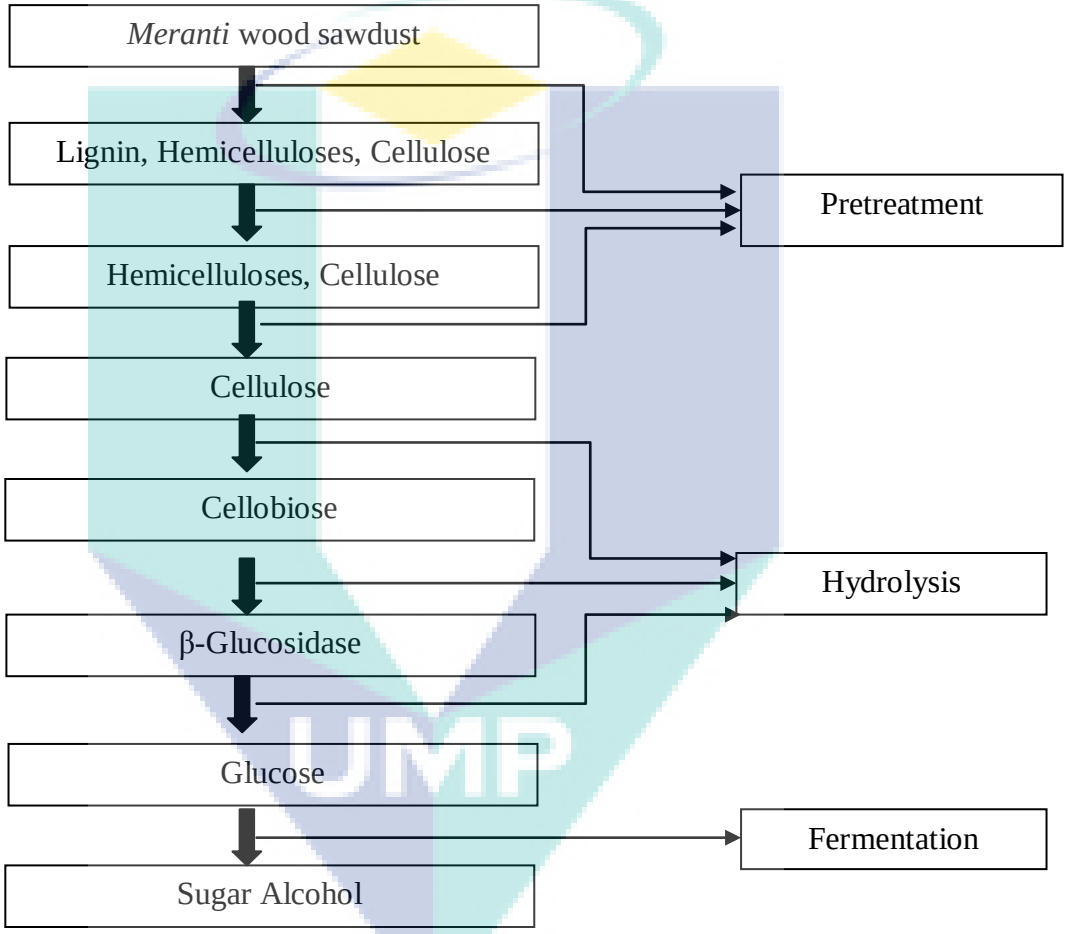


Figure 3.1: Flowchart of whole experimental procedure

3.2 RAW MATERIAL (WOOD SAWDUST)

To achieve the objective of the research, the material that was being used in this research must be the come from waste. Wood sawdust is one of the wastes in our industry. There are many type of biomass that we can use in research based on the cellulose content, but in this research we used sawdust because of the abundant factor and also easy to get. As had been discussed earlier in chapter 2, cellulose content in hardwood type was high than softwood. Due to the abundant wood sawdust in industry especially in Kuantan, we were used *meranti* sawdust as raw materials. *Meranti* is one of the hardwood type and we used *meranti* because nowadays in Kuantan, the production from sawmills are highly being based on the *meranti* wood was high. All the chemicals were being analytically pure and purchased locally. Table 3.1 shows the comparison of the chemical compound between hardwood and softwood type.

Table 3.1: Comparison of the chemical compound between hardwood and softwood

Wood Type	Cellulose (%DW)	Hemicellulose (%DW)	Lignin (%DW)	Extractives (%DW)	Ash (%DW)
Hardwood	40.1	27.8	23.1	7.2	1.8
Softwood	19.0	18.9	44.8	14.8	2.5

M*eranti* wood sawdust was collected from Gambang Sawmills (M) Sdn Bhd, Gambang Kuantan, Pahang. The sawdust was taken by bulk to make sure the qualities of the materials were same until the end of the research.

3.3 PRETREATMENT PROCESS

Once the sawdust was being taken from industry, it needed to be dried under the sunlight about five hours to make sure the bacterial or other microorganisms were not react with it and also to eliminate water content in *meranti* wood sawdust. This is because the sawdust taken from the sawmill still wet and full of the moisture content. Section 3.3.1 describes the physical treatment that being used to prepare the *meranti* wood sawdust to the chemical pretreatment and also to store it. In the section 3.3.2 describes the pre-delignification process that being applied to *meranti* wood sawdust to remove all the oily content, grease and dirt content that probability come from sawmill machines and also environment. Section 3.3.3 describes the first stage of the pretreatment that being applied to the *meranti* wood sawdust to remove the lignin content. Besides, the section 3.3.4 describes the second stage of the pretreatment and here the design of experimental software was being applied to optimize the removal of the lignin and also hemicelluloses. The flow of the pretreatment procedures conducted in this study was being summarized in Figure 3.2.

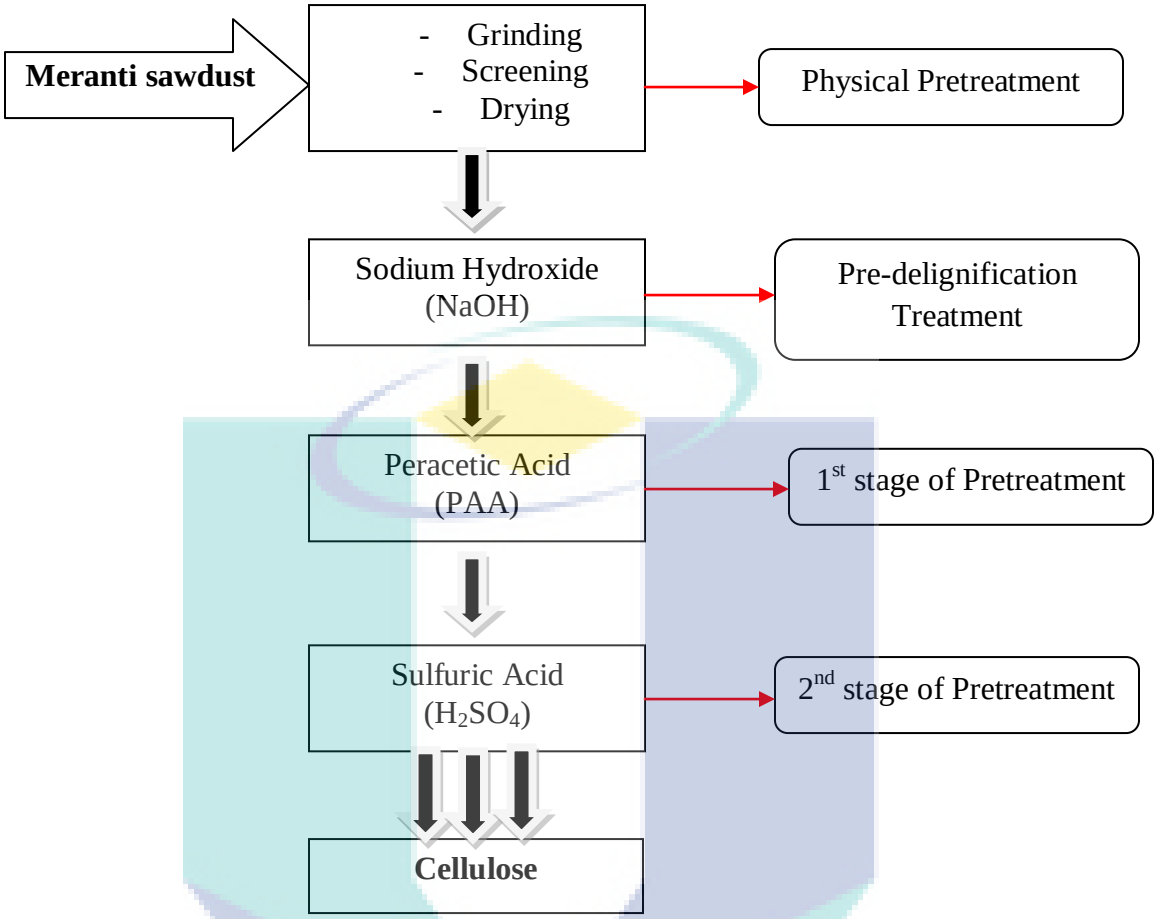


Figure 3.2: Flowchart of whole pretreatment procedure

3.3.1 PHYSICAL PRETREATMENT

There are many type of physical pretreatment that were being discussed in chapter 2 (literature review). In this work, physical treatment was being used as the first treatment to enhance the removal of lignin and also hemicelluloses. After *meranti* wood sawdust was dried under sunlight about five hours, the materials were grinding using grinder (model) to get the uniform size of the sawdust (≤ 0.5mm). Then, the materials were screening using sieve machine (model) to make sure the particle size that would be used in this work are uniformly (≤ 0.5mm). This screening process was to make sure the entire particles that bigger than 0.5mm were removed from the raw materials. Then, the *meranti* wood sawdust were drying for the second time in oven with temperature was set at 60^0C

for the 48 hours or until the moisture content was equal or below than 10%. The moisture content was determined using weight loss equation that shows below:

$$Moisture \quad content \quad (\%) = \frac{M_{initial} - M_{dried}}{M_{initial}} X100\% \tag{3.1}$$

After the target of the moisture content was achieved, whole of the samples were keep in vacuum bag until further process in room temperature. The details of the physical treatment as shown in Figure 3.3.

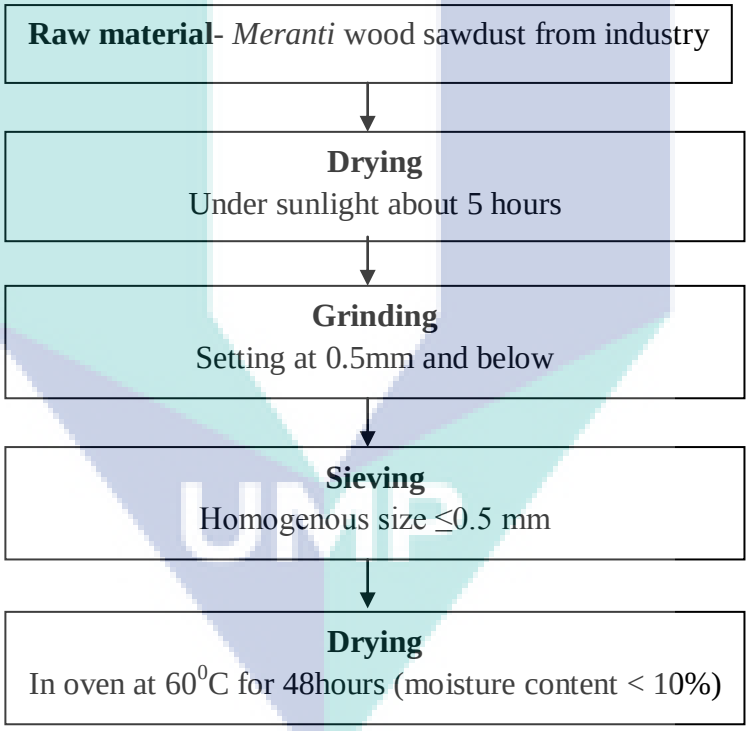


Figure 3.3: Physical treatment for the preparation of *meranti* sawdust fiber

3.3.2 PREDELIGNIFICATION PROCESS

A series of experiments were being conducted as preliminary study in order to investigate the potential improvement to remove the lignin content in biomass materials. Predelignification process was applied to make sure all the oil content, dirt, grease, and the strange chemicals that exist from sawmill machines or environment were removed. Sodium hydroxide (NaOH) is the principal strong base used in the chemical industry. Traditionally, NaOH was used in soap making (saponification). Nowadays, in the pulp and paper industry, NaOH was used as the agent to separate lignin from cellulose fibers and bleaching the brown pulp. Otherwise, in biodiesel manufacturing, NaOH was used as a catalyst for the trans-esterification of methanol and also triglycerides.

In this research, NaOH was being used in pre-delignification process to bleach and also to remove the dirt and oil that come from mill machine. 18% (w/w) of NaOH was used and the experiment was done at 110^0C for 90 minutes to bleach and also to pre-break off the link of the lignin, hemicelluloses and cellulose. Based on the previous research, this kind of the condition will recover maximum of the cellulose recovery and also enhance the probability of the enzymatic hydrolysis (Zhao et. al., 2008). The details of the predelignification process shown in figure 3.4.

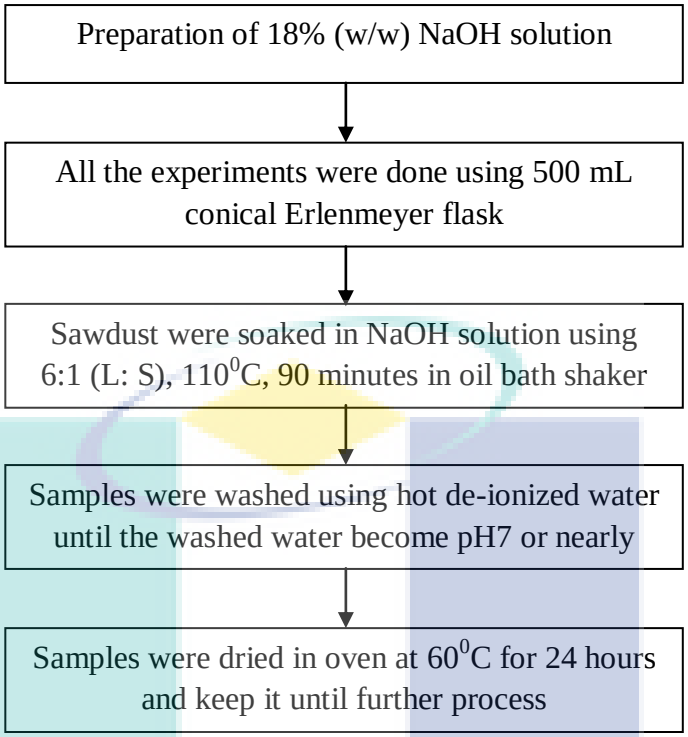


Figure 3.4: Pre-delignification process of *meranti* wood sawdust

3.3.3 PRETREATMENT (FIRST STAGE)

The first stage of pretreatment was being done to remove lignin content using peracetic acid (PAA). Previous study was done (Zhao et al., 2008) and proved that peracetic acid (PAA) can be remove lignin content in biomass materials highly. Peracetic acid (CH_3COOOH) that used in this process was prepared by self. Before the reaction between acetic acid (CH_3COOH) and hydrogen peroxide (H_2O_2) to produce peracetic acid (CH_3COOOH), certain volumes of acetic acid (AA) and sulfuric acid (SA) as catalyst were mixed homogenously. The certain volumes of 30% hydrogen peroxide (H_2O_2) were being added and mixed homogenously. The initial volume ratio of acetic acid and hydrogen peroxide was 1:5. The system was kept at a constant temperature (30^{0}C) in a water bath that located in fume hood because hydrogen peroxide and acetic acid have a very strong smell. This reaction was being operated for the 24 hours (Zhao et.

al., 2008). In this research, 50% (v/w) of peracetic acid (CH_3COOOH) was used and operated at 80^0C for 90 minutes using 6:1 (L: S) ratio (Zhao et al., 2008). The details of the peracetic acid preparation shown in figure 3.5 and first stage of pretreatment have been shown detailed in figure 3.6.

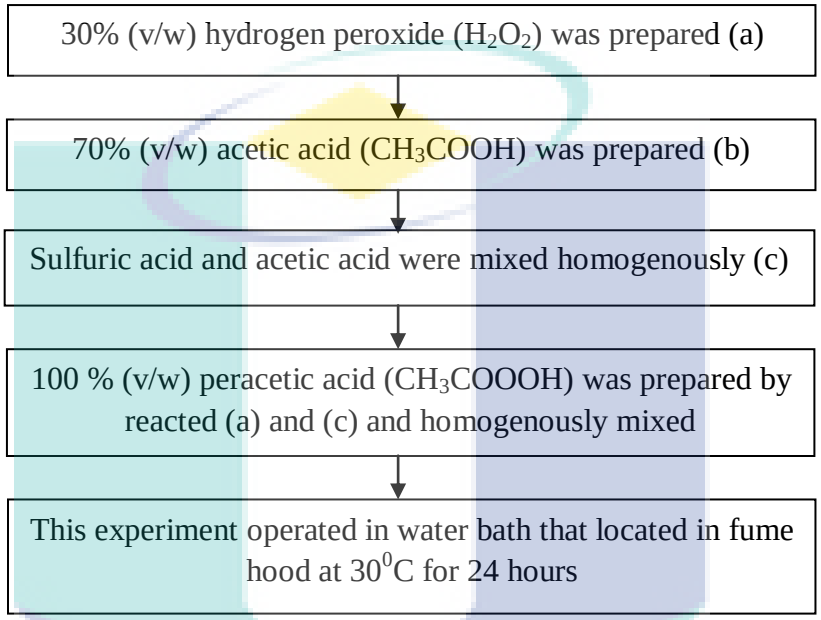


Figure 3.5: Preparation of Peracetic acid (CH_3COOOH)

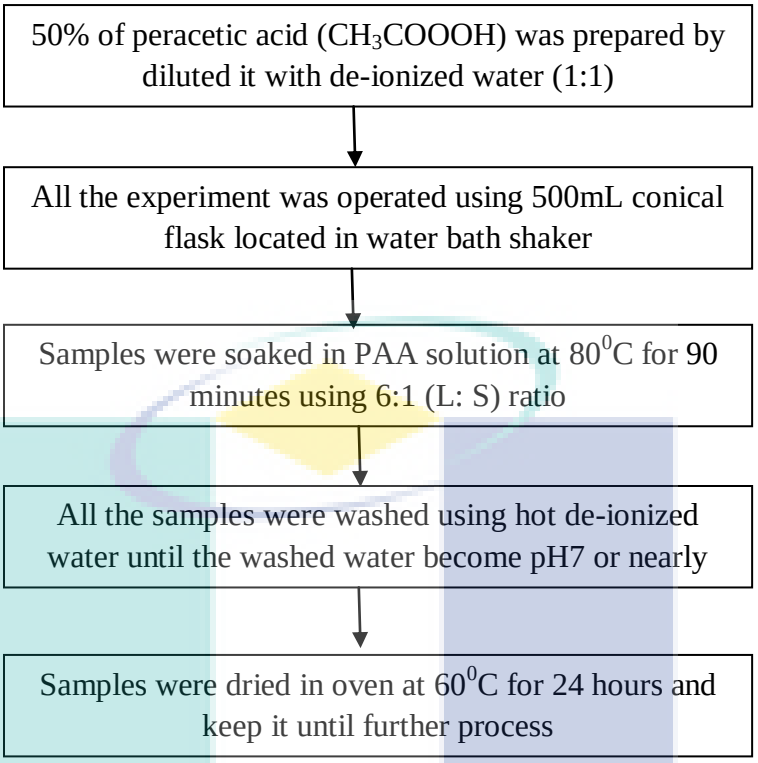


Figure 3.6: First pretreatment of the *meranti* wood sawdust

3.3.4 PRETREATMENT (SECOND STAGE)

In the second stage of pretreatment process, diluted sulfuric acid was being used and at this phase, design of experimental software (design expert version 6.0.8) was used to optimize the removal of lignin and hemicelluloses content. The variables that play role in this stage are temperature, time and also concentration of sulfuric acid. The optimization criteria will be discussed details in section 3.6.

Previous research (Zhao et al., 2008) had proved that the highest removal of lignin and hemicelluloses was operated at 120^0C for 120 min with 1.0% (w/w) sulfuric acid loaded. Because of that, preliminary experiment (one factor at a time, OFAT) was done to double prove the previous finding was correct and also to narrow down the limits

of the variables. The limits of variables will be used in the response surface methodology (RSM). The details of the preliminary experiment shows in figure 3.7 and the second stage pretreatment flow shows in figure 3.8. The table 3.2 shows the details of the variables and data that need to be run.

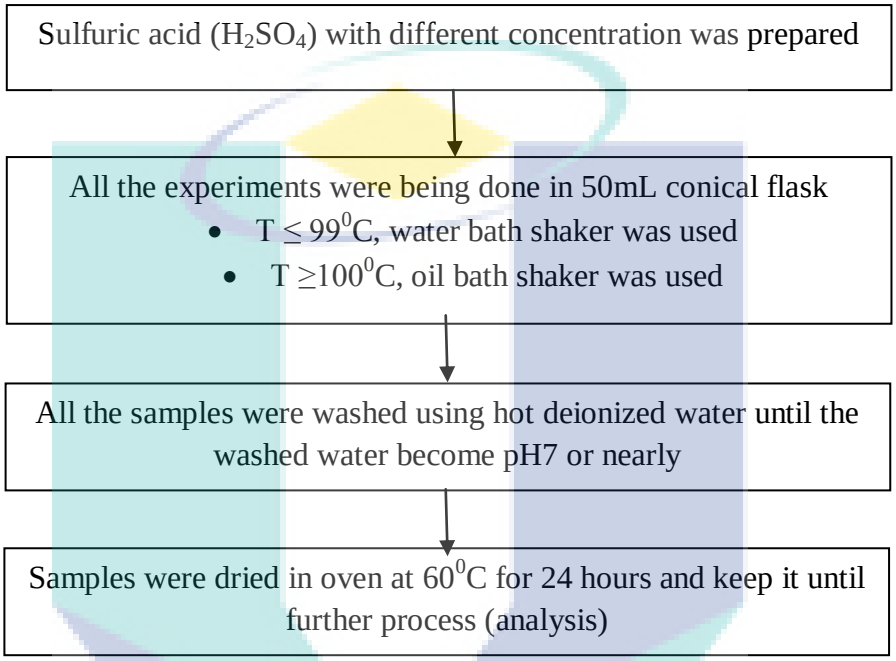


Figure 3.7: Preliminary study of the second stage of pretreatment

Table 3.2: The number of preliminary experiment at second stage treatment

No	Temperature (^{0}C)	Time (minutes)	Concentration % (w/w)
1	100	120	1.0
2	110	120	1.0
3	120	120	1.0
4	130	120	1.0
5	140	120	1.0
6	120	60	1.0
7	120	90	1.0
8	120	150	1.0
9	120	180	1.0
10	120	120	0.1
11	120	120	0.5
12	120	120	1.5
13	120	120	2.0

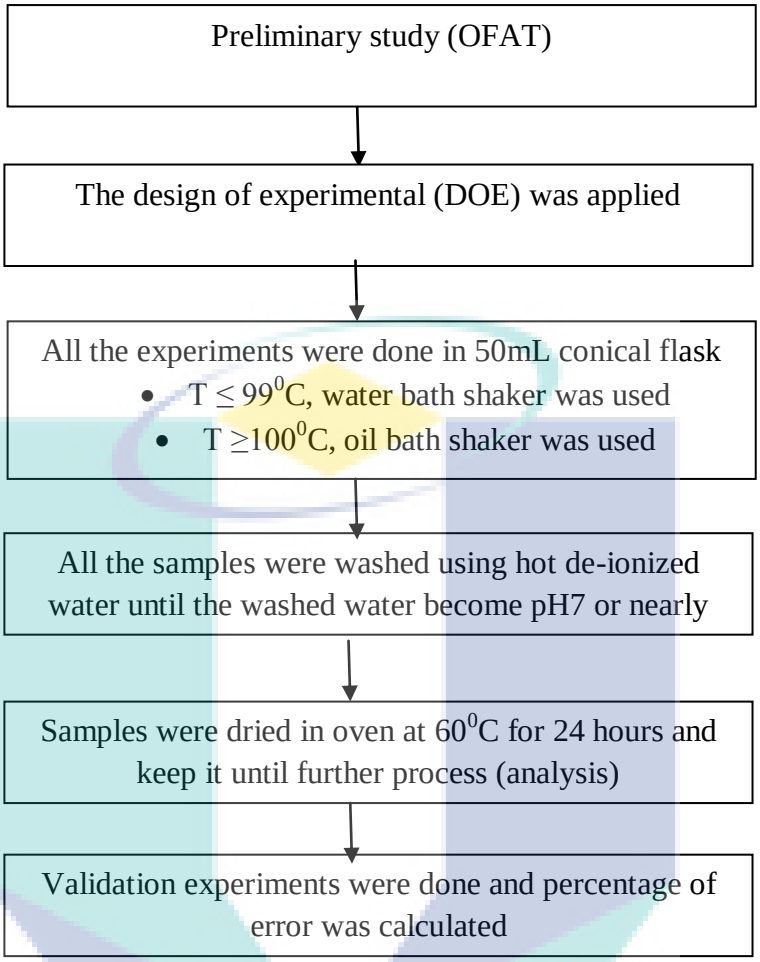


Figure3.8: The second stage of the pretreatment process (DOE application)

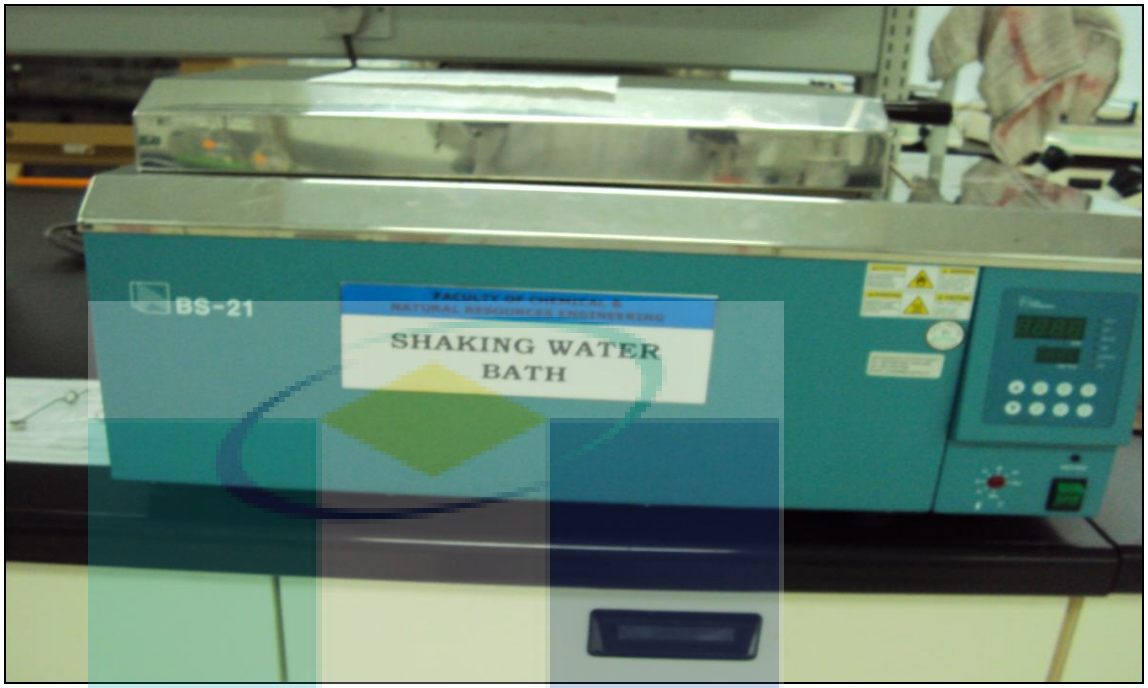


Figure 3.9: Water Bath Shaker

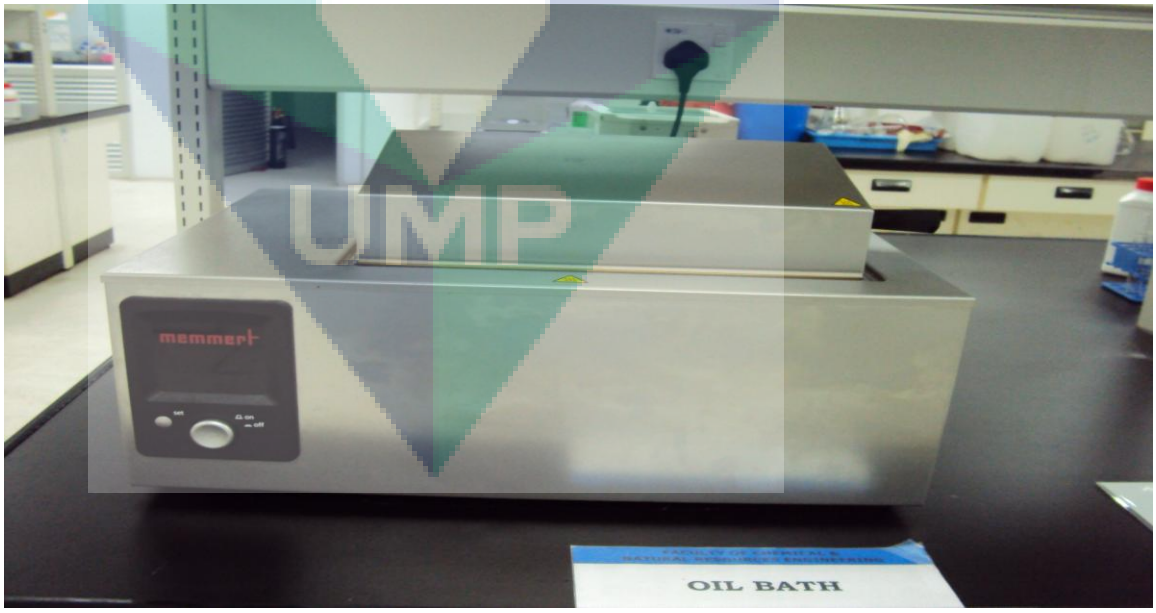


Figure 3.10: Oil Bath Shaker

3.4 ENZYMATIC HYDROLYSIS

The samples that were being pretreated in second pretreatment would be used in this process. There are many type of hydrolysis that we could used to degrade the polysaccharides to monosaccharide, but in this research, enzymatic hydrolysis was used as a process to degrade the cellulose fibers to glucose because of the environment friendly factors. To optimize the yield of the glucose, design of experimental software version 6.0.8 was used. In this work, two types of the enzyme were used; Cellulase (C6105) from *Trichoderma Reesei* and Cellobiase (C2730) from *Aspergillus Niger* and it have been purchased from Sigma Aldrich (M) Sdn Bhd.

Saccharification (hydrolysis) process was being held by enzymatic digestion of pretreated *meranti* wood sawdust. It was carried out in 250mL Erlenmeyer conical flask with 100mL working volume of sodium citrate buffer solution. According to the previous research, the high yield glucose production was carried out at 50^0C for 24 hours using sodium citrate buffer solution (pH5.0). In this phase, the one factor at a time (OFAT) study (preliminary study) was applied to narrow down the low and high limits of the variables that can used it for the central composite design (CCD) software. In figure 3.11, the preparation of the sodium citrate buffer solution shows in details. Table 3.3 shows the preliminary experiment that needs to be run based on the previous research. Besides, figure 3.12 show the flowchart of OFAT study of the hydrolysis process in details and figure 3.13 show the enzymatic hydrolysis process with DOE application.

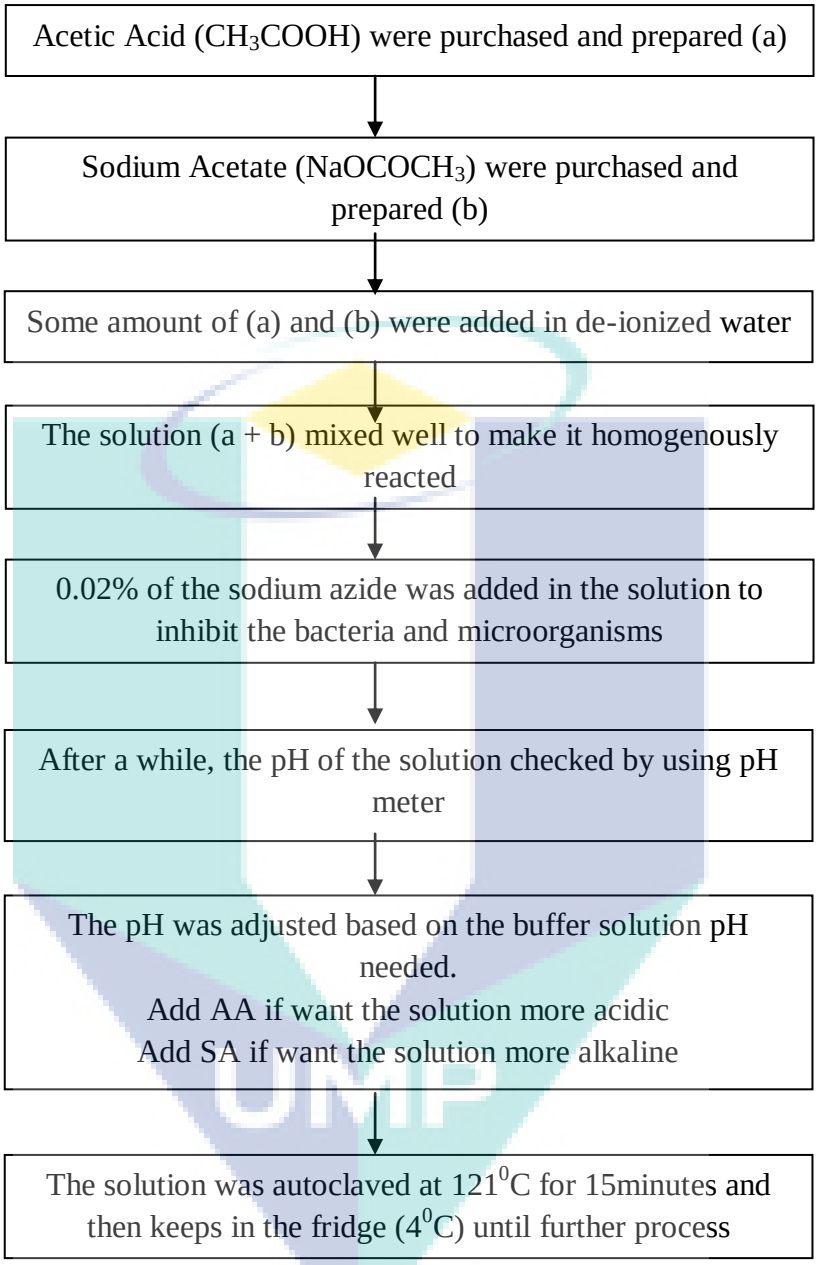


Figure 3.11: Preparation of the sodium citrate buffer solution

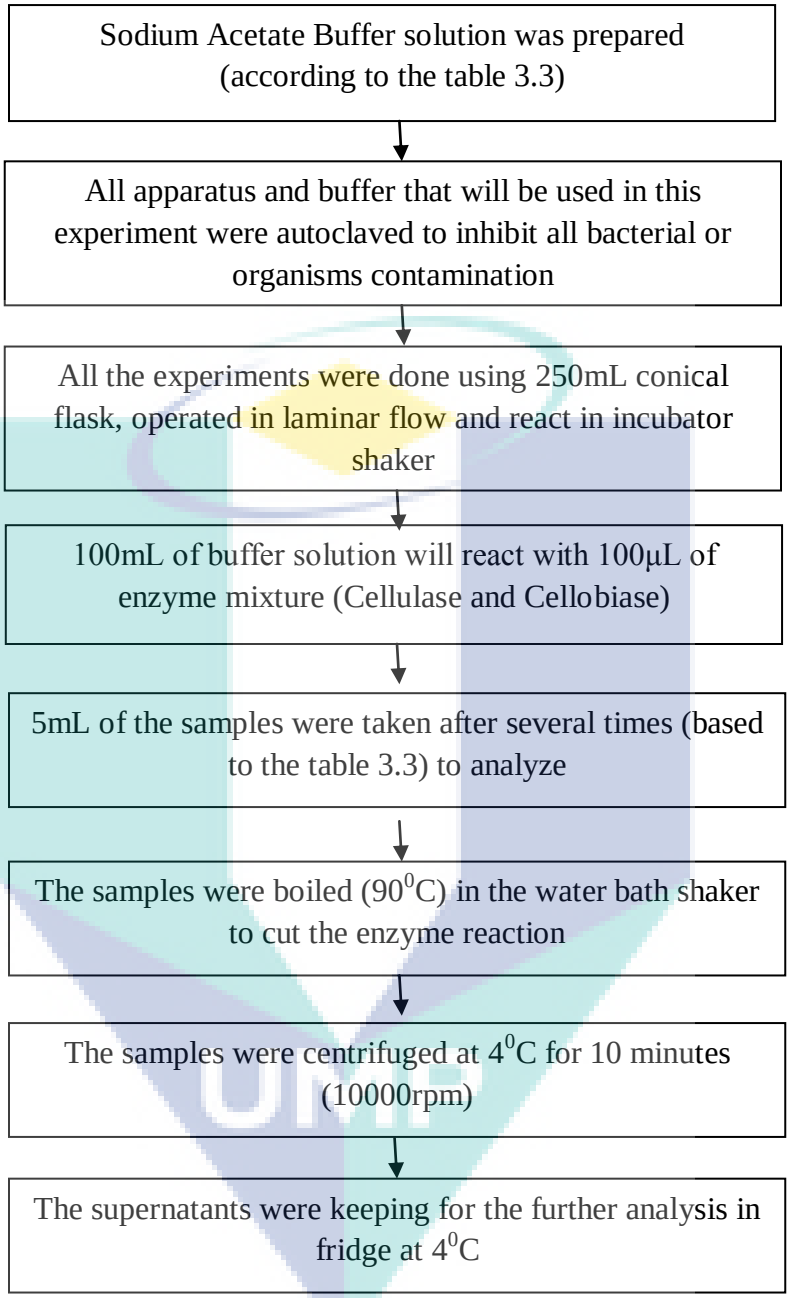


Figure 3.12: Preliminary (OFAT) study of the enzymatic hydrolysis process

Table 3.3: The number of preliminary experiment for hydrolysis process

No	Temperature (^{0}C)	Time (hours)	pH
1.	50	3	5.0
2.	50	6	5.0
3.	50	12	5.0
4.	50	18	5.0
5.	50	21	5.0
6.	50	24	5.0
7.	50	30	5.0
8.	50	36	5.0
9.	50	42	5.0
10.	50	48	5.0
11.	46	24	5.0
12.	47	24	5.0
13.	48	24	5.0
14.	49	24	5.0
15.	51	24	5.0
16.	52	24	5.0
17.	50	24	4.7
18.	50	24	4.8
19.	50	24	4.9
20.	50	24	5.1
21.	50	24	5.2
22.	50	24	5.3

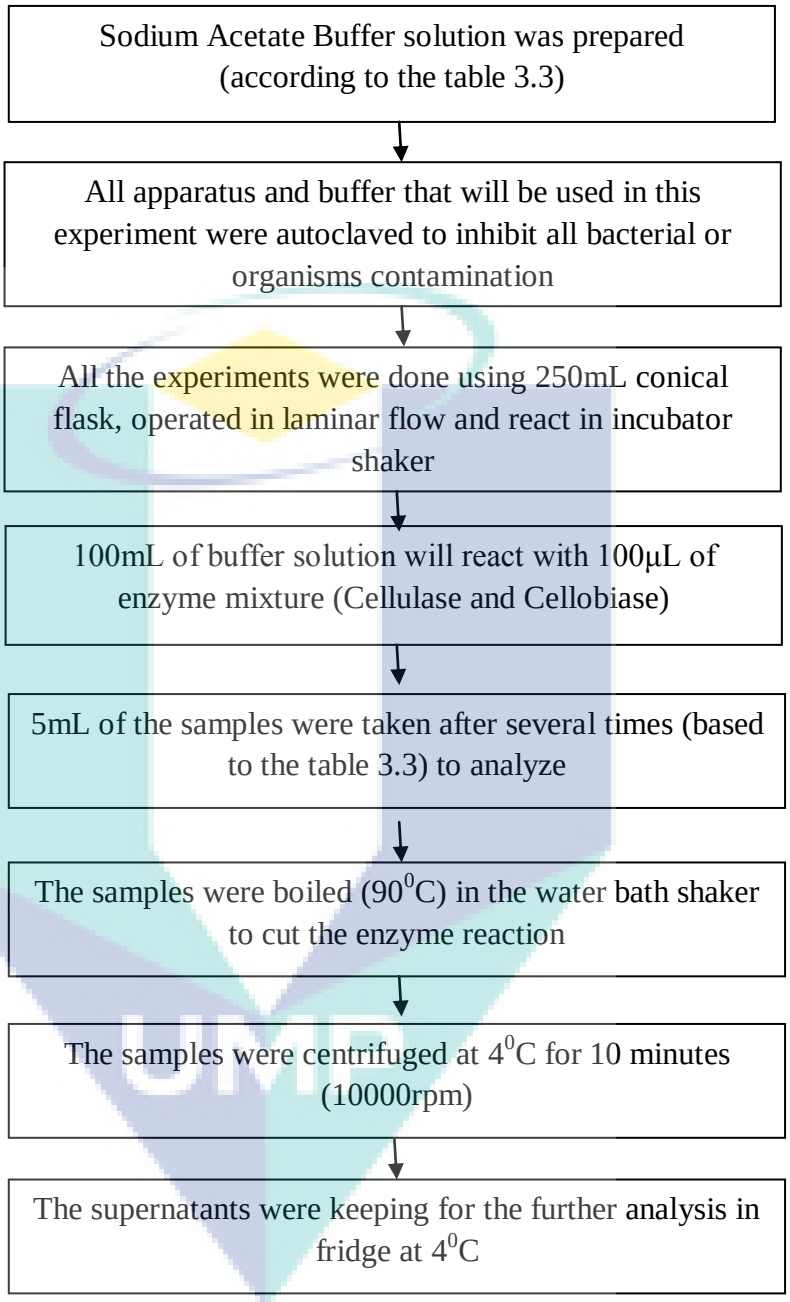


Figure 3.13: The enzymatic hydrolysis process (DOE application)

3.5 FERMENTATION

In this phase, commercial glucose was being used to do the preliminary study of the fermentation process. The glucose was being purchased from Sigma Aldrich (M) Sdn. Bhd. with 90% purity. In this process, the anaerobic fermentation was being applied which the carbon dioxide (CO_2) as a major source to make sure the bacteria will react with the substrate to produce sugar alcohol. Here, *Lactobacillus Plantarum* (BAA 793; NCIMB 8826) was used as bacteria which were purchased from America type Culture Collection (ATCC). The details of the propagation procedure of the bacteria were shown in figure 3.14 (ATCC, 2005). The details of the fermentation process were shown in figure 3.15. Routinely, *Lactobacillus plantarum* were grown in MRS medium (Ladero et. al., 2007). The MRS agar and broth were purchased from Merck (M) Sdn. Bhd.

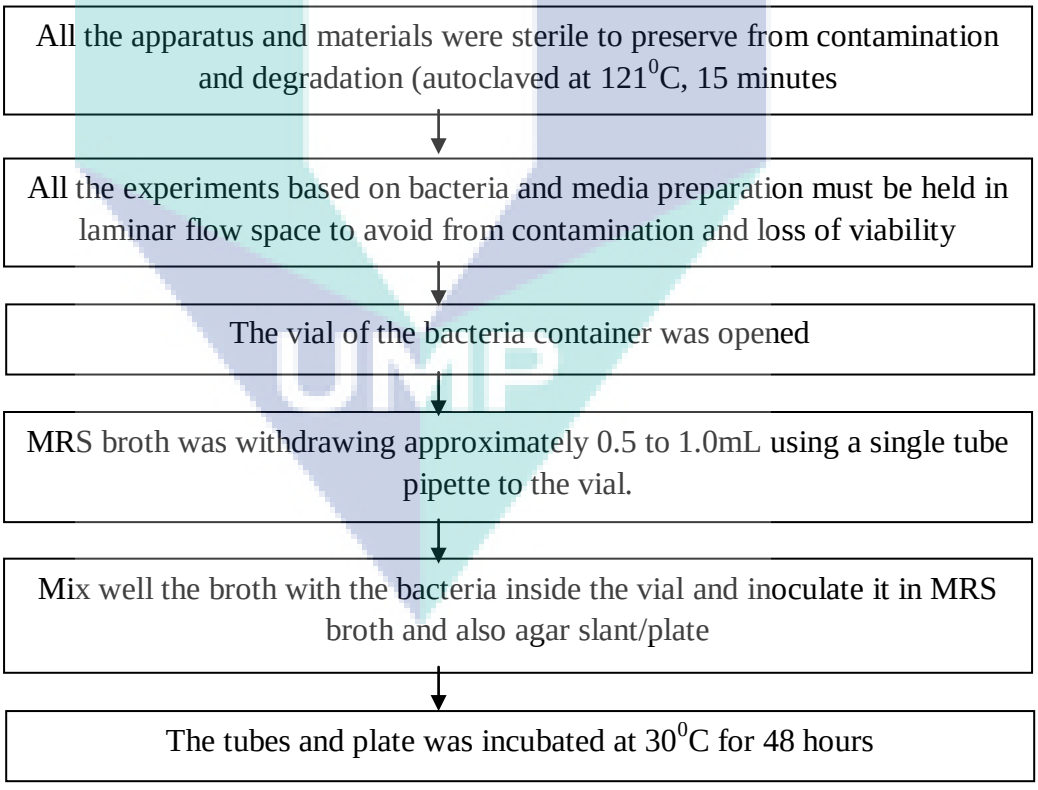


Figure 3.14: The propagation procedure of the bacteria

All the apparatus and materials were sterile to preserve from contamination and degradation (autoclaved at 121^0C, 15 minutes

↓

All the experiments based on bacteria and media preparation must be held in laminar flow space to avoid from contamination and loss of viability

↓

The inoculums from stock were added in the fermentation medium and substrate (glucose)

↓

The fermentation was carried out at 30^0C (incubator shaker), 150rpm and supplied with 20% of CO_2 about 2-5 days.

↓

Every single 6hours, the samples were taken to analyze the sugar alcohol that produces from the fermentation and also to get the profile growth of the bacteria.

Figure 3.15: The flowchart of fermentation of the glucose by *lactobacillus plantarum*

Figure 3.16: Incubator shaker

3.6 OPTIMIZATION BY USING RESPONSE SURFACE METHODOLOGY

Response surface methodology (RSM) is a collection of statistical and mathematical technique useful for developing, improving and optimizing processes. It also has important applications in the design, development and formulation of new products, as well as in the improvement of existing product designs. The most extensive applications of RSM are in the industrial world, particularly in situations where several input variables potentially influence some performance measure or quality characteristic of the product or process (Myers and Montgomery, 2002).

In order to attain the optimization of the lignin degradation from lignocellulosic fibers and glucose yield from hydrolysis process, an optimization process was being performed using Design Expert software version 6.0.8 Stat Ease Inc., USA, to set up a response surface methodology (RSM) or central composite design (CCD) to find the optimum value for each parameters. This design allowed us to include the interactions between two or more factors of the process parameters into the statistical model.

The model is a mathematical equation of higher order and the order depending on the number of influences and their complexity. It is possible that the coefficient depends not only on one parameter in a linear functional correlation; but a quadratic or even cubic, and all these influences were described as separate terms with specific weighing. In the practical application of central composite design (CCD) it is necessary to develop an approximating model for the true response surface. A first order response surface model that might substitute of the chosen parameters into the resulting model enables us to calculate a predicted response as shown in equation 3.2.

$$Y = \beta_0 + \sum_{j=1}^{4} \beta_j X_j + \sum_{j=1}^{4} \beta_{jj} X_j{}^2 X_i X_j \qquad (3.2)$$

where y is the predicted response value, β is the regression coefficient, a weighing factor which is a number calculated by the statistical program to fit the experimental data, and χ is an experimental factor influencing the processes.

3.6.1 DESIGN OF EXPERIMENT (DOE): EXPERIMENTAL PLANNING

For the experimental design of the lignin removal in second stage pretreatment of the *meranti* wood sawdust, time of the soaking in the sulfuric acid (H_2SO_4), temperature of the process, and concentration of sulfuric acid were being chosen as the parameters that will most likely influence the removal of the lignin in samples. For the experimental design of the glucose production from enzymatic hydrolysis process, time of the reaction between enzyme and substrates, temperature of the reaction and pH of the buffer were chosen as the parameters that will most likely influence the production of the glucose in cellulose substrates. The arrangement of experiments that must be carried out as being arranged by design expert V6.0.8 is shown in table 3.6 and table 3.7. The response surface methodology produced a set of 20 experiments, representing the core data for building the model of the second stage of pretreatment condition.

The low and high levels for all the independent process variables were based on prior screening (one factor at a time) experiments. The table 3.4 and table 3.5 show the values for the concentration of sulfuric acid, time and temperature (pretreatment) used by the software and also the values for the pH, temperature and time (hydrolysis) to generate the experimental design.

Table 3.4: The experimental range of the variables process

No	Variable	Unit	Low Range	High Range
1.	Temperature	^{0}C	110	130
2.	Time	Minute	90	150
3.	Concentration (w/w)	%	0.5	1.5

Table 3.5: The experimental range of the variables process

No	Variable	Unit	Low Range	High Range
1.	Temperature	^{0}C	49	51
2.	Time	hour	21	30
3.	pH	-	4.9	5.1

The optimization process was based on central composite design (CCD) which is the best design that applies response surface methodology (RSM). RSM is a method that basically involves three major steps; design of experiments using statistical approach, generation of coefficient based on mathematical model and response prediction and finally model adequacy check.

The equation model was tested with an analysis of variance tool, ANOVA analysis with 99% degree of confidence. The RSM output such as contour plot and 3D graphical surface plot yielded the optimum and most influencing variable for the second stage of pretreatment to attain maximum delignification results. According to the central composite design (CCD), the total number of experiment combination is $2^k + 2k + n_0$, where k is the number of independent variables and n_0 is the number of repetition of the experiments at the centre point. In this study, n_0 is performed six times.

Table 3.6: Arrangement of experiment using central composite design (CCD), second stage of pretreatment

Run No.	Type	Factors		
		Time (min)	Temperature (^{0}C)	Concentration (%)
1.	Fact	150	110	0.5
2.	Center	120	120	1.0
3.	Fact	150	110	1.5
4.	Fact	150	130	0.5
5.	Center	120	120	1.0
6.	Fact	90	110	0.5
7.	Fact	90	130	0.5
8.	Fact	90	130	1.5
9.	Fact	90	110	1.5
10.	Center	120	120	1.0
11.	Fact	150	130	1.5
12.	Center	120	120	1.0
13.	Axial	120	120	0.6
14.	Axial	120	103.18	1.0
15.	Axial	170.45	120	1.0
16.	Axial	120	120	1.84
17.	Axial	120	136.82	1.0
18.	Center	120	120	1.0
19.	Center	120	120	1.0
20.	Axial	69.55	120	1.0

Table 3.7: Arrangement of experiment using central composite design (CCD), enzymatic hydrolysis process

Run No.	Type	Factors		
		Time (hour)	Temperature (^{0}C)	pH
1.	Fact	30	49	5.1
2.	Fact	21	51	4.9
3.	Fact	21	49	5.1
4.	Fact	30	51	5.1
5.	Fact	30	49	4.9
6.	Fact	21	51	5.1
7.	Center	25.5	50	5.0
8.	Fact	21	49	4.9
9.	Center	25.5	50	5.0
10.	Fact	30	51	4.9
11.	Center	25.5	50	5.0
12.	Center	25.5	50	5.0
13.	Axial	25.5	51.68	5.0
14.	Center	25.5	50	5.0
15.	Axial	25.5	50	5.17
16.	Center	25.5	50	5.0
17.	Axial	25.5	50	4.83
18.	Axial	25.5	48.32	5.0
19.	Axial	17.93	50	5.0
20.	Axial	33.07	50	5.0

The variables X_i were being coded according to equation 3.3 below and subsequently being constructed into equation 3.4, a model supposedly to predict the response Y_u according to X_i.

$$X_i = (X_1 - X_0)/\Delta X_i; \qquad i = 1,2,3.....k \tag{3.3}$$

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_{ui} + \sum_{i=1}^{k} \beta_{ii} X_{ui} + \sum\sum_{1\langle j}^{k} \beta ij X_{ui} X_{uj} \tag{3.4}$$

where Yu = predicted response, β_0 = offset term, β_i = linear term, β_{ii} = squared term and β_{ij} = interaction term.

It was being found that a total of 20 experiments were necessary to optimize the lignin removal and also glucose production to attain maximum yield of sugar alcohol in fermentation process. The experiments were performed in triplicate to make sure the error percentages were below than 0.05%. The regression equations were optimized by iteration method to obtain the optimum values. The design of experiment was carried out using design expert version 6.0.8.

3.7 INSTRUMENTS AND TECHNIQUES TO ANALYSIS

Several characterizations techniques have been conducted in order to analyze the properties and characteristics of raw material and also production of three major processes (pretreatment, hydrolysis, and fermentation) were operated in this research. The involved characterization techniques are listed as below:

1) Fourier Transform Infrared Spectroscopy (FTIR)
2) Scanning Electron Microscope (SEM)
3) Ultraviolet Visible Spectroscopy (UV VIS)
4) High Performance Liquid Chromatography (HPLC)

3.7.1 DETERMINATION OF KAPPA NUMBER

In this research, Kappa number was being calculated to determine the removal of lignin in *meranti* wood sawdust. 0.1gm of samples were added into a mixture of 20mL 0.02mol/L $KMnO_4$ (potassium permanganate) with 5.0mL of 2.0mol/L H_2SO_4 (sulfuric acid) and mix well for 3 minutes. Then the solid samples were separated from the solution through filtration while the filtrate were measured using Ultraviolet Visible Spectroscopy (UVVIS) at 546 nm. The equation of the Kappa number was being shown as below:

$$K = \frac{a}{w}\left(\frac{A_0 - A_e}{A_0}\right) \tag{3.5}$$

Where;

α = volume of $KMnO_4$ used in solution

w = weight of moisture free sample used

A_0 = spectral intensities at time=0 (before sample added)

A_e = spectral intensities at the end of reaction

$$Lignin \quad content \quad (wt\%) = 0.15K \tag{3.6}$$

$$Lignin \quad \deg radation = \frac{lignin \quad untreated - lignin \quad treated}{lignin \quad untreated} \tag{3.7}$$

3.7.2 DINITROSALICYLIC COLORIMETRIC METHOD (DNS)

This method tests for the presence of free carbonyl group (C=O), the so-called reducing sugars. This involves the oxidation of the aldehyde functional group present in, for example, glucose and the ketone functional group in fructose. Simultaneously, 3,5-dinitrosalicylic acid (DNS) is reduced to 3-amino,5-nitrosalicylic acid under alkaline oxidations:

$$Aldehyde\ \ group \xrightarrow{oxidation} carboxyl\ \ group \qquad (3.8)$$

$$3,5-dinitrosalicylic\ \ acid \xrightarrow{reduction} 3-a\min o,5-nitrosalicylic\ \ acid \qquad (3.9)$$

Due to the dissolved oxygen can interfere with glucose oxidation, sulfite, which itself is not necessary for the color reaction, is added in the reagent to absorb the dissolved oxygen (Wang, 2005).The details on how to prepare DNS solution (1%) was shown in figure 3.17 and The details of the method were shown in figure 3.18.

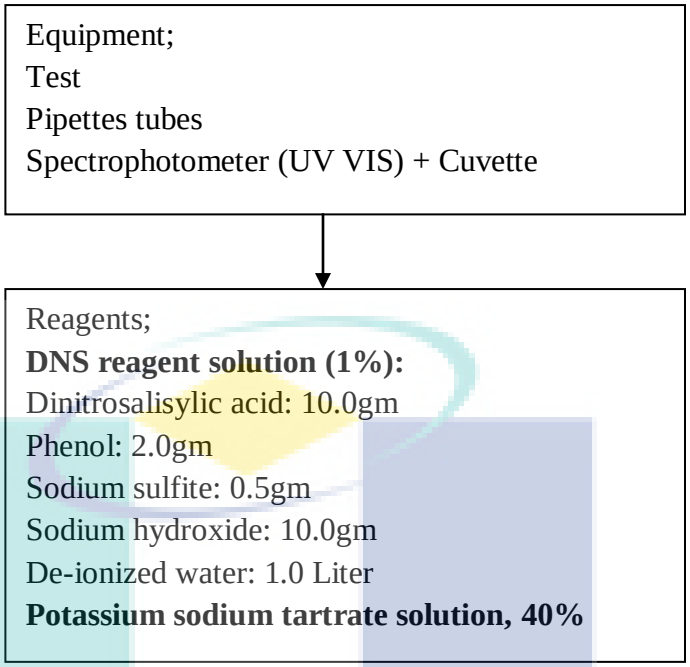


Figure 3.17: Preparation of DNS solution (1%)

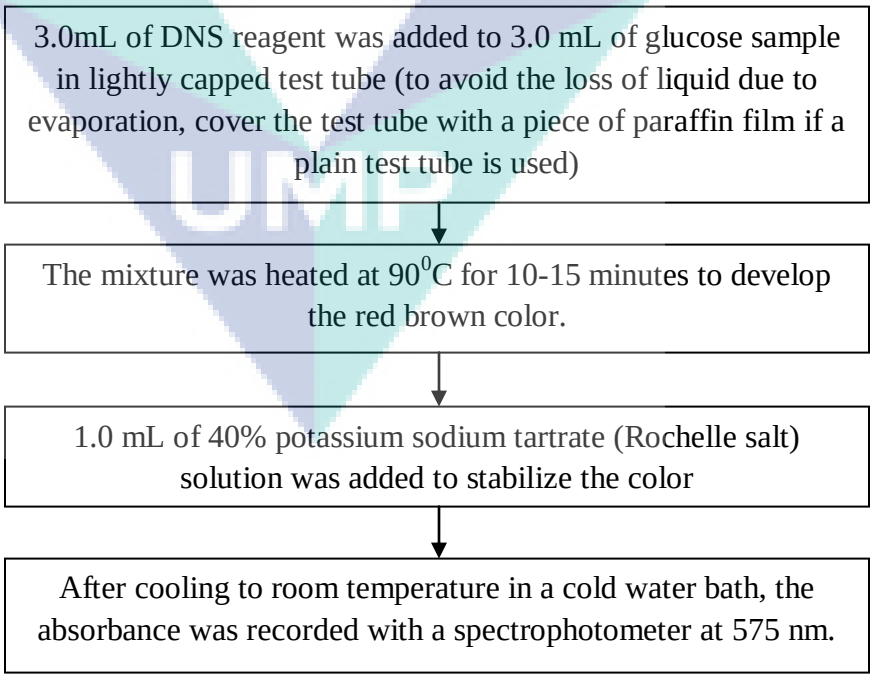


Figure 3.18: The procedures of the DNS method

3.7.3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

In the present work, a simple and a sensitive HPLC method was developed for quantitative determination of sugar alcohol compound. The HPLC that used in this work is HPLC-RID, (Agilent, 1200 series). The column that was being used was RCM Monosaccharide, 300 X 7.8 mm with water as a mobile phase and operated at 75^0C with retention time is 40 minutes. This method using RI as a detector and the flow rate of the mobile phase was about 0.6 mL/min.

Figure 3.19: High Performance Liquid Chromatography (HPLC)

3.7.4 FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

Mechanism reaction and esterification morphology of meranti sawdust, cellulose fibers, glucose and sugar alcohol were analyzed by means of Fourier Transform Infrared Spectroscopy (FTIR), (MODEL: THERMO) using a standard KBr pellet technique. The solid from sample was cut or homogenized particle from sample were weighing about

0.2gm. The standard of KBr and sample were putted in the container and placed it int the FTIR test area. The KBr being analyzed using FTIR software as a background and then followed by materials as samples. Each spectrum was recorded with 32 scans in frequency range from 4000cm^{-1} to 400cm^{-1} with resolution 4cm^{-1}.

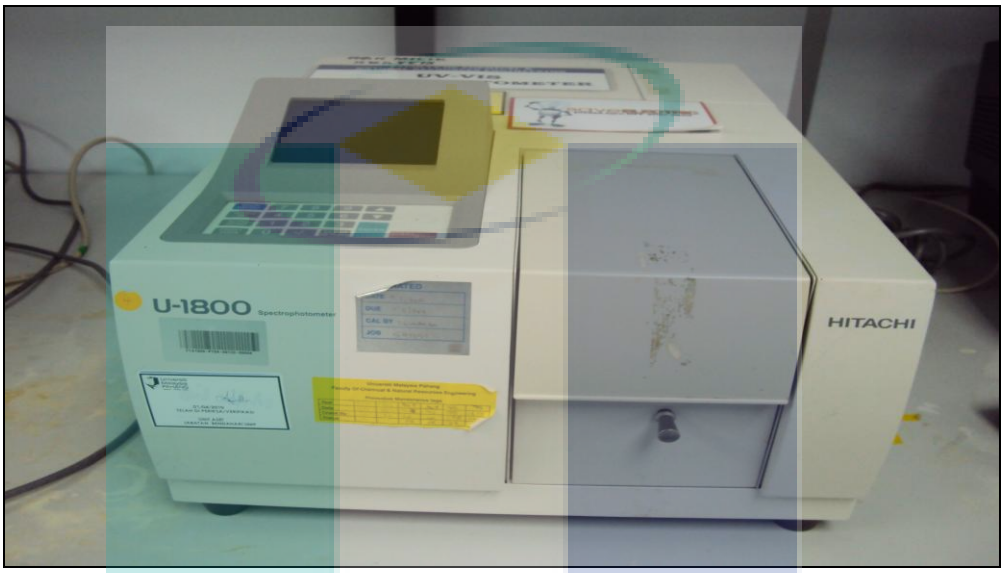


Figure 3.20: Ultra Violet Visible Spectroscopy (UV VIS)

3.7.5 SCANNING ELECTRON MICROSCOPE (SEM)

Scanning Electron Microscope (SEM) was used to analyze the morphological structure of *meranti* wood sawdust and cellulose fibers before and after treatment (MODEL: ZEISS). Samples of air dried cellulose fibers were being fixed to a metal base specimen holder using a double sided sticky carbon tape and then taken for microscope image using EVO 50 (ZEISS brand) at 300KV or larger. By observing the surface of *meranti* wood sawdust fiber and cellulose, the microstructures of materials were being compared.

CHAPTER 4

RESULT AND DISCUSSION

A series of preliminary studies were being performed prior to the investigation of the lignin degradation based on *meranti* wood sawdust to enhance enzymatic hydrolysis and also fermentation process to produce sugar alcohol. The details of discussions are elaborated in this chapter which is divided into subsections; details included the pretreatment of the *meranti* wood sawdust, enzymatic hydrolysis of the *meranti* sawdust pretreated and fermentation process of the glucose to produce sugar alcohol. Furthermore, the characterization of *meranti* wood sawdust, gluocse and sugar alcohol also respectively being discussed and that characterization was being obtained from Fourier Transform Infrared (FTIR), Ultraviolet Visible Spectroscopy (UV-VIS) and Scanning Electron Microscopic (SEM).

4.1 RAW MATERIAL (MERANTI WOOD SAWDUST) COMPOSITION

As indicated in the methods for biomass analysis, the composition of raw material, *meranti* wood sawdust was being determined by the Forest Research Institute of Malaysia (FRIM) laboratory, using in house (FRIM) method. The chemical composition of the *meranti* wood sawdust used in this study was represented in Table 4.1. The initial composition of *meranti* wood sawdust was determined as follows: 48.1% cellulose, 33.9% lignin, 71.7% holocellulose, 0.4% ash and 12.4% pentosan. Holocellulose was being accounted about 71.7% of the dry material which give *meranti* wood sawdust a

very promising substrate for cellulose fiber recovery and sugar alcohol production. The lignin content in *meranti* wood sawdust was about 33.9% and it's very high than previous research reported that the range of the biomass such as corn stover was (17-19%) (Kim et al., 2005, Lu et al., 2009). This means that there was a need to optimize the lignin removal to enhance the enzyme digestibility. Lignin is the one of the component in biomass that will be blocked the enzyme from reacted with the substrate and then hydrolysis process will not held and then the major hypothesis will not achieved successfully.

Table 4.1: Chemical composition of *meranti* wood sawdust

Composition	Percentage (%)	Test Method
Lignin	33.9	T222 om-02, Acid Insoluble Lignin in wood and pulp*
Holocellulose	71.7	In-house method*
Cellulose	48.1	T203 os-74, Alpha, beta, and gamma cellulose in pulp*
Ash	0.4	T211 om-02, Ash in wood, pulp, paper and paperboard*
Pentosan	12.4	In house method*

*In house method by Forest Research Institute of Malaysia, FRIM laboratory

In Table 4.1, the cellulose content of the *meranti* wood sawdust was 48.1% based on weight percentage. This percentage shows that *meranti* wood contained more cellulose fiber than other hemicelluloses and lignin. According the previous research that had been done by Perez (perez et al., 2007), the cellulose content in hardwood type was 40.1% and this percentage shows that our meranti wood had higher cellulose content than other hardwoods. The lignin content was 33.9% and this percentage were higher and here, that's mean needed the most effective pretreatment to remove it soon. Table 4.2 shows us

the comparison of the chemical composition between *meranti* wood sawdust and other biomass. According to the Perez, (Perez et al., 2007), the lignin content in the hardwood was 27.8%. This percentage shows that the lignin content in *meranti* wood sawdust was higher than other hardwood types.

Table 4.2: Comparison of chemical composition between biomass

Biomass type	Cellulose (%)	Holocellulose (%)	Lignin (%)	Ash (%)
Meranti wood[a]	48.10	71.70	33.90	0.40
Crofton Weed Stem[b]	37.14	66.22	16.42	4.52
Sugarcane bagasse[b]	44.98	76.76	18.45	1.38
Corn Stover[b]	37.68	65.30	18.38	4.66
Wheat Straw[b]	40.40	66.00	22.34	6.04
Cotton Stalk[b]	31.10	41.80	27.90	6.00

[a] Forest Research Institute of Malaysia (FRIM)

[b] Zhao et al., 2008

In Table 4.2, it could be seen that the holocellulose and cellulose content in the *meranti* wood sawdust were become similar to those sugarcane bagasse but much higher content than others. According to same table, can see that the *meranti* wood has much lignin content than others and we need to do the optimization in pretreatment to make sure the lignin content will remove highly and maximum. Recently, using the agricultural residues such as wheat straw and corn stalk to produce fermentable sugars for further production of bioethanol has attracted much interest (Zhao et al., 2008). The wood sawdust has abundant in industries like sawmill and logging, large biomass and strong adaptability characteristics. It is estimated that the biomass yield of the wood sawdust in Malaysia especially in Kuantan area can be as much as 48 ton per year (residue). This number will be increase yearly. Therefore, it is possible to utilize the wood sawdust as a

potential biomass feedstock for fermentation sugars production. However, the wood sawdust maybe contains some chemicals which may inhibit the enzyme activity and growth of microorganisms, so it is necessary to treat the wood sawdust to remove the unnecessary substances and enhance the enzymatic digestibility.

4.2 PRETREATMENT PROCESS OF MERANTI WOOD SAWDUST

The pretreatment is a necessary step to alter some structural characteristics of lignocelluloses, increasing glucan and xylan accessibility to the enzymatic attack. As it has been mentioned, these structural modifications of the lignocelluloses are highly dependent on the type of pretreatment employed and have a great effect on the enzymatic hydrolysis (Kumar et al., 2009; Alvira et al., 2010). The goals of the pretreatment are to decompose the polymeric components of the wood and form monomer sugar (Yat et al., 2008), and also to enhance enzymatic conversion of the cellulose fraction, and hopefully, obtain a higher sugar alcohol yield in the last fermentation step.

As discussed details in chapter 2 and 3, the pretreatment process was applied to prepare the *meranti* wood sawdust for enzymatic hydrolysis and also to remove lignin and hemicelluloses content. In this work, physical and chemical treatments were applied to make sure the lignin and hemicelluloses were removed highly and the percentage of cellulose recovery was maximized. Besides, the response surface methodology (RSM) was applied to optimize the lignin degradation in second stage pretreatment of *meranti* wood sawdust. In order examined of the materials properties, there are needed to study the characterization of the materials before and after the treatment.

4.2.1 PHYSICAL PRETREATMENT OF MERANTI WOOD SAWDUST

The chemical composition was not the sole factor influencing the enzymatic hydrolysis. Physical properties and cellulose microstructure were among the potential factors influencing enzymatic hydrolysis (Xu et al., 2007). Because of that, physical pretreatment method such as comminution (mechanical reduction in biomass particulate size) and steam explosion can be classified into mechanical and non-mechanical pretreatment. Physical forces used in mechanical pretreatments can subdivide lignocellulosic material into fine particles which are substantially susceptible to acid or enzymatic hydrolysis. Non-mechanical physical pretreatments cause decomposition of lignocellulosics by exposing them to harsh external forces other than mechanical forces (Yat et al., 2008).

In this work, *meranti* wood sawdust were chopped and milled to homogenize the size to become less than 0.5mm. With this uniform size, the further treatments become more effectiveness and prepare the sawdust to treat and then recover the cellulose fibers. The objective of the using milling (cutting the lignocellulosic) biomass into smaller pieces) process in this work is a reduction of particle size and crystallinity. The reduction in particle size leads to an increase of available specific surface area and reduction of the degree of polymerization (DP) (Palmowski and Muller, 1999; Hendriks and Zeeman, 2009). The milling causes also shearing of the biomass.

The increase in surface area, reduction of degree of polymerization (DP), and the shearing, are all the factors that increase the total hydrolysis yield of the lignocelluloses in most cases by 5-25% (depends on kind of biomass, kind of milling and duration of the milling), but also reduces the technical digestion time by 23-59% (thus an increase in hydrolysis rate) (Delgenés et al., 2002; Hartmann et al., 1999; Hendriks and Zeeman, 2009). A particle size reduction below 40 mesh however has little effect on the hydrolysis yield as well as hydrolysis rate of the biomass (Chang and Holtzapple, 2000; Hendriks and Zeeman, 2009).

4.2.2 CHEMICAL PRETREATMENT OF MERANTI WOOD SAWDUST; ONE FACTOR AT TIME (OFAT) APPLICATION

Chemical pretreatment refers to the process of using chemicals to remove or modify key chemical components that interfere with biomass cellulose saccharification, mainly hemicelluloses and lignin (Zhu and Pan, 2010). In this research, chemical pretreatment were carried out to remove the lignin and hemicelluloses content and also to recover the cellulose fibers. For the stating work, pre-delignification was done using sodium hydroxide (NaOH) to prepare the wood sawdust for the first and the second stage of pretreatment. Recent research has shown that the delignification is not the only way to remove the recalcitrance of lignin. Physically blocking lignin, chemical modification of lignin and lignin preserving pretreatments are less expensive but promising (Pan et al, 2005; Zhu and Pan, 2010).

Alkali-PAA (peracetic acid) pretreatment (first stage) was conducted under mild conditions (below 90^0C) which mainly caused removal of hemicelluloses and lignin. However, it was known that removal of lignin was much more helpful for increasing the enzymatic digestibility (Zhao et al., 2007; Zhao et al., 2008; Zhao et al., 2009). The role of the pre-delignification stage and first stage pretreatment partially removed lignin and swelled the fibers, which also could result in increase of enzymatic digestibility. In order to further investigate the effectiveness of alkali-PAA pretreatment, a comparison of this process was shown in Table 4.3.

Table 4.3: The comparison between alkali and PAA stage

	After alkali stage	After PAA stage
Biomass loading, % (w/w)	10.0	10.0
Temperature (^{0}C)	90	75
Yield of solid recovered (%)	72.4	58.3
Total lignin content (%)	10.4	5.3
Degree of delignification (%)	69.3	84.4

Comparatively, after PAA treatment, the degree of delignification was increased by 77.5% from total of lignin (after alkaline) and the lignin content was decreased to 5.1%. This result indicated PAA was a very selective delignification agent and this chemical treatment will proceed with second stage of treatment using dilute sulfuric acid as an agent to remove hemicelluloses and lignin.

Diluted acid pretreatment of lignocellulosic biomass has received considerable research attention over the years (Sun and Cheng, 2002; Mosier et al., 2005; Zhao et al., 2008). It predominantly removes hemicelluloses, which increases the accessible surface area of the biomass. There are primarily two types of dilute acid pretreatment processes: high temperature (T greater than 160^{0}C) and low temperature (T less than 160^{0}C). In this study, diluted sulfuric acid was used to treat the *meranti* wood sawdust at a temperature below than 160^{0}C, so it was called a "mild" sulfuric acid pretreatment process. The factors affecting sulfuric acid pretreatment mainly include the particle size (D), concentration of acid (C), the liquid solid ratio, temperature (T), reaction time (t) and so on.

In this stage, one factor at a time (OFAT) study was used to narrow down the lower and upper limits of the variables that had been chosen in this work. The variables that used in this stage are reaction time (t), temperature (T) and concentration of sulfuric

acid (C). As discussed in previous chapter, the Kappa number equation was used to calculate the degradation of lignin and lignin content in sample. Table 4.4 – Table 4.6 shows the lignin content in samples based on the OFAT study. There are fifteen experiments were run and based on the Kappa number, the lignin content calculated.

Table 4.4: Study the effected of temperature (T) for the lignin degradation (%)

Temp (^{0}C)	t (min)	C (%)	a(mL)	w(gm)	A_0	A_e	Kappa No	Lignin Content	Lignin Degradation (%)
100	120	1.0	20.0	0.0847	2.620	2.500	10.8150	1.6223	69.41%
110	120	1.0	20.0	0.1104	2.620	2.563	3.9413	0.5912	88.85%
120	120	1.0	20.0	0.1138	2.620	2.597	1.5428	0.2314	95.64%
130	120	1.0	20.0	0.0987	2.620	2.543	5.9553	0.8933	83.15%
140	120	1.0	20.0	0.1355	2.620	2.420	11.2673	1.6901	68.13%

Table 4.5: Study the effect of time (t) for the lignin degradation (%).

Temp (^{0}C)	t (min)	C (%)	a(mL)	w(gm)	A_0	A_e	Kappa No	Lignin Content	Lignin Degradation
120	60	1.0	20.0	0.0838	2.620	2.500	10.9312	1.6397	69.08%
120	90	1.0	20.0	0.1080	2.620	2.252	26.0107	3.9016	26.42%
120	120	1.0	20.0	0.1138	2.620	2.597	1.5428	0.2314	95.64%
120	150	1.0	20.0	0.1005	2.620	2.240	28.8633	4.3295	18.35%
120	180	1.0	20.0	0.1001	2.620	2.167	34.5456	5.1818	2.27%

Table 4.6: Study the effect of acid concentration (C) for the lignin degradation (%)

Temp (^{0}C)	t (min)	C (%)	a(mL)	w(gm)	A_0	A_e	Kappa No	Lignin Content	Lignin Degradation
120	120	0.1	20.0	0.0965	2.620	2.240	30.0597	4.5090	14.96%
120	120	0.5	20.0	0.0924	2.620	2.290	27.2628	4.0894	22.88%
120	120	1.0	20.0	0.1138	2.620	2.597	1.5428	0.2314	95.64%
120	120	1.5	20.0	0.0978	2.620	2.430	14.8301	2.2245	58.05%
120	120	2.0	20.0	0.1101	2.620	2.456	11.3706	1.7056	67.83%

Figure 4.1 shows the percentage of lignin degradation effected by temperature. From the graph, could conclude that the maximum lignin degradation was at T=120^{0}C, it is 95.64% from the total lignin (5.3024% w/w). At the end of experiment, the lignin content in the sample was 0.2312% w/w and this value was decrease from the first experiment. The main issued when the experiments at the level of T= 130^{0}C and 140^{0}C, the lignin degradation percentage were decreased. This is because, at this high temperature, the chemical structures in biomass samples were inconsistent and the lignin component can't degrade usually. Concerning about the cellulose recovery it can be increased by using temperature not higher than 120^{0}C. At other point, a higher temperature may further increase the cellulose conversion to enzymatic digestibility soon, but more energy consumption and a higher pressure will be needed (Zhao et al., 2008). Besides, one potential cause of increased enzymatic digestibility at higher dilute sulfuric acid pretreatment temperature is the glass transition of lignin which occurs between 120^{0}C to 140^{0}C and creates easier access to the pores of the cellulose. However it has also been shown that re-deposition of lignin mobilized under the conditions of these experiments (temperature greater than 150^{0}C, pH less than pH7) could have negative impact on cellulose digestion (Selig et al., 2007; Jensen et al., 2010) but the results of this analysis suggest effects of the former observation.

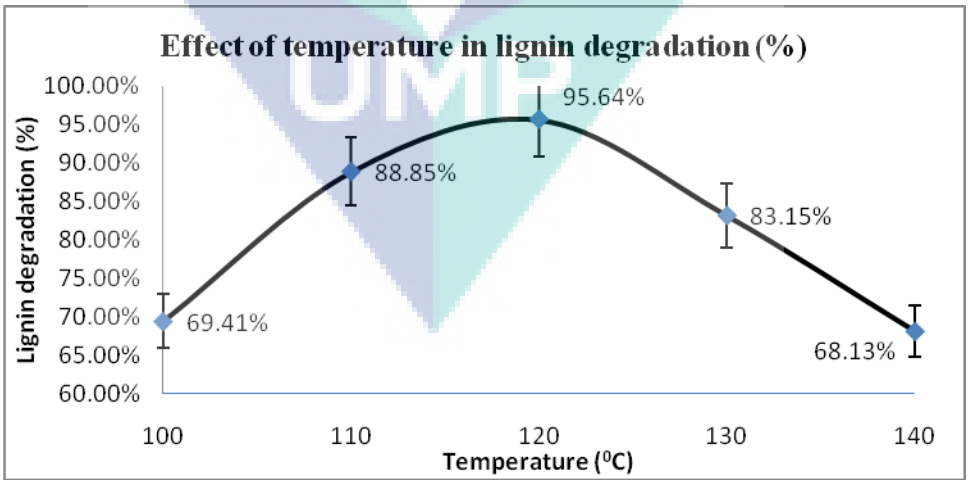


Figure 4.1: Study the lignin degradation based on temperature effect

Figure 4.2 shows the percentage of lignin degradation effected by time. The lignin degradation was 95.64% at time = 120 minutes (2 hours). From the graph, when the reaction time higher than 120 minutes, the degradation of lignin was decreased. It is because, when the reaction time were longer than maximum point, the chemical structure of the biomass were break down and they need time to starting back the removal of lignin. Pretreatment time significantly affected all the response variables and beside that, the cellulose recovery also needs to be noted.

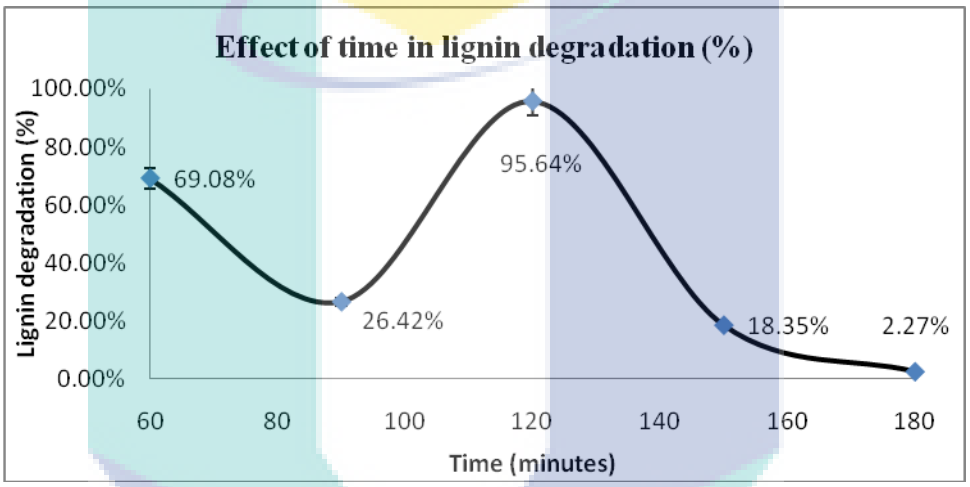


Figure 4.2: Study the lignin degradation based on time effect

Figure 4.3 shows the lignin degradation based on acid concentration effected. From the starting point, the lignin was degraded slowly and achieved the 'optimal' point at 1.0% of acid concentration. After that, the graph slowly decreased. The decrease observed because the high concentration of acid will affected the cellulose recovery and because of that, the lignin degradation also decrease. In this experiment, the result not only mention about lignin degradation but also cellulose recovery percentage because from this recovery, we can measure the sugar production in enzymatic digestibility soon. The higher lignin content along with the characteristic rigid structure of hardwoods cause *meranti* wood sawdust to remain resistant to enzymatic attack after first treatment since the structure remains more recalcitrant than that of other biomass (Galbe and Zacchi, 2002; Jensen et al., 2010).

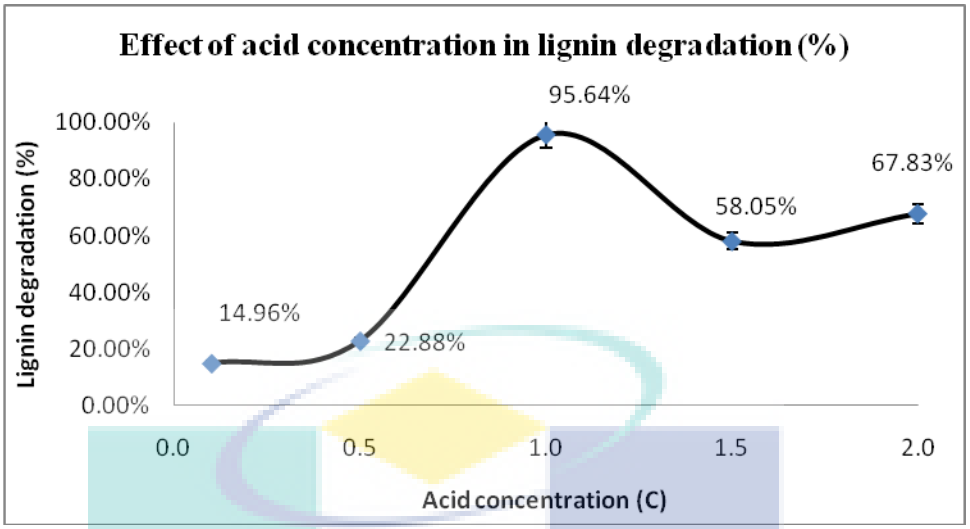


Figure 4.3: Study the lignin degradation based on acid concentration effect

4.2.3 CHEMICAL PRETREATMENT OF MERANTI WOOD SAWDUST; RESPONSE SURFACE METHODOLOGY (RSM) APPLICATION

A central composite design (CCD) was employed to reduce the total number of experiments needed to determine the best combination of parameters for optimization of the process. The statistical software "Design Expert", version 6.0.8 was used for the central composite design and to analyze the experimental data obtained. The conditions for each experiment have been shown in chapter 3. Table 4.7 shows the experimental layout and results of central composite design.

Table 4.7: Experimental layout and results of central composite design (CCD)

Standard	Run	Block	Factor Variables			Response
			Time (min)	Temp (^{0}C)	Conc (C)	Lignin degr. (%)
1	6	1	90.0	110.0	0.5	14.14
2	1	1	150.0	110.0	0.5	8.31
3	7	1	90.0	130.0	0.5	26.64
4	4	1	150.0	130.0	0.5	12.58
5	9	1	90.0	110.0	1.5	18.2
6	3	1	150.0	110.0	1.5	19.55
7	8	1	90.0	130.0	1.5	56.31
8	11	1	150.0	130.0	1.5	53.51
9	5	1	120.0	120.0	1.0	80.2
10	2	1	120.0	120.0	1.0	95.4
11	10	1	120.0	120.0	1.0	86.4
12	12	1	120.0	120.0	1.0	95.4
13	20	1	69.5	120.0	1.0	83.83
14	15	1	170.5	120.0	1.0	17.04
15	14	1	120.0	103.2	1.0	37.78
16	17	1	120.0	136.8	1.0	48.48
17	13	1	120.0	120.0	0.2	2.15
18	16	1	120.0	120.0	1.8	16.28
19	19	1	120.0	120.0	1.0	90.3
20	18	1	120.0	120.0	1.0	75.4

The three significant variables reaction time, temperature and concentration of acid were further optimized using response surface methodology and results on the effect of variable factors towards lignin degradation, the response variables, are shown in table 4.8. In this design, the PRT, pretreatment reaction time was set at the center point settings (120 minutes), due to its low significances for lignin removal while the pH was maintained at 1.0. Fit summary output analysis indicated that the quadratic model was statistically significant to represent the lignin degradation response. The adequacy of a quadratic model was examined by F test, "Prob>F" and the determination coefficient R^2.

As can be inferred in table 4.8, the computed F and Prob>F were 26.7662 and <0.0001, respectively, which implied that the model was highly significant with low probability. Results obtained adequately suggesting that the present mathematical model was in good prediction of the experimental results and as a matter of fact the terms in the model have a significant effect of the response. In a similar manner, the multiple correlation coefficient of R^2 was calculated to be 0.9679, indicating a good agreement existed between the experimental and predicted value as well as depicting that 96.79% of the variability in the response could be well explained by the model while only 3.21% of the total variation was poorly described by the model.

Moreover, the "lack of fit" value was found insignificant (Prob>F= 0.4091) which denoted that the model was desirably fit. Second order effect of acid concentration (C^2) was found to be the most significant factor to have largest effect towards the lignin degradation efficiency. This was followed by the second order effect of reaction time (A^2), the second order effect of temperature (B^2), the main effect on pH (C) and the main effect of temperature (B). Moreover, the main effect of time (A), the two level interaction between time and temperature (AB), the two level interaction between time and concentration of acid (AC) and the two level interaction between temperature and concentration of acid (BC) were found to be responsible for the secondary effect on the lignin degradation.

Table 4.8: ANOVA for response surface quadratic model (partial some of square), response; lignin degradation (%)

Source	Sum of Squares	DF	Mean Squares	F Value	Prob>F
Model	18971.6862	9	2107.9651	26.7662	< 0.0001[a]
A	55.2674	1	55.2674	0.7018	0.4265
B	835.7530	1	835.7530	10.6121	0.0116
C	880.5933	1	880.5933	11.1815	0.0102
A^2	3802.9841	1	3802.9841	48.2889	0.0001
B^2	2767.2203	1	2767.2203	35.1372	0.0004
C^2	9332.9103	1	9332.9103	118.5059	< 0.0001
AB	19.1580	1	19.1580	0.2433	0.6351
AC	42.5042	1	42.5042	0.5397	0.4835
BC	382.2613	1	382.2613	4.8538	0.0587
Residual	630.0383	8	78.7548		
Lack of Fit	353.4033	4	88.3508	1.2775	0.4091[b]
Pure Error	276.6350	4	69.1588		
Cor Total	19769.4062	18			
Std. Dev.	8.87		R^2	0.9679	
Mean	44.95		Adjusted R^2	0.9317	

Values of "prob>F" less than 0.0500 indicate model are significant.

[a] significant

[b] not significant

It should be noted that other model such as the two level interactions of the all factor were insignificant (Prob>F more than 0.0500 indicate model terms are insignificant and therefore can be eliminated for model improvement (Reduced Quadratic Model). In conjunction, the backward elimination procedure was employed to eradicate the insignificant terms and NOVA results of this reduced quadratic model is tabulated in Table 4.9. The results revealed that the model significance was improved (F=39.0549 instead of the 26.7662 for previous model) with the second order effect of all variables appeared as the primary main effect contributor allowed by the two level interaction of temperature and acid concentration (BC), main effects (B) and (C). Hence in this study the ranking is as follows: A^2>B^2>C^2>B>C>BC. The coefficient of determination R^2 for

lignin degradation was 0.9647 indicating good correlation existed between the experimental and predicted values. The multiple regression equations for lignin degradation using reaction time (A), temperature (B) and acid concentration (C) as the main variable were follows:

Final empirical model in terms of coded factors:

$$Lignin\ \ Degradation = +86.26 - 2.58 \times A + 7.82 \times B + 8.03 \times C - 22.10 \times A^2 - 14.33 \times B^2 - 26.33 \times C^2 + 6.91 \times B \times C \quad (4.1)$$

Final empirical model in terms of actual factors:

$$Lignin\ \ Degradation = -2370.5106 + 5.8066 \times time + 33.8032 \times temperature + 60.7640 \times concentration\ \ of\ \ acid - 0.0246 \times time\ \ - 0.1434 \times temperature^2 - 105.3021 \times concentration\ \ of\ \ acid^2 + 1.3825 \times temperature \times concentration\ \ of\ \ acid \quad (4.2)$$

Table 4.9: ANOVA for response surface reduced quadratic model (partial some of square), response; lignin degradation (%)

Source	Sum of Squares	DF	Mean Squares	F Value	Prob>F
Model	18910.0239	7	2701.4320	39.0549	< 0.0001[a]
A	55.2674	1	55.2674	0.7990	0.3924
B	835.7530	1	835.7530	12.0826	0.006
C	880.5933	1	880.5933	12.7308	0.0051
A^2	3802.9841	1	3802.9841	54.9802	< 0.0001
B^2	2767.2203	1	2767.2203	40.0060	< 0.0001
C^2	9332.9103	1	9332.9103	134.9270	< 0.0001
BC	382.2613	1	382.2613	5.5264	0.0406
Residual	691.7005	10	69.1701		
Lack of Fit	415.0655	6	69.1776	1.0003	0.5247[b]
Pure Error	276.6350	4	69.15875		
Std. Dev.	8.32		R^2	0.9647	
Mean	44.95		Adjusted R^2	0.9400	

Values of "prob>F" less than 0.0500 indicate model are significant.

[a] significant

[b] not significant

The above empirical model equations are mathematical correlation model that can be employed to predict and optimize the lignin degradation within the range of variable factors of this experiment. Analyses on normal probability plot of the residuals (figure 4.4) depicted nearly a straight line residuals distribution, which donating errors are evenly distributed and therefore support adequacy of the least-square fit, while results illustrated in figure 4.5 revealed that the models proposed are distinctively adequate and reasonably free from any violation of the independence or constant variance assumption, as studentized residuals are equally tabulated within the red line of the x-axis.

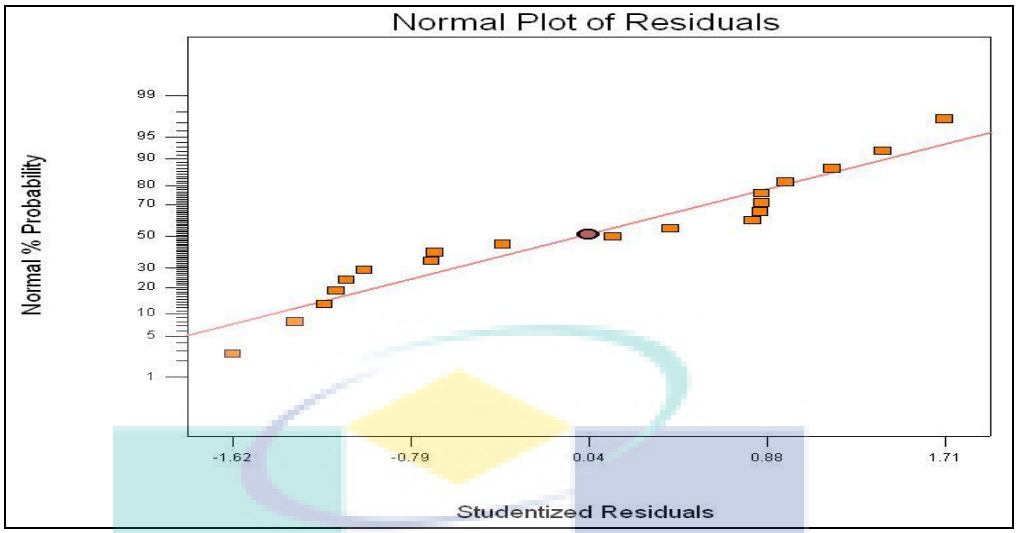


Figure 4.4: Normal probability plot of residuals for lignin degradation

The effect of the reaction time, temperature and concentration of acid process variables on lignin degradation was further analyzed using simulated three dimensional response surface and contour plots according to the backward quadratic model. The effect of temperature and concentration of acid on lignin degradation depicted in figure 4.6 and figure 4.7 demonstrated that the lignin degradation increased when pH changed from 0.5 to 2.0 and as temperature increase from 110^0C to 130^0C.

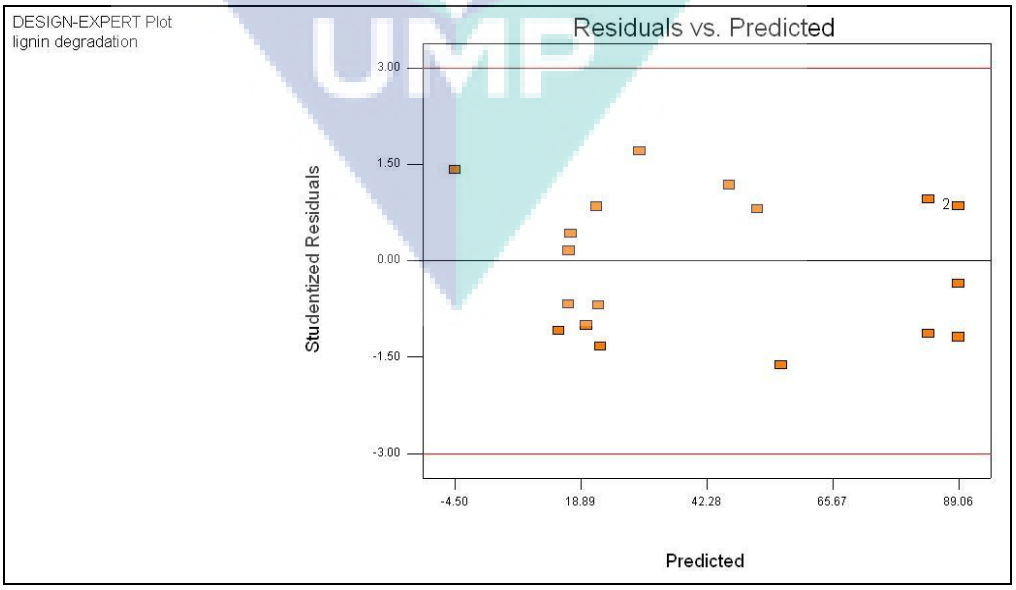


Figure 4.5: Plot of residual against predicted response of lignin degradation

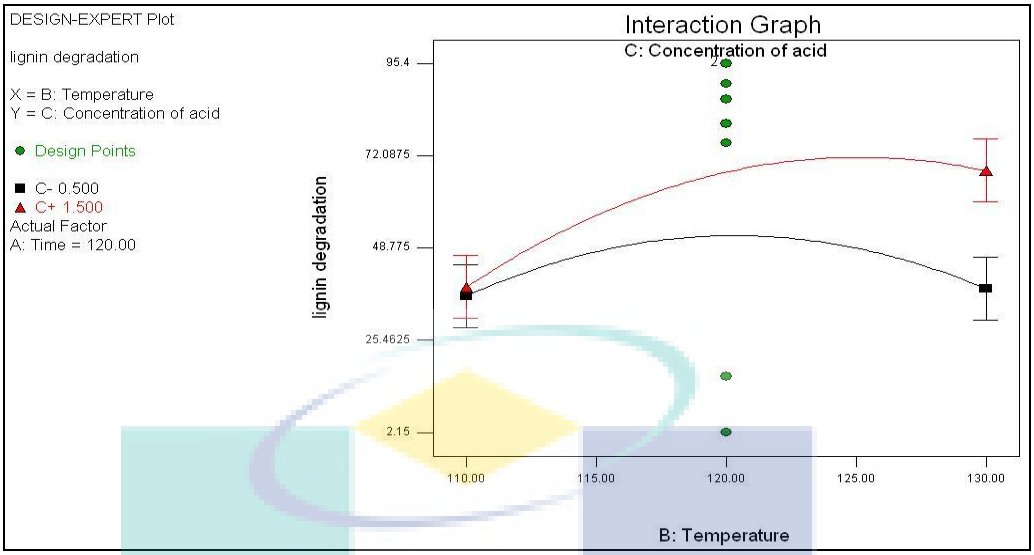


Figure 4.6: Interaction graph of lignin degradation from the model equation: effect of temperature and concentration of acid

Based on the result, the maximum lignin degradation of 95.4% is obtained when temperature and acid concentration is 120^0C and 1.0%, respectively. In fact the slope of the increase is dependent on temperature and concentration of acid, and appears to be gradually steep at the higher level conditions.

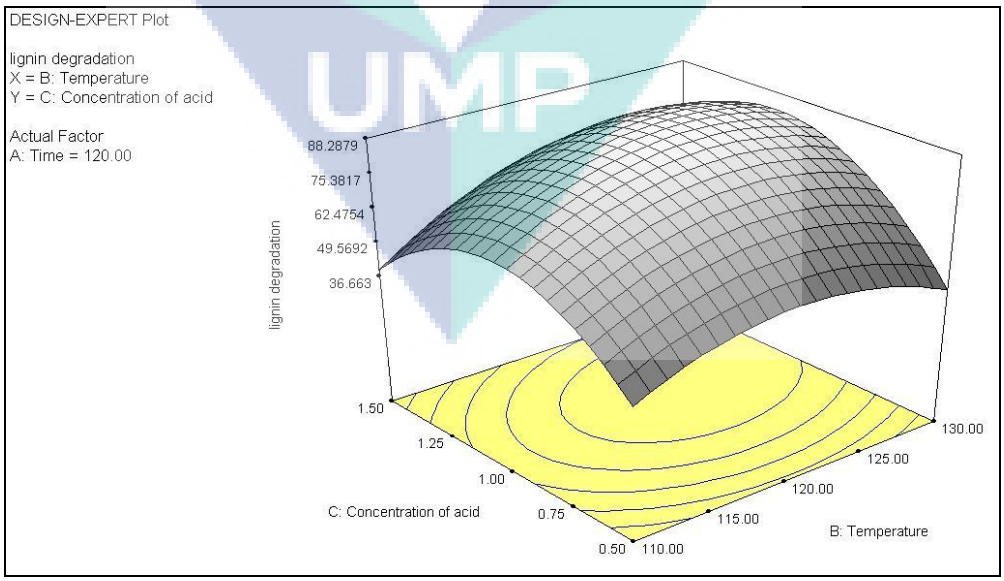


Figure 4.7: Three dimensional graph (3D) of lignin degradation from the model equation: effect of temperature and concentration of acid

4.2.4 CHEMICAL PRETREATMENT OF MERANTI WOOD SAWDUST; VALIDATION OF EMPIRICAL MODEL ADEQUACY

Adequacy of the developed empirical model needs to be verified or validated in order to confirm the prediction accuracy, which is generated by the regression equation in predicting the lignin degradation efficiency at any particular reaction time, temperature and acid concentration within the range of level defined previously.

Experimental rechecking was performed using conditions that were previously used and combined with the additional experiments which have not been tried before but was within the limits tested previously (Table 4.10). The obtained actual values and its associated predicted values from the selected experiments were compared for further residual and percentage error analysis. The percentage error between actual and predicted value of the response (lignin degradation) over a selected range of operating levels are calculated based on equation 4.3 and 4.4.

Table 4.10: Results of operating conditions with experimental design confirmation runs

No	Run factor			Lignin Degradation			
	Time (min)	Temp (^{0}C)	Conc (C)	Predicted	Actual	Residual	%Error
1	124	119	0.8	78.25	80.95	2.69	3.33%
2	101	125	1.2	80.51	79.55	-0.96	-1.21%
3	103	113	1.3	60.89	63.42	2.53	3.99%
4	147	117	1.1	63.09	61.25	-1.84	-3.00%
5	133	112	1.1	66.96	67.11	0.16	0.23%
6	122	121	1.4	77.11	76.24	-0.87	-1.13%
7	129	122	0.9	81.44	85.07	3.63	4.27%
8	134	111	1.2	57.99	60.19	2.20	3.66%
9	122	112	1.0	71.34	71.47	0.12	0.17%
10	148	126	1.5	51.90	54.13	2.23	4.12%

$$\mathrm{Re}sidual = (Actual\ \ value - \mathrm{Pr}edicted\ \ value) \quad (4.3)$$

$$\%Error = \frac{\mathrm{Re}sidual}{Actual\ \ value} \times 100\% \quad (4.4)$$

Results of table have shown that the percentage error is ranging from 0.17% to 4.27% for lignin degradation, respectively. Thus implied that the empirical model developed was considerably accurate for responding term (lignin degradation) as percentage error between the actual and predicted values were well within the value of 5%, suggesting that the model adequacy is reasonably within the 95% of the prediction interval. By this means further analysis with regards to ideal operational process for optimal lignin degradation would be based on this developed model.

4.2.5 CHEMICAL PRETREATMENT OF MERANTI WOOD SAWDUST; PROCESS OPTIMIZATION

Considering the previous discussion, main effect of reaction time, temperature and acid concentration were found more dominant than their interaction effect with respect to lignin degradation. Nevertheless one cannot simply neglect the importance of various interaction effects, for that interactions among them had only little influence on response because any individual factor that involve in interactions have to be considered jointly.

Table 4.11: Result of optimum operational conditions for *meranti* wood sawdust

Factor	Proposed optimal conditions	Lignin degradation	
		Predicted (%)	Actual (%)
time (min)	118	88.3704	86.0799 (2.29%)
Temp (^{0}C)	123		
concentration (C)	1.1		

Utilizing the optimum mode capability of the software enables the prediction of response complete with 95% prediction interval. Optimization procedure has been conducted for the *meranti* wood sawdust pretreatment and the prediction results of the empirical model are tabulated in Table 4.11. Result has shown optimum reaction time, temperature and acid concentration for optimal lignin degradation of *meranti* wood sawdust. The result of the lignin degradation was determined to be 118 minutes, 123^0C and pH1.1 respectively. Under this proposed optimized conditions, the maximum value of the lignin degradation from the model was 88.3704%. In order to confirm the predicted optimization conditions, experimental confirmation run were performed by employing the suggested model conditions. Apparently, the optimal value of 86.0799% was obtained. It is worth to note result of the experimental carried out here adequately implied that the proposed mathematical models suggested are reasonably accurate and reliable as most of the actual values for the confirmation runs are well within 95% prediction interval, hence it could be reliably be employed for the prediction of optimum degradation of lignin process conditions with respect to optimal production of high quality of cellulose production and enzymatic digestibility.

4.2.6 CHARACTERIZATION OF MERANTI WOOD SAWDUST

Infrared spectroscopy is frequently used for investigating the structure of constituents and chemical changes in samples that have been treated. According to the Pandey reported (Pandey 1999; Xu et al., 2007), the specific band positions of each constituents in his study of chemical structure of wood. Figure 4.8 (untreated) and figure 4.10 (treated) shows the FTIR spectroscopy of *meranti* wood sawdust. The FTIR spectroscopy was used to study the changes of chemical structure of *meranti* wood sawdust after the pretreatment applied. In this study, FTIR spectroscopy was used as a complementary technique to monitor functional groups of pretreatment samples.

Based on figure 4.8 and figure 4.10, the region from 4000cm^{-1} to 2000cm^{-1} does not give really useful information since it is merely signifies the O-H and aliphatic C-H stretching frequencies (Alawar et al.,2009). The major peaks at that region are considered to be broad bands at 3400cm^{-1} to 3200cm^{-1} as attributed to hydroxyl groups in phenolic and aliphatic structure, small peak from alkyne and the bands centered between 2900cm^{-1} to 3000cm^{-1} predominantly arising from C-H stretching for polymer matrix group. The remaining region, 1900cm^{-1} to 500cm^{-1}, is more informative and the results that will be discussed further confined to this region. In addition, formation of stretched peaks at 1800cm^{-1} to 1500cm^{-1} indicated the presence of conjugated carboxyl group (C=O) for lignin group. When the comparison between FTIR spectra of untreated and treated samples was occurred, the band intensities at all lignin peaks of the untreated sample were higher than those of all pretreated samples. This again proves the delignification effect of the two stages of pretreatment.

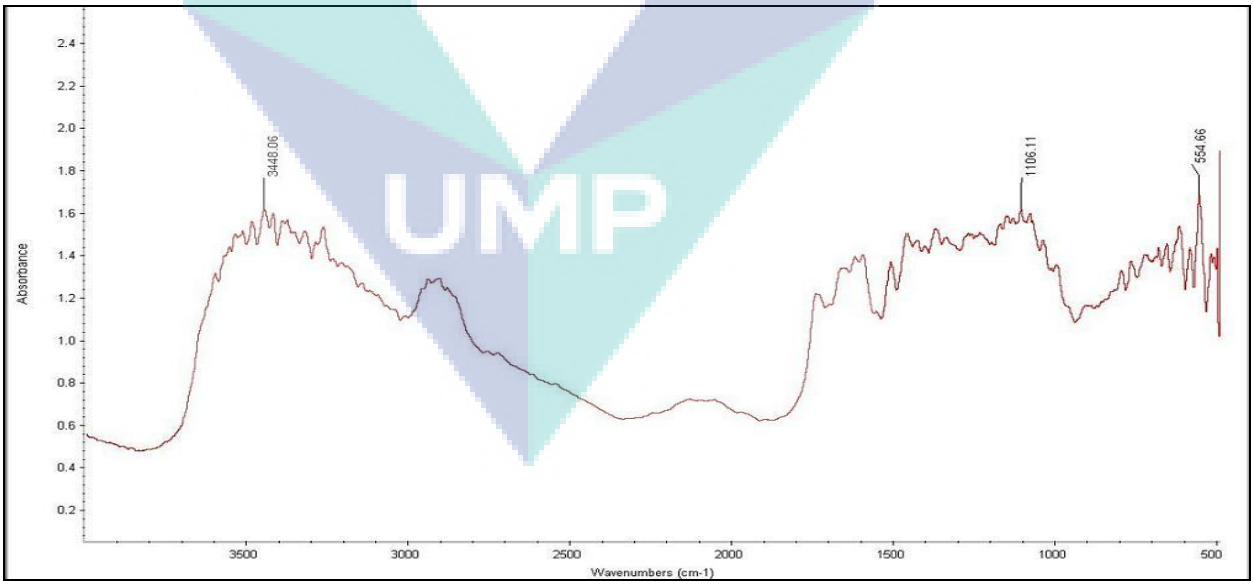


Figure 4.8: FTIR spectra of *meranti* wood sawdust (untreated)

Because a large fraction of hemicelluloses and lignin was removed by pretreatment, there were some physical changes in the *meranti* wood sawdust. For this reason, SEM pictures ao pretreated and untreated *meranti* wood sawdust were produced. The Figure 4.9 (untreated) and Figure 4.11 (treated) shows the SEM image for the *meranti* wood sawdust. Some previous literature reviews have revealed that constituents like pectin and hemicelluloses were hydrolyzed by the action of ion in alkaline solutions whereas lignin was removed during additional steps using sodium chloride or hydrogen peroxide. The effect of two stages of pretreatment of biomass fiber was evaluated by using SEM micrograph as depicted in figure 4.9 and 4.11.

Figure 4.9 shows *meranti* wood sawdust fiber surface without any pretreatment are rough and cellulose tightly held. As comparison, Figure 4.11 shows the *meranti* wood sawdust topography after second stage of pretreatment. It is clearly seen that untreated *meranti* wood sawdust surface is rough due to high residual of lignin content. By applying the all of the treatment, the cell walls ruptured which breakdown the fiber bundles, removing the surface impurities such as lignin, hemicelluloses and other leading to enhance the porosity, the effective surface area and surface charge, ultimately to improve the enzymatic digestibility. Moreover, elimination of lignin and impurities constituents are exposed further hydroxyl and carboxyl groups, thus exposing micro-fibrils which tend better packing of cellulose chain (Rahman et al., 2009). Besides, the untreated meranti wood sawdust exhibited rigid and highly ordered fibrils, while the fibers of pretreated samples by two stages of pretreatment were distorted. The micro-fibrils were also separated from the initial connected structure and fully exposed, thus increasing the external surface area and porosity.

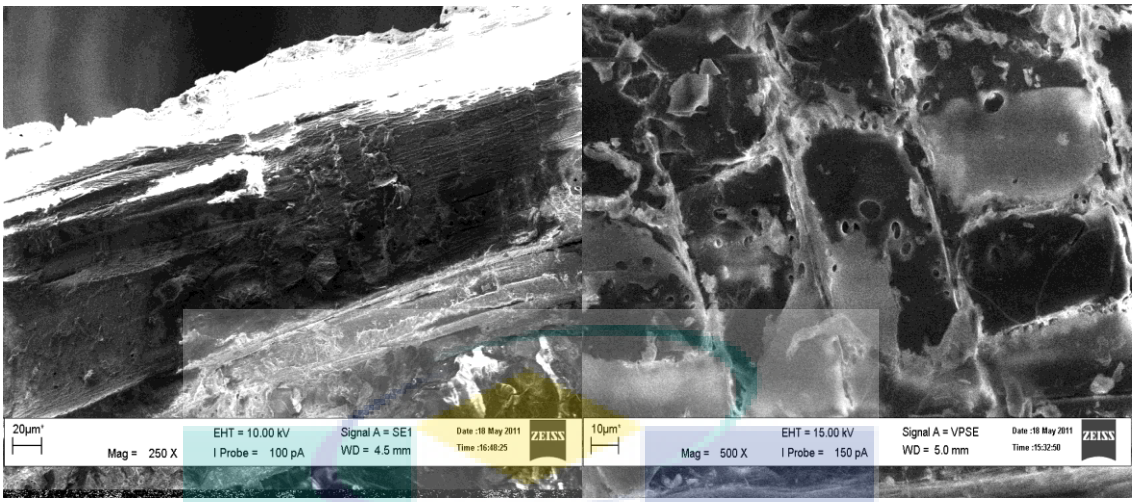


Figure 4.9: SEM image of *meranti* wood sawdust (untreated)

Furthermore, some researchers reported that applying pretreatment might change the crystallinity of natural fibers such as sugarcane, jute, coir and flax (Bledzki and Gassan, 1999; Luz et al., 2008). The increase of the percentage crystallinity index of pretreatment of fibers occurs because the removal on the cementing materials, which leads to better packing of cellulose chain. It is concluded that SEM micrographs confirmed the chemical treatment and its influence in the morphological aspects of fibers, thus strongly affected the enzymatic digestibility.

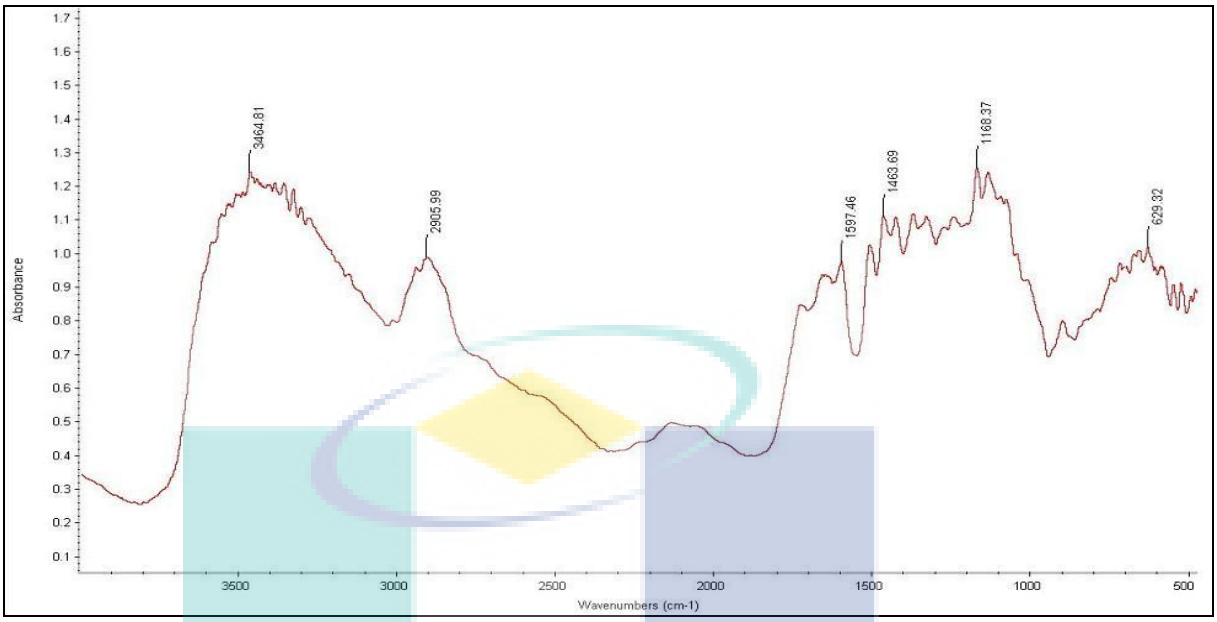


Figure 4.10: FTIR spectra of *meranti* wood sawdust (after treatment by sulfuric acid)

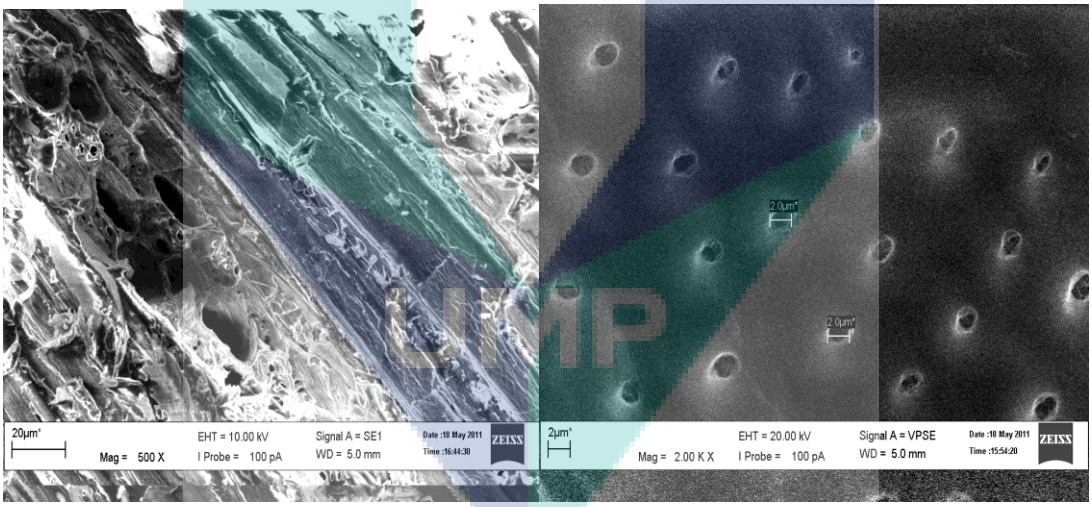


Figure 4.11: SEM image of the *meranti* wood sawdust (after treatment by sulfuric acid)

4.3 HYDROLYSIS PROCESS OF PRETREATED MERANTI WOOD SAWDUST

After completed the pretreatment stage, the pretreated *meranti* wood sawdust need to been hydrolyses to perform monomer sugar, glucose especially. Enzymatic hydrolysis of pretreated *meranti* wood sawdust was carried out by *Cellulase* enzyme, mixture with *β-Glucosidase* enzyme. The various parameters such as hydrolysis time, temperature of the incubator shaker and pH were optimized to achieve the maximum glucose production that produce from hydrolysis process of the pretreated *meranti* wood sawdust.

4.3.1 CHARACTERIZATION OF GLUCOSE

FTIR spectroscopy was used to study the changes of chemical structure in samples. In this study, the FTIR spectroscopy was used as a complementary technique to characterize the structure of glucose that had been produce from enzymatic hydrolysis process. Glucose is one of the sugars that produced from enzymatic hydrolysis process. As we already discussed in chapter 2 and 3, glucose is the monosaccharide that had been hydrolyzes from polysaccharide, and enzyme was used as a catalyst in that reaction. The Figure 4.12 shows the FTIR spectra of the glucose. From the FTIR spectra, formation of the stretches peaks at 1463.0cm^{-1} which corresponding the carbonyl group (-C=O) of the glucose while the hydroxyl (O-H) - phenolic aliphatic group of the glucose was 3400.0 cm^{-1}. The prominent band at 1172.02cm^{-1}, 1071.6cm^{-1} and 1009.49cm^{-1} are attributed the C-C stretching or C-OH bending in sugar production.

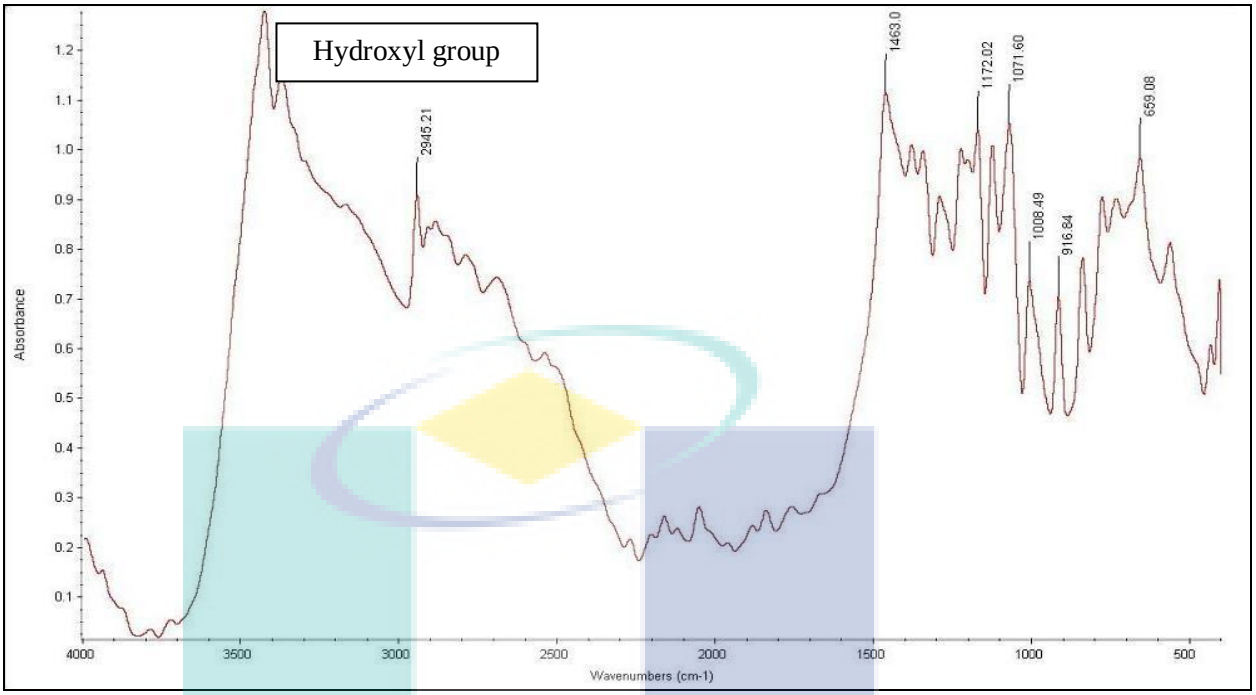


Figure 4.12: FTIR spectra of glucose (enzymatic hydrolysis production)

4.3.2 KINETIC STUDY OF CELLULASE ENZYME

The price of commercial enzymes used for the saccharification of pretreated lignocellulosic materials represents a significant part in the overall cost of the biomass to other product from fermentation process. Reduction the production cost may affect the valuable of the process and because of that; the preliminary study of the enzyme was done to decrease the cost usage of the enzymes. An enzyme is a catalytic protein and it remarkable lowers activation energy of a given reaction. Although all enzymes are proteins, many enzymes contain non-protein components as well for example, carbohydrates, phosphates, metallic ion, lipids or organic moieties, etc (Whitaker, 1972; Pandey et al., 2006). On the kinetic basis, the enzymes are catalytic agents that increase the conversion rate of substrate into product. As without enzyme the reaction rate is generally negligible, the quantification of enzyme activity is based on the measurement of the reaction rate (Avecado et al., 2002; Pandey et al., 2006). Cellulases, a multi-enzyme system that de-polymerized cellulose to give soluble sugars were used in this

research field. Because of that, the kinetic study was figure out to know the activity of these specific enzymes.

To calculate the enzyme reaction based on the sugar production, Figure 4.13 shown the calibration curve that we need to figure out the equation of the concentration glucose. From the graph, the coefficient R^2 was 0.9938 and the equation was y=0.0026x + 0.1634; where x-axis is a glucose production and y-axis is an absorbance of the UV VIS at 540nm. This equation will be used to the enzyme activity and also the stability of enzyme.

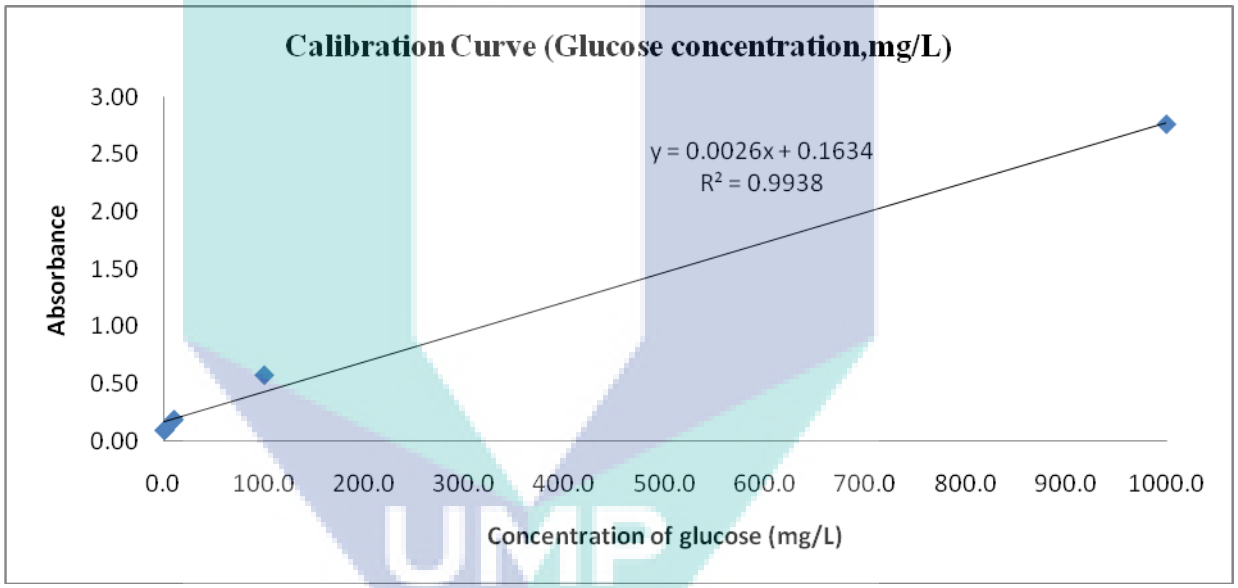


Figure 4.13: The Calibration curve of cellulose concentration

While the use of enzymes in industrial scales is wide-spread from food industries to pharmaceutical ones, the understandings of factors influencing enzyme activity are crucial for the control and manipulation of products of enzymatic reactions. Some of the factors that influence desired enzymatic reactions can be manipulated with relative ease, while some of them are not. The accuracy of the determination of enzyme activity can be achieved only on the basis of defined conditions of measurement. The conditions of measurement need to take a number of factors into account, amount them the measurement of temperature, pH, substrates and enzymes. To define the optimal

conditions for enzyme action, it is necessary to carry out a number of experiments varying the value of the studied parameter knowing that often exist interdependence between them.

Because of that, the experiment of the enzyme activity based on the effect of the temperature was carried out. Figure 4.14 shows the reaction between enzyme and the certain temperature. From the graph, the optimal point of the enzyme activity was at 50^0C, this is because, the production of the sugar was increase proportionally and when achieved the optimal point, and the production was dropped down. Enzyme catalyzed reactions, like all chemical reactions increase in rate with rises in temperature (Wiseman 1985; Pandey et al., 2006). Temperature generally has dual effects on the enzyme reaction. The increase in the temperature level raises the reactivity of the enzyme substrate complex and also inactive the enzyme. It is not surprising that the optimal temperature of an enzymatic process also depends on the operation time, and its definition must take into account this double effect of temperature; activity and stability with time (will discussed later).

For many proteins, denaturation begins to occur at 45^0C to 50^0C and is severe at 55^0C (Bailey and Ollins, 1986; Doran, 1998; Pandey et al., 2006). Only a few enzymes can be heated to above 100^0C and still retain activity. From the graph, the decreased line mean the enzyme activity was slow and because of that, the sugar production also decreased. This is because, the *Cellulase* and *β-Glucosidase* enzyme were starting to denature at 60^0C and above. And at 80^0C, the sugar productions become zero because the enzymes completely denatured. The enzymes actually present a range of temperature, sometimes narrow, in which the catalytic activity is maximal.

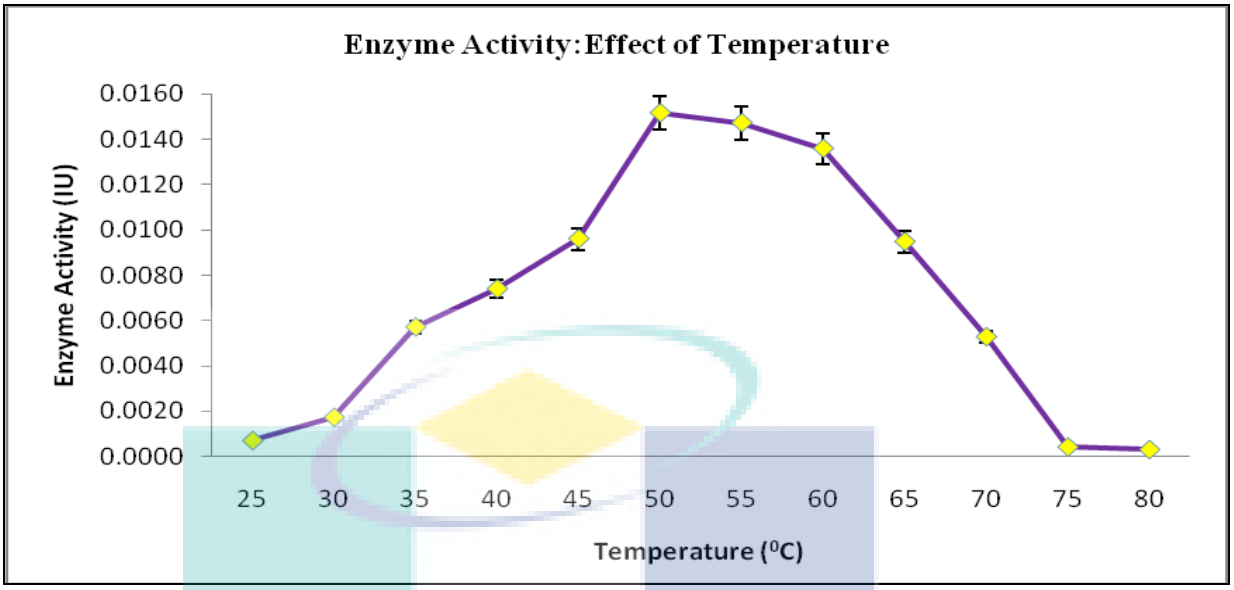


Figure 4.14: The Enzyme Activity based on effect of temperature (IU)

All enzymes have an optimum pH range of their activities, which is often very narrow. The optimum pH does not depend only on the nature and ionic strength of the buffer, in general also depends on the temperature and substrate concentration (Bergmeyer, 1983; Scriban, 1984; Pandey et al., 2006). A define optimum pH for enzyme actiivity is usually observed because, like other proteins, enzymes have a number of ionisable groups so that pH changes may modify the confirmation of the enzyme, the binding of the substrate and the catalytic activity of the group present in the active site of the enzyme. Actually, the optimum pH varies largely among the enzymes. For example, the optimum pH of pepsin that acts in the acid medium of stomach is about 1.5, while the optimum of arginase, the enzyme that breaks the amino acid arginine, is about 9.7. But for the majority of enzymes the optimum pH is between 4 and 8 (Montgomery et al., 1994; Pandey et al., 2006).

Figure 4.15 shows the enzyme activity based on the effect of pH. From the graph the optimal pH of the enzyme activity is 5.0. This is because, the sugar production increased and maximum at that point. The formula on how to calculate the enzyme activity (IU) was shown in equation 4.5. The molecular weight of the glucose is 180 g/mole and the dilution factor is 2. Meanwhile, the incubation time of this experiment

was set at 60 minutes at 50^0C. As we know, the *Cellulase* and *β-Glucosidase* enzyme are from lactic acid bacteria. So, they are an actived enzyme especially in acidic buffer. From the graph, we can see the production of sugar was produce early at the pH 3 and then increase proportionally with increasing the pH value. When achieved the optimal point at pH5, the sugar production starting to decrease highly at pH8. This is because, the enzymes cannot react in alkaline buffer and will be denatured.

$$Enzyme \quad Activity\,(IU) = \left(\frac{amount \quad of \quad sugar \quad produce(\mu g)}{molecular \quad weight\,(sugar)} \right) \times \left(\frac{Dilution \quad factor}{Incubation \quad time} \right) \tag{4.5}$$

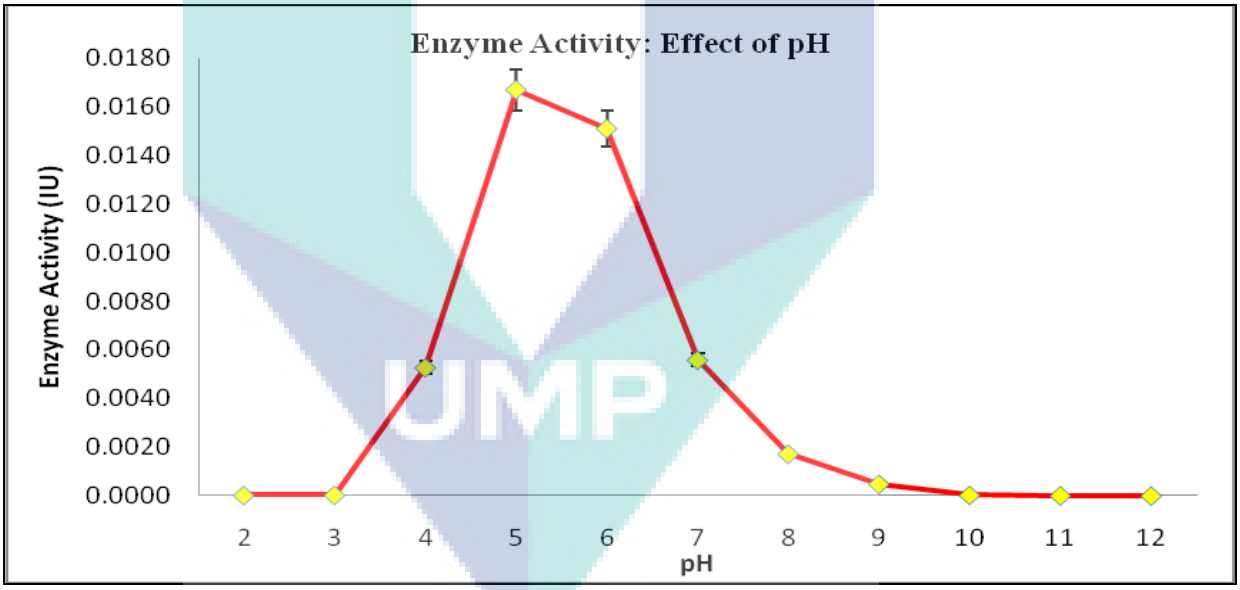


Figure 4.15: The Enzyme Activity based on effect of pH (IU)

Reaction time may affected enzymatic reactions many ways ranging from quality to quantity of the reaction products (Mu et al., 1998; Yadav and Gupta, 2000; Chen et al.,2002; Chang et al.,2003; Pandey et al., 2006). Reaction time sometimes can be substantially reduced for a given enzymatic reaction by adding more enzyme activity. In some instance, even the same enzyme from different sources, however, behave

differently (Rostometal, 1998; Pandey et al., 2006). Actually, reaction time affect the enzyme catalysis of various reactions. Similarly, reaction time can be crucial in accessing viability of an enzyme for its industrial applications. Figure 4.16 shows the enzyme activity based on the effect of the reaction time. After achieved the optimal point, the activity of enzyme starting to inactive proportionally with time or in other words, the enzyme activity were decreased as soon as prolong the time after optimal point. This is because, the substrates decrease and already hydrolysed to sugar production and as we know, the enzyme activity was unproportional with time reaction. So, that why the graph decreased after achieved the optimal point.

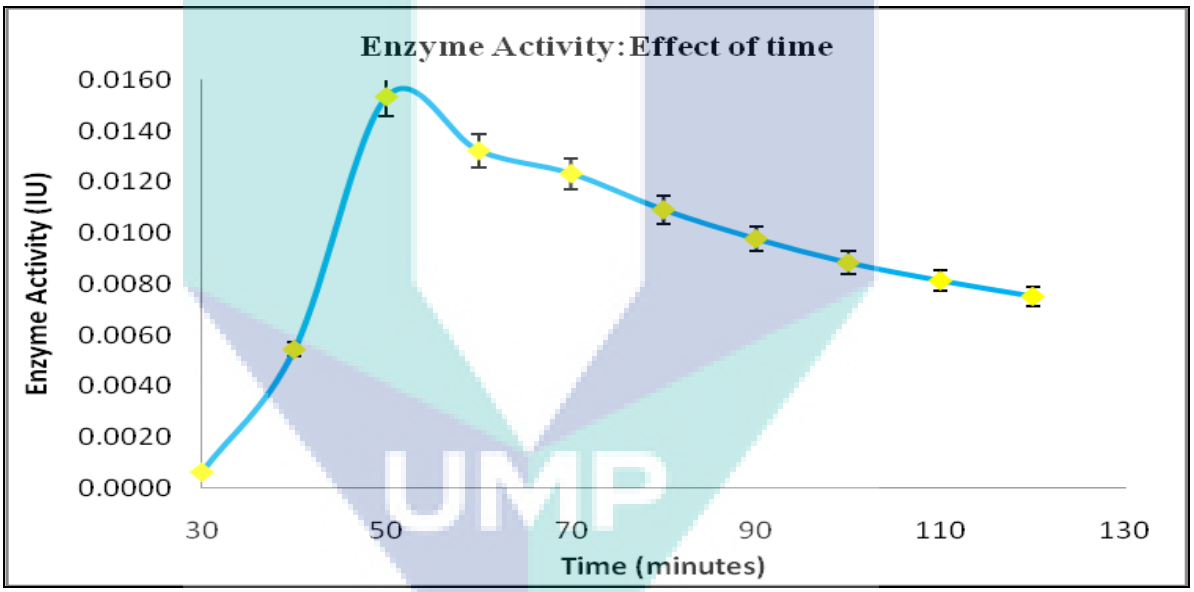


Figure 4.16: The Enzyme activity based on effect of time (IU)

The most important property of an enzymes is its catalytic capacity or activity. The catalytic capacity depends on the protein structure, i.e. the native structure of the protein that results from numerous weak interactions (Illanes, 1994; Pandey et al., 2006). It is, therefore, obvious that a multitude of physical and chemical parameters can and do cause perturbations in the native protein's geometrical and chemical structure with concomitant reductions in activity. This phenomenon called enzyme denaturation. Depending on the magnitude of the denaturating agent, the quaternary, tertiary or

secondary structure may be affected. The quaternary structure alteration is often reversible; the change in the tertiary structure is often irreversible leading to a total or partial activity loss; and the alteration of the secondary structure is irreversible, leading to a coagulation effect and total inactivation of the enzyme (Pandey et al., 2006).

Figure 4.17 shows the satbility of enzyme affected by thermal. In this experiment, the enzymes were incubate at different temperatures for 10 minutes before reacted with substrates for 60 minutes.From the graph, the optimal point of enzyme stability (affected by thermal) was at 50^0C, and then decrease slowly. At temperature higher that 60^0C, the enzyme was denatured and sugar production was not produce. This is because, as we know, enzyme will be denatured at high temperature and for *Cellulase* and *β-Glucosidase* enzyme, the denatured temperature was at 60^0C and above. At 60^0C, the reaction still occurred but in very small amount because the enzymes started to denature.

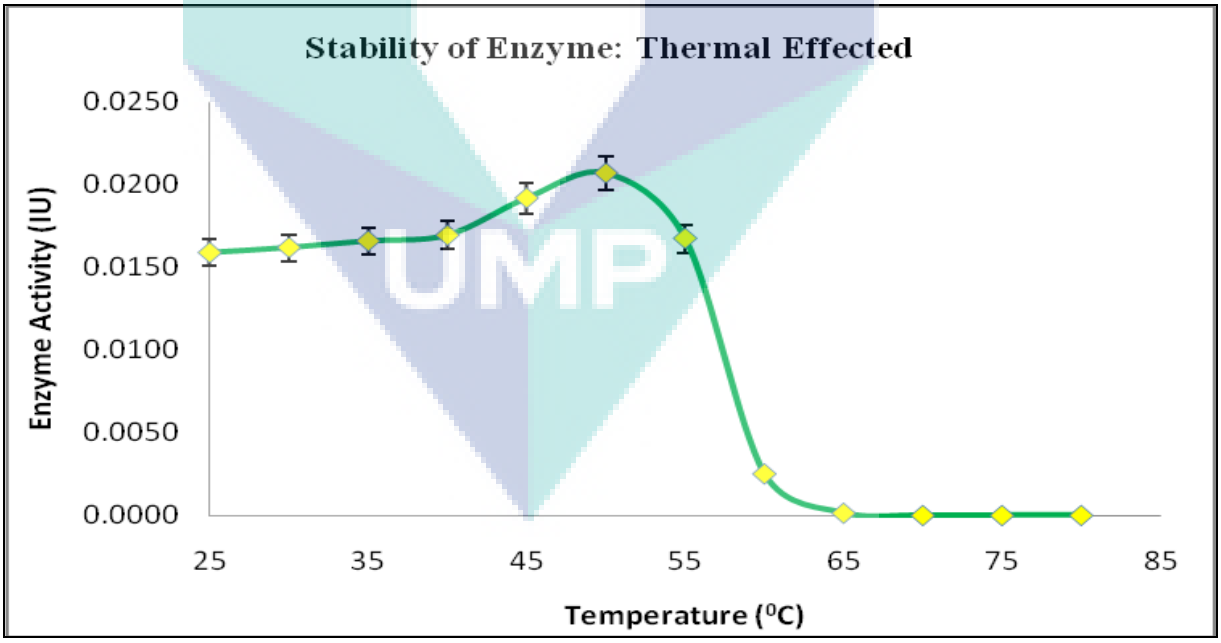


Figure 4.17: Stability of Enzyme,thermal effected (IU)

Enzyme stability loss depends on the length of time the enzyme has been maintained at the unfavourable pH such that the optimum operational pH is generally a

compromise between effects on enzyme activity and enzyme stability. The enzyme activity, as proposed by the International Union of Biochemistry, is expressed as International Units (IU) of enzyme activity. One unit is defined as the amount of enzyme that catalyses the transformation of one micromole of substrate per minute under defined environmental conditions. These conditions are; saturated substrate concentration, optimal pH and temperature (Acevedo et al., 2002; Pandey et al., 2006).

Figure 4.18 shows the stability of enzyme affected by pH. Before reaction held between substrate and enzyme, the enzymes were incubated in different pH of buffer (alkaline, neutral, acidic) for two hours at room temperature. Then, the reaction starting when the substrate added in the experiment. From the graph, we can see, the denatured enzymes were at pH lower than pH4 and higher than pH8. This reason already discussed for figure 4.15. As we know, as an acidic enzymes, the *Cellulase* and *β-Glucosidase* enzyme cannot react in very acidic condition and alkaline condition. At neutral level (condition), it's still can reacted, but their activity was not active as in acidic condition (pH 5-pH6).

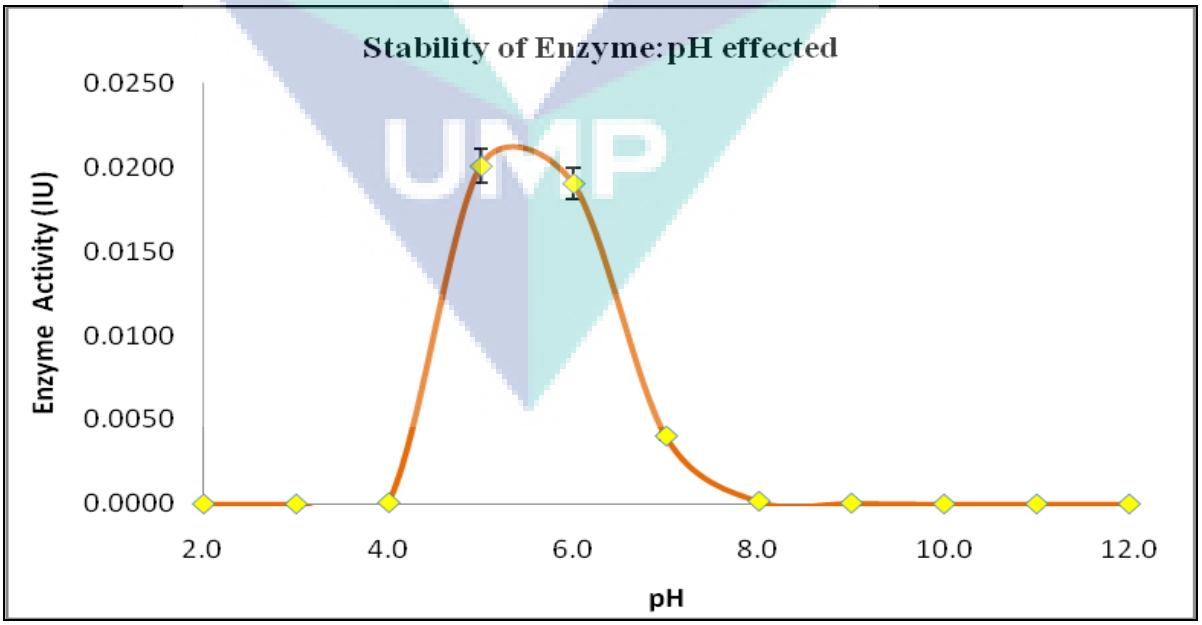


Figure 4.18: Stability of Enzyme, pH effected (IU)

Figure 4.19 shows the stability of enzyme affected by substrate concentration. Substrate concentration is the one of the most important factors affecting the velocity of an *in vitro* enzyme catalysed reaction. However, it is difficult to study the effect of substrate concentration due to the fact that substrate varies with reaction time while substrate is converted into product. With increasing substrate concentration, the rate of its conversion become greater until it approaches its optimum value (Nelson and Cox, 2002; Pandey et al., 2006). This graph will be used as a model to calculate the velocity, V_{max} and also the K_m, the Michaelis constant.

Mathematical models were used in searching for optimal operating conditions and/or creating optimal microenvironments for enzymes in order to optimize their effectiveness. Recently, the study of various kinds of models of enzymatic reactions attain of great interest in research, as well as in the industrial application of these biocatalysts (the enzymes), for optimal operation points, and to enhance our knowledge about the process. In this experiment, we used Henri-Michaelis-Menten (H-M-M) equation for discussed this section.

A mathematical model of the kinetics of single-substrate-enzyme-catalyzed reactions was first developed by V.C.R. Henri in 1902 and by L. Michaelis and M.L. Menten in 1913. Kinetics of simple enzyme-catalyzed reactions are often referred to as Michaelis-Menten kinetics or saturation kinetics. The qualitative features of enzyme kinetics are similar to Langmuir-Hinshelwood kinetics.

$$E + S \underset{k2}{\overset{k1}{\rightleftarrows}} [ES] \xrightarrow{k3} E + P \qquad (4.6)$$

Equation 4.6 shows that the ES complex is established rather rapidly and the rate of the reverse reaction of the second step is negligible. The assumption of an irreversible second reaction often holds only when product accumulation is negligible at the beginning of the reaction. Based on the Michaelis-Menten hypothesis, the coversion of

[ES] into E+P is too slow when compared to the break down of [ES] given E+S. This means that $k_3 << k_2$ and that E and [ES] are in equilibrium:

$$k_1[E][S] = k_2[ES] \text{ or } \frac{[E][S]}{[ES]} = \frac{k_2}{k_1} = K_s \quad (4.7)$$

Where K_s is the dissociation constant of [ES].

The conversion of [ES] into E+P, thus the rate limiting step, and the velocity equation may be written as follows:

$$v_0 = k_3[ES] \quad (4.8)$$

The total amount of enzyme present in the reacting mixture $[E_0]$ is the sum of the amount of free [E] and bound [ES] enzymes:

$$[E_0] = [E] + [ES] \quad (4.9)$$

Substituting [E] derived from equation (4.9) into (4.7) and isolating [ES]:

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

If we substitute [ES] derived from equation (4.8) into (4.10), we obtain:

$$v_0 = \frac{k_3[E_0]\,[S]}{[S] + K_s} \quad (4.11)$$

If substrate concentartion is increased ($S_1<S_2<S_3<\ldots\ldots<S_{n-1}<S_n$), the initial velocities could also increase. However, the higher the [S] the lower the increase of v_0, until reaching the stage in which no incrase is observed. At this point, the enzyme-substrate complex [ES] is at maximum, i.e., all the enzyme is completely saturated with the substrate and is present in the [ES] form. The initial limiting velocity is reached:

$$v_{max} = k_3[ES]_{max} = k_3[E_0] \tag{4.12}$$

Substituting the expression $k_3[E_0]$ in equation (4.11) by the value obtained in equation (4.12), we have:

$$v_0 = \frac{v_{max}[S]}{[S] + K_s} \tag{4.13}$$

From the graph (figure 4.19), the V_{max} was 0.0234 IU and the $0.5V_{max}$ was 0.0117IU. After we got the value of the $0.5V_{max}$, the value of K_m can be identified. From the graph, the K_m was 0.9624mg/L (substrate).

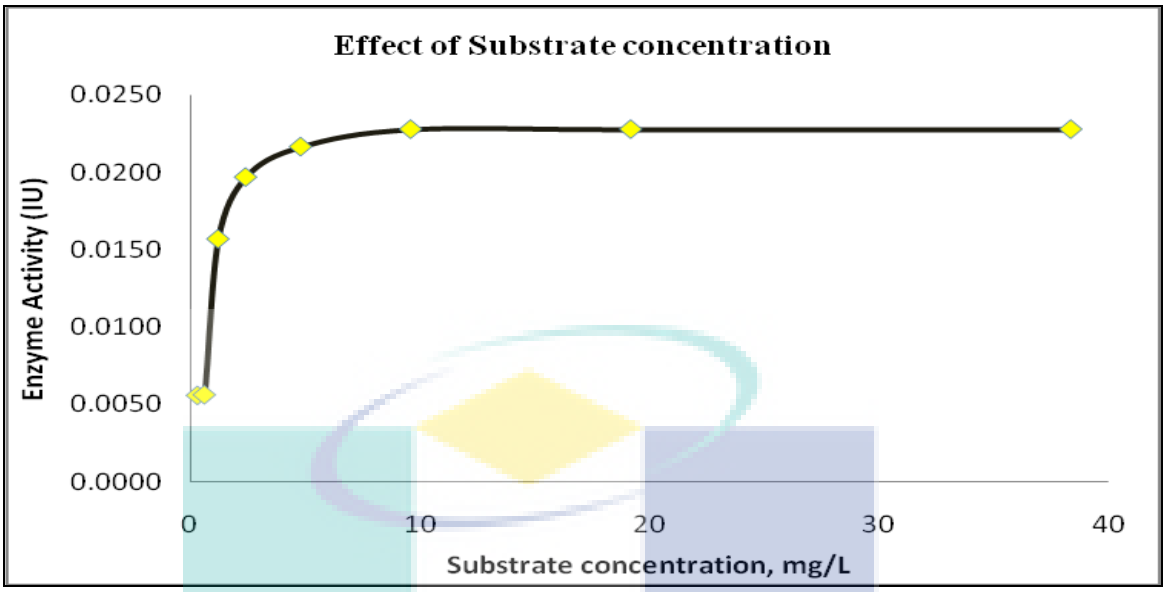


Figure 4.19: Stability of Enzyme, effect of substrate concentration (IU)

The Figure 4.20 shows the Double-reciprocal plot (Lineweaver-Burk plot). The graph can be linearized in double-reciprocal form:

$$\frac{1}{v} = \frac{1}{V_m} + \frac{K_m}{V_m}\frac{1}{[S]} \qquad (4.14)$$

From the graph, the value of the slope is 48.555 represent the K_m/V_m, the y-axis intercept represent the value of the $1/V_m$, 40.87 and the x-axis intercept represent the value of 1/[S] or $1/K_m$, 0.841726 (calculated from the equation).

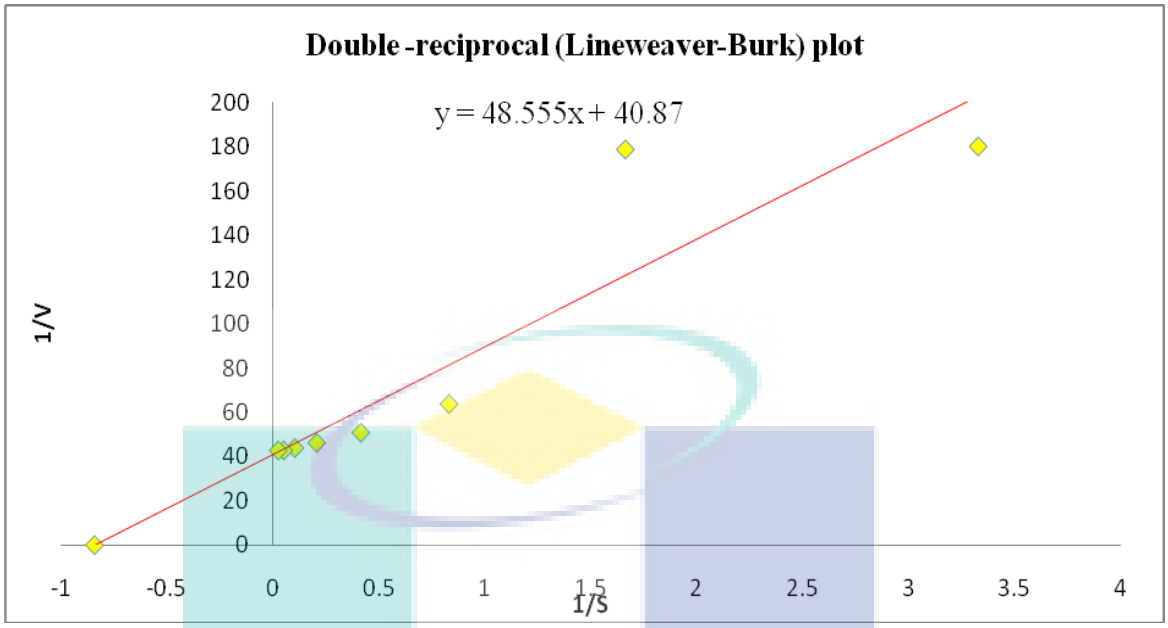


Figure 4.20: Duoble-reciprocal (Lineweaver Burk) plot

4.3.3 STUDY THE EFFECT OF pH ON GLUCOSE PRODUCTION

From the previous subsection discussion, the activity of enzyme was studied and the optimal parameters of *Cellulase* activity were founded to achieve the maximum of sugar production. In this section, the effect of the all parameters; reaction time, temperature and pH to the sugar production will discussed in details. Figure 4.21a and Figure 4.21b shows the effect of pH buffer for all pretreated *meranti* wood sawdust on parameters of a higher glucose production with *Cellulase* enzyme. The use of a higher pH buffer (up to 5.2) gave hydrolyzates with a lower glucose production because as we discussed earlier, *Cellulase* enzyme can react with substrate at pH acidic, but all the pH in these experiments were acidic point but the most maximum yield of glucose was produced at pH5.0. From the graph trend also we can see the glucose production increased linearly and when it achieved the optimal point, the yield become decreased and at certain point, the enzyme become denature (because of temperature was high or pH closed to neutral point). From the graph also, the yield at temperature 50^0C and 52^0C were enclosed. Truth, there are several factors which can contribute to the low degree of

sugar production in enzymatic hydrolysis of *Cellulase* enzyme. These factors may include the decrease in the reactivity of cellulosic material (pretreated *meranti* wood sawdust) in the course of hydrolysis (Lee and Fan, 1983; Sinitsyen et al., 1991; Yu et al., 1997), different kinds of enzyme inactivation (Reese, 1980; Gusakov et al., 1992; Yu et al., 1997), non-specific adsorbtion of Cellulase enzyme into lignin (Chernoglazov et al., 1988; Ooshima et al., 1990; Yu et al., 1997) and end-product inhibition (Holtzapple et al., 1990; Ramos et al., 1993; Yu et al., 1997).

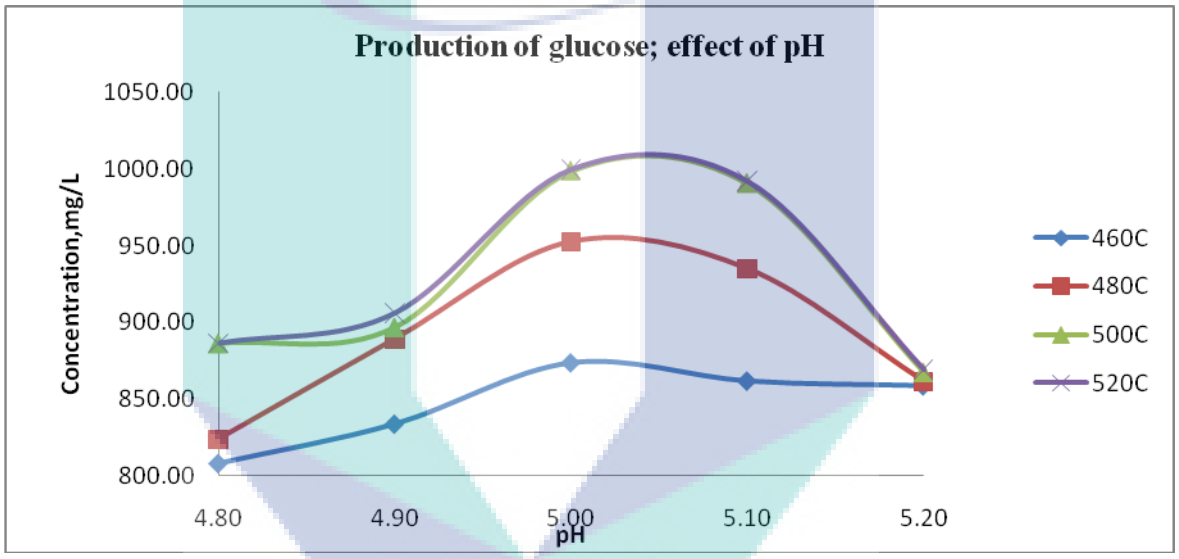


Figure 4.21a: Effect of pH on glucose yield at different temperature

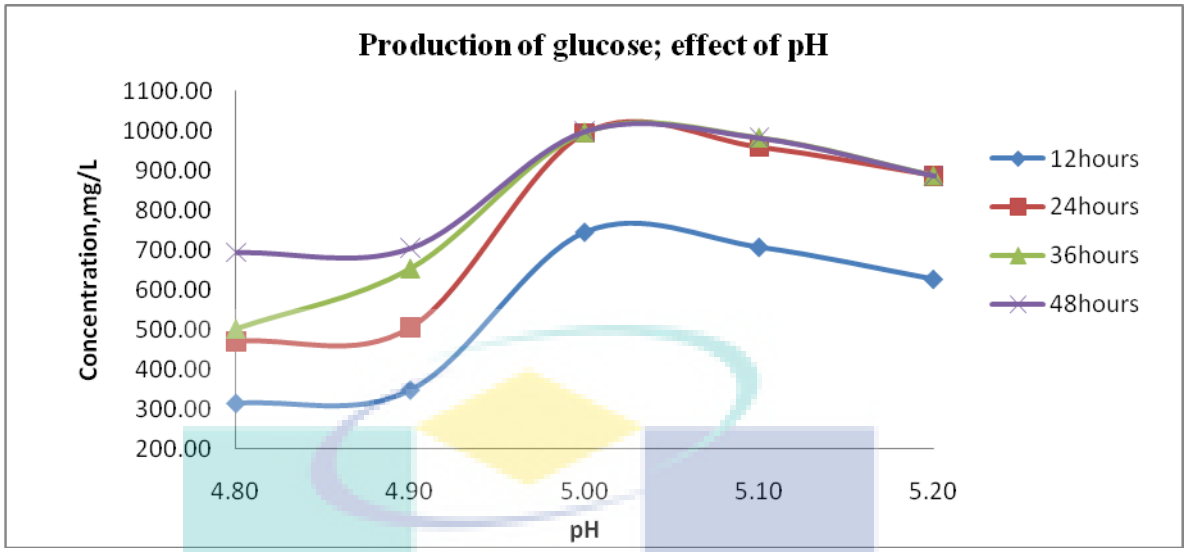


Figure 4.21b: Effect of pH on glucose yield at different time

4.3.4 STUDY THE EFFECT OF TIME ON GLUCOSE PRODUCTION

In the separate experiments (Figure 4.22a and Figuure 4.22b), we found that the sugar yield of enzymatic hydrolysis of *Cellulase* enzyme were increased proportionally with time. From the graph, the highest yield of the sugar (glucose) was at pH 5.0 when the time arrived to 24 hours. After 24hours, the glucose still produced but the amount produced was slowly. This kind of trend was found because the reactivity of all pretreated *meranti* wood sawdust materials was signifivantly reduced after achieved the optimal time. The graph pattern also shows clearly that at pH5.0, cellulase enzyme were optimized for their activity when arrived at 24hours. From this result it can be considered that glucose production and/or its cellulase enzyme efficiency was decreased when pH shifted. Thus the enzyme charge are disrupted (Hu et al., 2004; Ariffin et al., 2006; Angsana et al., 2009), then enzymes undergoes changes in its shape and hence becomes denatured and unable to catalyze the chemical reactions. On the other hand, the shift of pH values could be partially attributed to the limited activity rate of the enzymes.

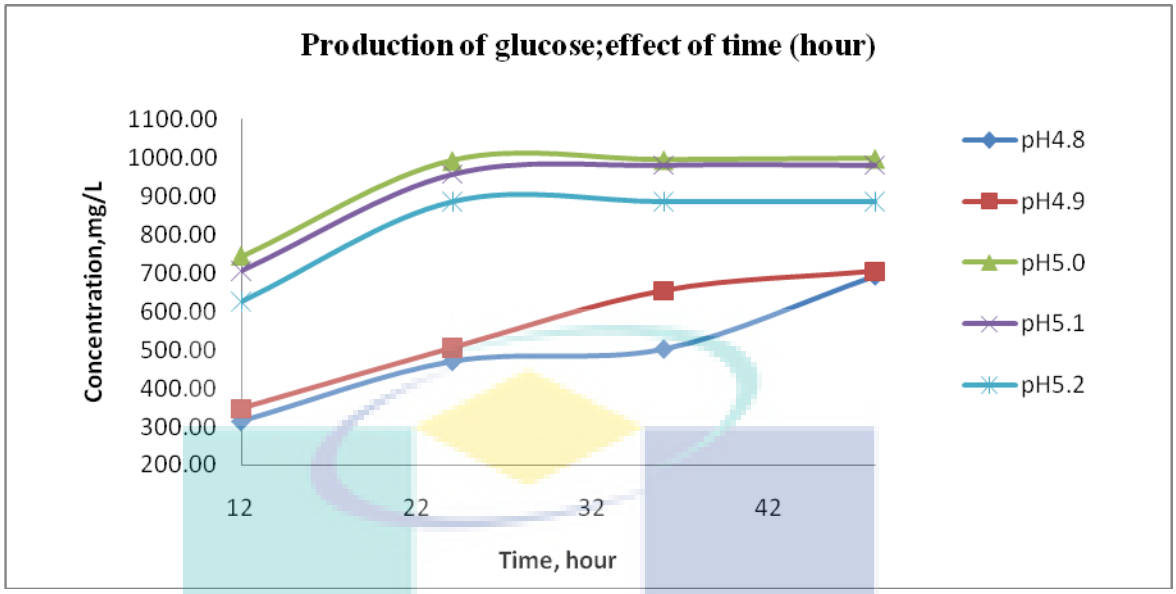


Figure 4.22a: Effect of time on glucose yield at different pH

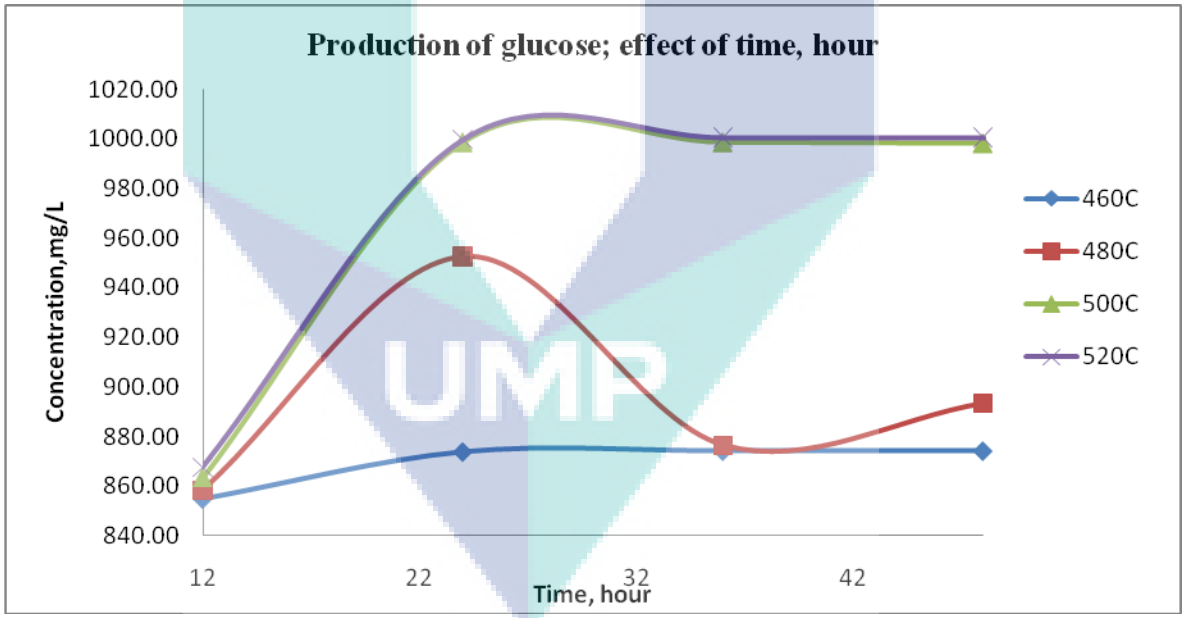


Figure 4.22b: Effect of time on glucose yield at different temperature

4.3.5 STUDY THE EFFECT OF TEMPERATURE ON GLUCOSE PRODUCTION

Figure 4.23a and Figure 4.23b illustras the effect of temperature on glucose production at different pH and time. As per discussed befored, the highest yield of the glucose production was at pH5.0 with temperature effect at 50^0C when the reaction time arrived 24 hours. At the point of Katz and Reese, the highest glucose yield was at pH4.5 with reaction time 50^0C and when time arrived at 5days and aboved (Katz and Reese, 1968). At the other point, Kahar was agreed with our results that the maximum yield of hydrolysis was achieved at that conditions (Kahar et al., 2010).

Actually, without the injection of *β-Glucosidase* enzyme, the hydrolysis will not completed react and cellobiose will not converted to glucose. That's why in our works, the *cellullase* enzyme was loading together with *β-Glucosidase* enzyme (Zhu and Pan, 2010). Furthermore, the lignin content that still remain in pretreated meranti wood sawdust also will affected the enzyme reactivity and glucose yield production also will decreased.

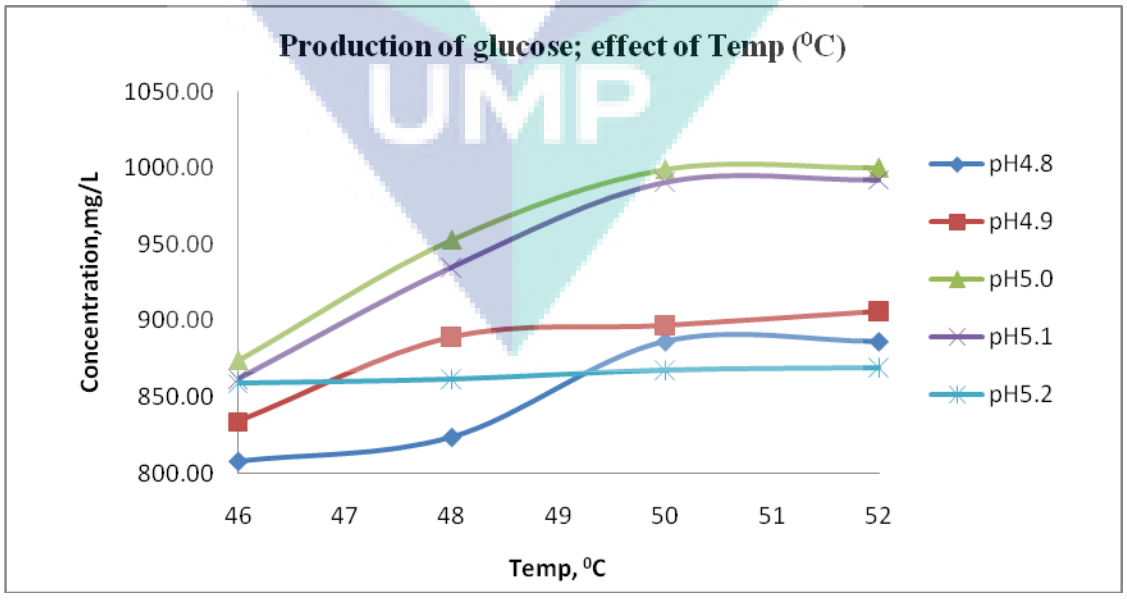


Figure 4.23a: Effect of temperature on glucose yield at different pH

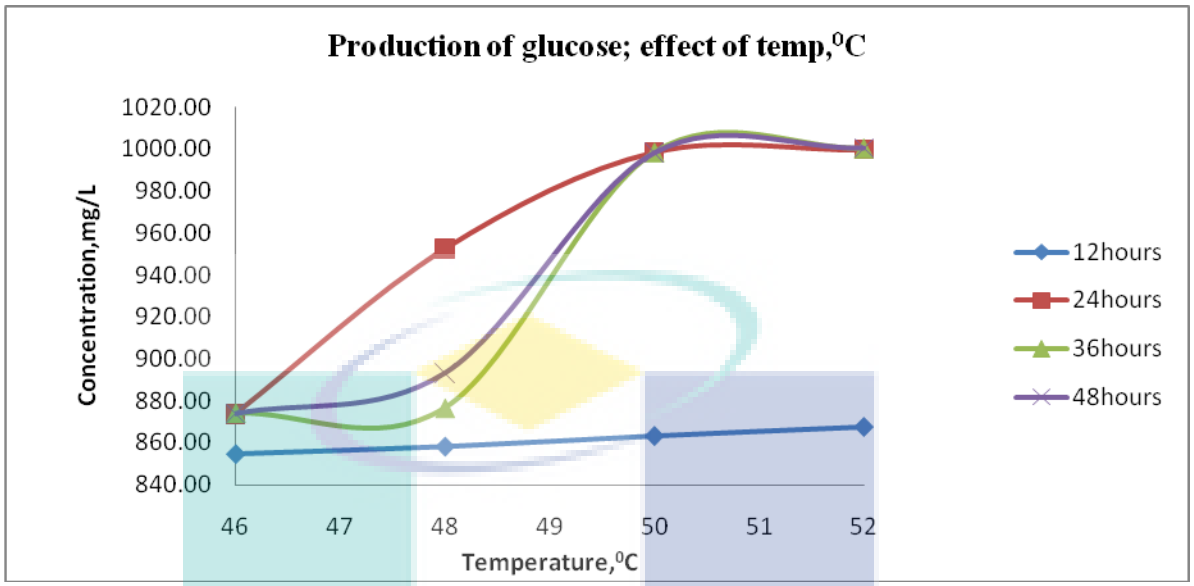


Figure 4.23b: Effect of temperature on glucose yield at different time

4.3.6 ENZYMATIC HYDROLYSIS OF PRETREATED MERANTI WOOD SAWDUST; ONE FACTOR AT A TIME (OFAT) STUDY

Experiments may be designed to investigate one factor at a time so that all other independent variables-factors are held constant. This is the so-called classical experimental design. A classical experiment means researching mutual relationship between variables of a system, under "specially adapted conditions" (Lazić, 2004). In this experiment, the OFAT study was done under three major variables of enzymatic hydrolysis. There are reaction time of hydrolysis (hour), temperature of the incubation shaker (^{0}C) and the pH of the buffer used. To calculate the sugar production of the enzymatic hydrolysis process, the calibration curve and equation that already discussed in enzyme activity section was used. There are based on the DNS analysis of sugar production.

Figure 4.24 shows the sugar production based on OFAT study of the reaction time (hour). The graph indicated that the optimal time with the optimal glucose production is

24 hours with the glucose production reading is 992.92 mg/L. When refer at the trend of the graph, the glucose production was decreased after 24 hours, but starting increased back after that time, This is because, all the experiments were run in one shaker at one time and when the samples were taken at time=24hours, some environmental error was occurred. This is cause of the enzyme activity and also the temperature of the incubator shaker that will affect the experiment running.

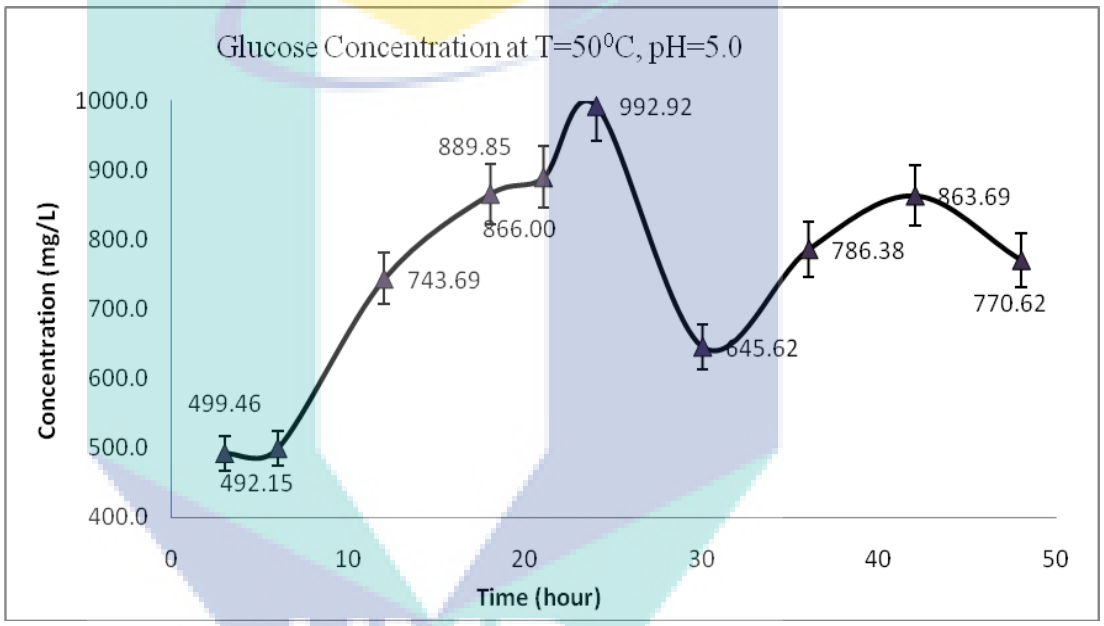


Figure 4.24: Sugar production based on effect of the reaction time (mg/L)

For the OFAT study of the temperature, the Figure 4.25 shows the details. Indicating from the graph, the highest production of sugar at optimal temperature (50^0C) was 998.69mg/L. The trend of the graph was stable and increased uniformly. After achieved the optimal point, the line starting go down and indicated that the enzymes were not react with substrate actively. This happen because, as already discussed at enzyme activity section, at high temperature, the enzymes starting to denature.

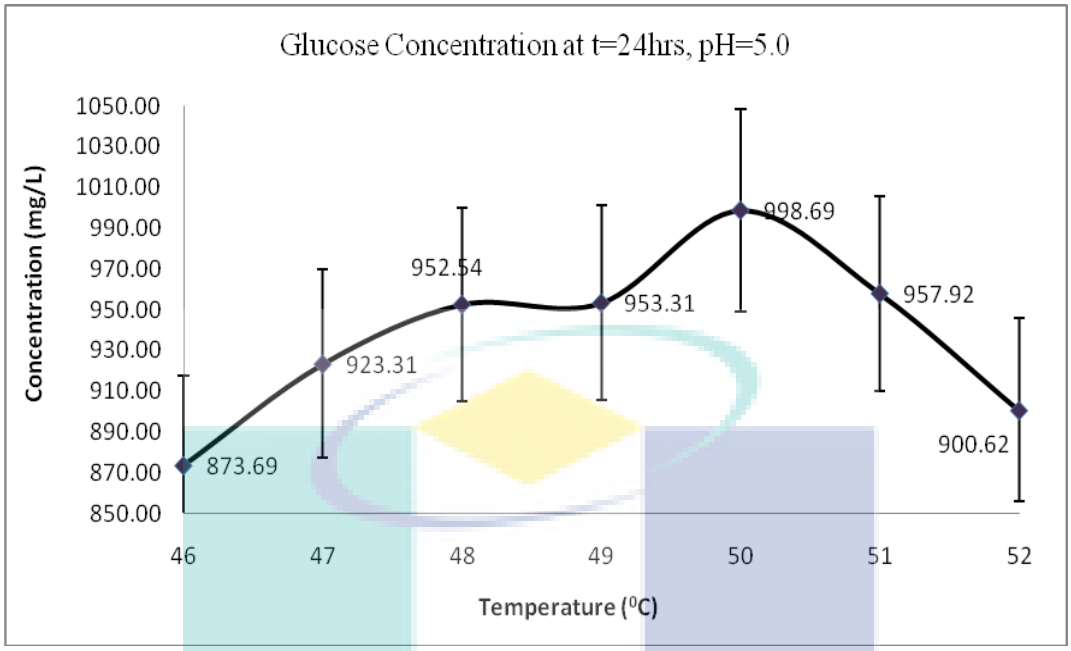


Figure 4.25: Glucose production based on effect of the temperature (mg/L)

Figure 4.26 shows the production of glucose based on pH affected. From the graph, the optimal pH was at pH5 with the highest glucose production, 984.92mg/L. *Cellulase* and *β-Glucosidase* enzyme is the one of the acidic enzyme that will be react actively in acidic condition. From the graph, the variables are in acidic condition but the most optimal pH was at pH5. At other pH, the enzyme still react, but compare with pH5, the others have more decreasing of the glucose production.

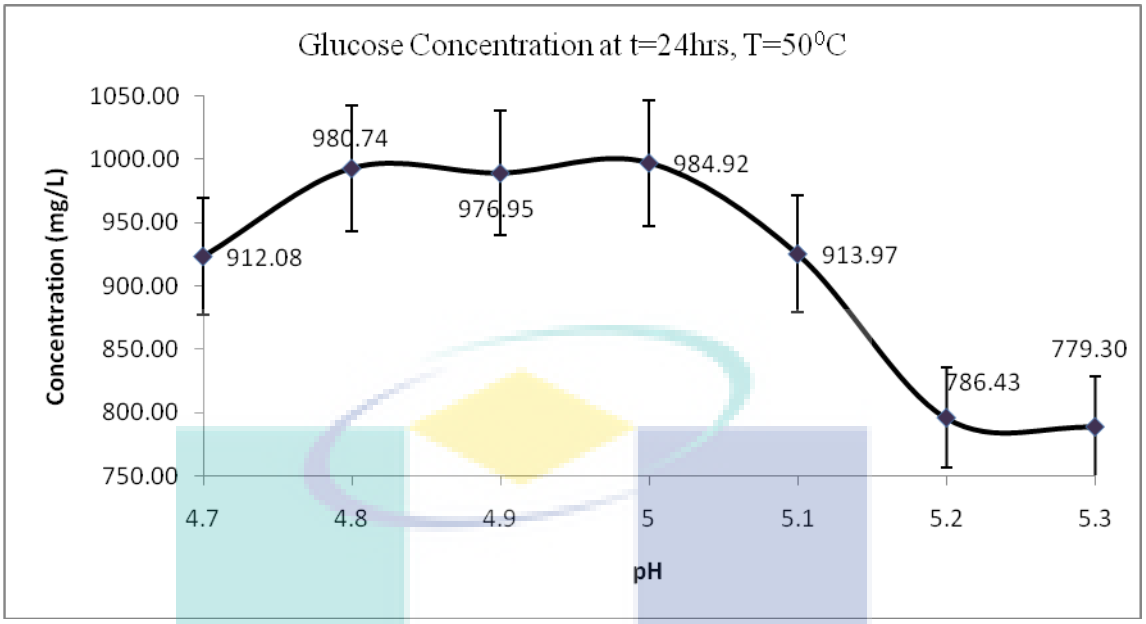


Figure 4.26: Glucose production based on effect of the pH buffer (mg/L)

4.3.7 ENZYMATIC HYDROLYSIS OF PRETREATED MERANTI WOOD SAWDUST; RESPONSE SURFACE METHODOLOGY (RSM) APPLICATION

A central composite design (CCD) was employed to reduce the total number of experiments needed to determine the best combination of parameters for optimization of the process. The statistical software "Design Expert", version 6.0.8 was used for the central composite design and to analyze the experimental data obtained. The conditions for each experiment already shown in previous chapter. The three significant variables reaction time of hydrolysis, temperature of incubator shaker and pH were further optimized using response surface methodology (RSM) and results on the effect of the variable factors towards the glucose production, the response variables, was shown in Table 4.12.

Table 4.12: Experimental layout and result of the response surface methodology (RSM)

Standard	Run	Block	Factor variables			Response (Glucose concentration, mg/L)
			Time (hr)	Temp (^{0}C)	pH	
6	1	Block 1	30.0	49.0	5.1	746.15
3	2	Block 1	21.0	51.0	4.9	1090.42
5	3	Block 1	21.0	49.0	5.1	757.92
8	4	Block 1	30.0	51.0	5.1	838.12
2	5	Block 1	30.0	49.0	4.9	757.92
7	6	Block 1	21.0	51.0	5.1	788.69
10	7	Block 1	25.5	50.0	5.0	979.58
1	8	Block 1	21.0	49.0	4.9	807.69
12	9	Block 1	25.5	50.0	5.0	1008.04
4	10	Block 1	30.0	51.0	4.9	957.92
11	11	Block 1	25.5	50.0	5.0	1021.81
9	12	Block 1	25.5	50.0	5.0	1013.96
16	13	Block 2	25.5	51.7	5.0	943.38
19	14	Block 2	25.5	50.0	5.0	1037.31
18	15	Block 2	25.5	50.0	5.2	761.62
20	16	Block 2	25.5	50.0	5.0	1033.31
17	17	Block 2	25.5	50.0	4.8	922.58
15	18	Block 2	25.5	48.3	5.0	767.92
13	19	Block 2	17.9	50.0	5.0	872.58
14	20	Block 2	33.1	50.0	5.0	876.58

Fit summary output analysis indicated that the quadratic model was statistically significant to represent the glucose response. The adequacy of a quadratic model was examined by F test, "Prob>F" and the determination coefficient R^2. As can be inferred in table 4.13, the computed F and Prob>F were 394.53 and <0.0001, respectively, which implied that the model was in good prediction of the experimental results and as a matter of fact the terms in the model have a significant effect on the response. In a similar manner, the multiple correlation coefficient of R^2 was calculated to be 0.9983, indicating a good agreement existed between the experimental and predicted value as well as depicting that 99.83% of the variability in the response could be well explained by the

model while only 0.17% of the total variation was poorly described by the model. Moreover, the "lack of fit" values was found insignificant (Prob>F=0.2951) which denoted that the model was desirably fit. The main effect of temperature (B) and pH (C) were found to be most significant factor to have the largest effect towards the glucose production efficiency and this was followed by the second order effect of time (A^2), the second order effect of temperature (B^2), the second order effect of pH (C^2) and the two level interactions between temperature and pH (BC). Moreover the main effect of time (A), the two level interactions between time and temperature (AB) and the two level interactions between time and pH (AC) were found to be responsible for the secondary effect on the glucose production.

From the Table 4.13 also noted that other model terms such as the main effect of time (A), the two level interaction with other variables (AB), (AC) and (BC) were found insignificant (Prob>F more than 0.0500 indicate model terms are insignificant) and therefore can be eliminated for model improvement (Reduced Quadratic Model). In conjunction, the backward elimination procedure was employed to eradicate the insignificant terms and ANOVA results of this reduced quadratic model is tabulated in Table 4.14.

Table 4.13: ANOVA for Response Surface Quadratic Model (Partial sum of squares)

Response: Glucose production

Source	Sum of Squares	DF	Mean Squares	F-Value	Prob>F
Model	182100.00	9	20234.08	394.53	< 0.0001[a]
A	*3.73*	*1*	*3.73*	*0.073*	*0.7963*
B	*33063.45*	*1*	*33063.45*	*644.68*	*< 0.0001*
C	*21453.1*	*1*	*21453.1*	*418.3*	*< 0.0001*
A_2	*25834.38*	*1*	*25834.38*	*503.72*	*< 0.0001*
B_2	*32275.5*	*1*	*32275.5*	*629.31*	*< 0.0001*
C_2	*37330.7*	*1*	*37330.7*	*727.88*	*< 0.0001*
AB	*156.94*	*1*	*156.94*	*3.06*	*0.1308*
AC	*80.07*	*1*	*80.07*	*1.56*	*0.258*
BC	*581.39*	*1*	*581.39*	*11.34*	*0.0151*
Residual	307.72	6	51.29		
Lack of Fit	*204.31*	*3*	*68.1*	*1.98*	*0.2951*[b]
Pure Error	*103.41*	*3*	*34.47*		
Cor Total	183800.00	16			
Std Dev.	7.1600		R^2	0.9983	
Mean	892.2000		Adjusted R^2	0.9958	

Values of "prob>F" less than 0.0500 indicate model are significant.

[a] significant

[b] not significant

Table 4.14 shows the ANOVA results after reduced quadratic model done. The results revealed that the model significances was improved (F=154.074 instead of 120.0306 for previous model) with the temperature (B) and pH (C) appeared as the primary main effect contributor followed by all the second order effect of variables (A^2), (B^2), (C^2) and the two level interactions (AB) and (BC). Hence in this study the ranking is as follows: B>C>A^2>B^2>C^2>AB>BC. In the case of the glucose production, the backwards elimination procedure was also selected for model improvement (Table 4.14). The co-efficient of determination R^2 for glucose production was 0.9979 indicating good correlation existed between the experimental and predicted values.

Table 4.14: ANOVA for Response Surface Reduced Quadratic Model (Partial sum of squares) Response: Glucose production

Source	Sum of Squares	DF	Mean Squares	F-Value	Prob>F
Model	182000.00	8	22753.33	410.72	< 0.0001[a]
A	*3.73*	*1*	*3.73*	*0.067*	0.8026
B	*33063.45*	*1*	*33063.45*	*596.83*	< 0.0001
C	*21453.1*	*1*	*21453.1*	*387.25*	< 0.0001
A^2	*44201.42*	*1*	*44201.42*	*797.88*	< 0.0001
B^2	*54839.03*	*1*	*54839.03*	*989.9*	< 0.0001
C^2	*63165.08*	*1*	*63165.08*	*1140.19*	< 0.0001
AB	*2383.07*	*1*	*2383.07*	*43.02*	0.0003
BC	*1432.96*	*1*	*1432.96*	*25.87*	0.0014
Residual	387.79	7	55.4		
Lack of Fit	*284.38*	*4*	*71.09*	*2.06*	0.2892[b]
Pure Error	*103.41*	*3*	*34.47*		
Cor Total	1.84E+05	16			
Std Dev.	7.4400		R^2	0.9979	
Mean	892.2000		Adjusted R^2	0.9954	

Values of "prob>F" less than 0.0500 indicate model are significant.

[a] significant

[b] not significant

The multiple regression equations for glucose production using time (A), temperature (B) and pH (C) as the main variable were as follows:

Final empirical model in terms of coded factors:

$$Glucose\ Production = +1025.72 + 0.59 \times A + 55.26 \times B - 44.51 \times C - 58.76 \times A^2 - 65.45 \times B^2 - 70.25 \times C^2 + 23.83 \times A \times B - 18.48 \times B \times C \quad (4.15)$$

Final empirical model in terms of actual factors:

$$Glucose\,Production = -380102 - 116.64189 \times Time + 7389.59441 \times Temperature + 79041.96533 \times pH - 2.90194 \times Time^2 - 65.45456 \times pH^2 + 5.29542 \times Time \times Temperature - 184.78283 \times Temperature \times pH \quad (4.16)$$

The above empirical model equations are mathematical correlation model that can be employed to predict and optimize the glucose production within the range of variable factors of this experiment. Analyses on normal probability plot of the residuals (Figure 4.27) depicted nearly a straight line residuals distribution, which denoting errors are evenly distributed and therefore support adequacy of the least square fit, while results illustrated in Figure 4.28 revealed that the models proposed are distinctively adequate and reasonably free from any violation of the independence or constancy variance assumption, as studentized residuals are equally tabulated within the red line of the x-axis.

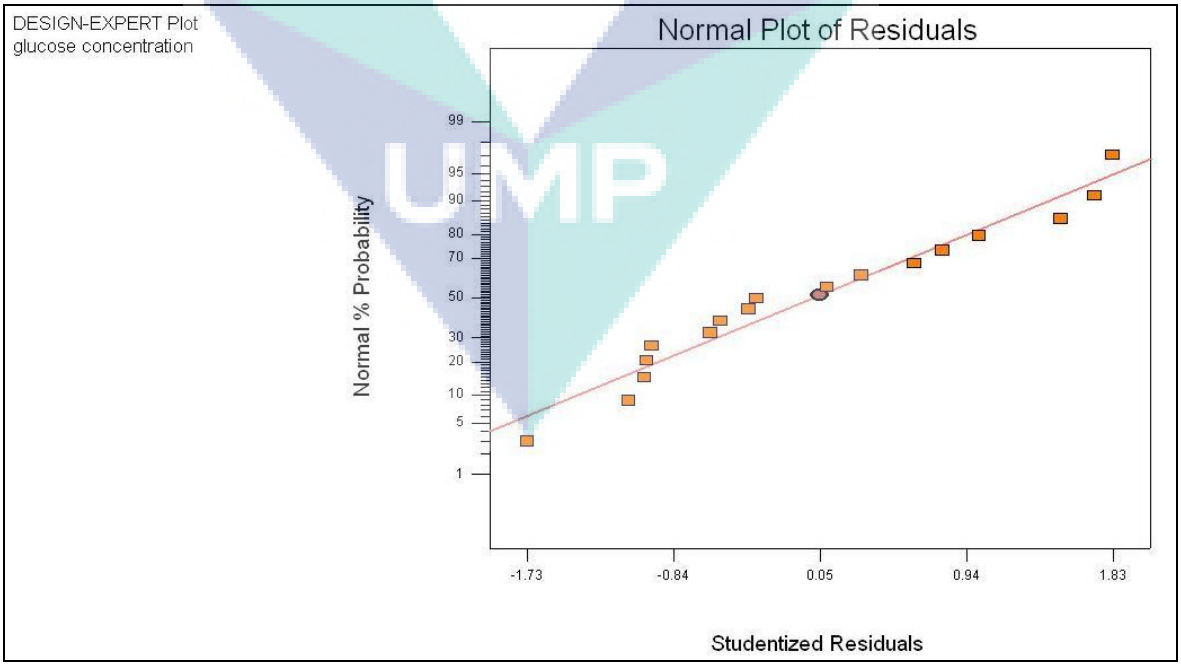


Figure 4.27: Normal probability plot of residuals for glucose production

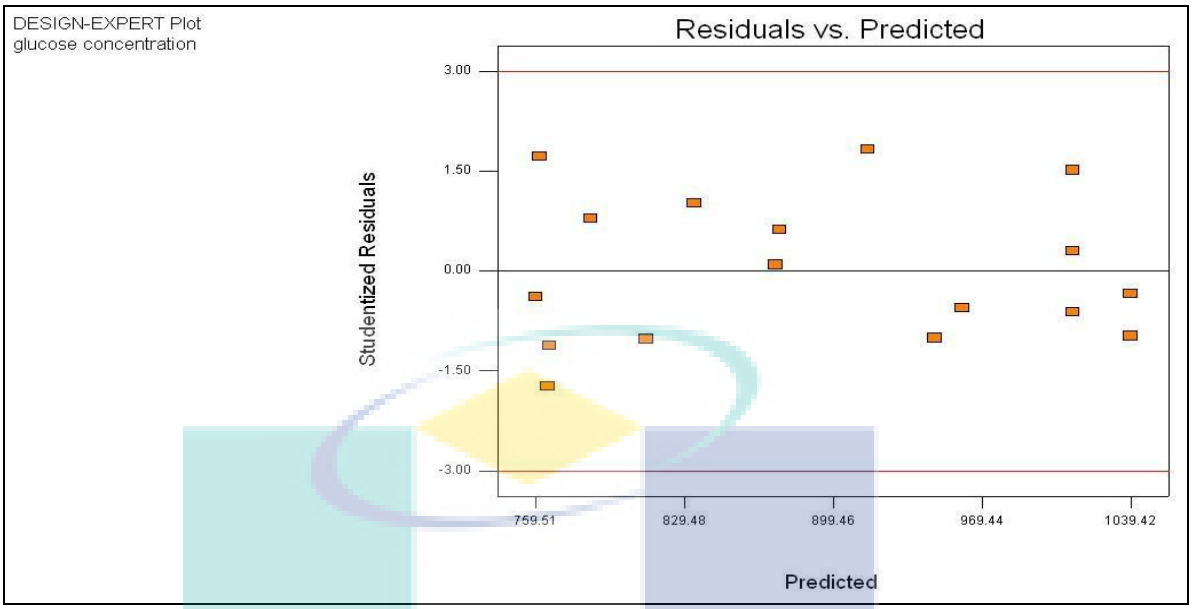


Figure 4.28: Plot of residual against predicted response of glucose production

The effect of reaction time, temperature and pH process variables on glucose production was further analyzed using simulated three dimensional response surface and contour plots according to the backward quadratic model (reduce model). The effect of time and temperature on glucose production depicted in Figure 4.29 and Figure 4.30 demonstrated that the glucose production increased when temperature change from 49^0C to 51^0C and as reaction time increased from 21 hours to 30 hours.

The surface and 3D plots of the effect of time and temperature on glucose production while keeping pH at the middle level. From the graph interaction or 3D, it was clearly shown that the glucose production was increased from 932.341mg/L at 21.0 hours to 981.174 mg/L at 30.0 hours when pH constantly maintained at pH5. The other site, the opposite result was observed with the decreasing of glucose production at 869.484mg/L at 21.0 hours to 823.00 mg/L at 30.0 hours. In the early, the production of glucose was increased but when achieved at middle point, the glucose production become decreasing uniformly.

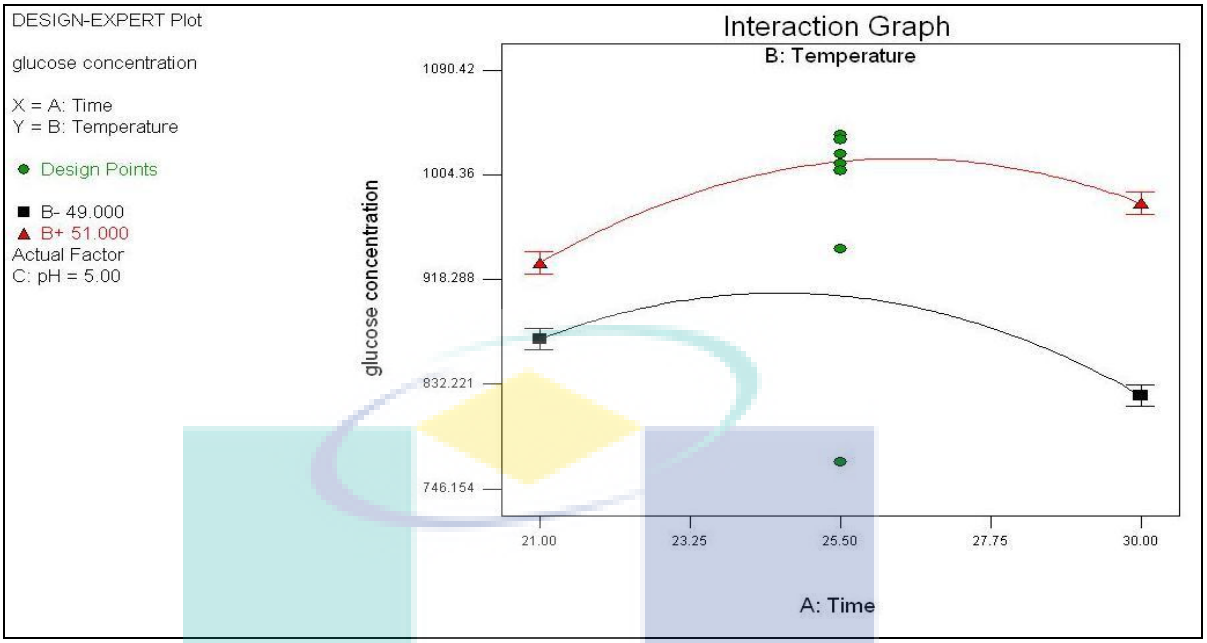


Figure 4.29: Interaction graph of glucose production from the model equation: effect of time (hour) and temperature (^{0}C)

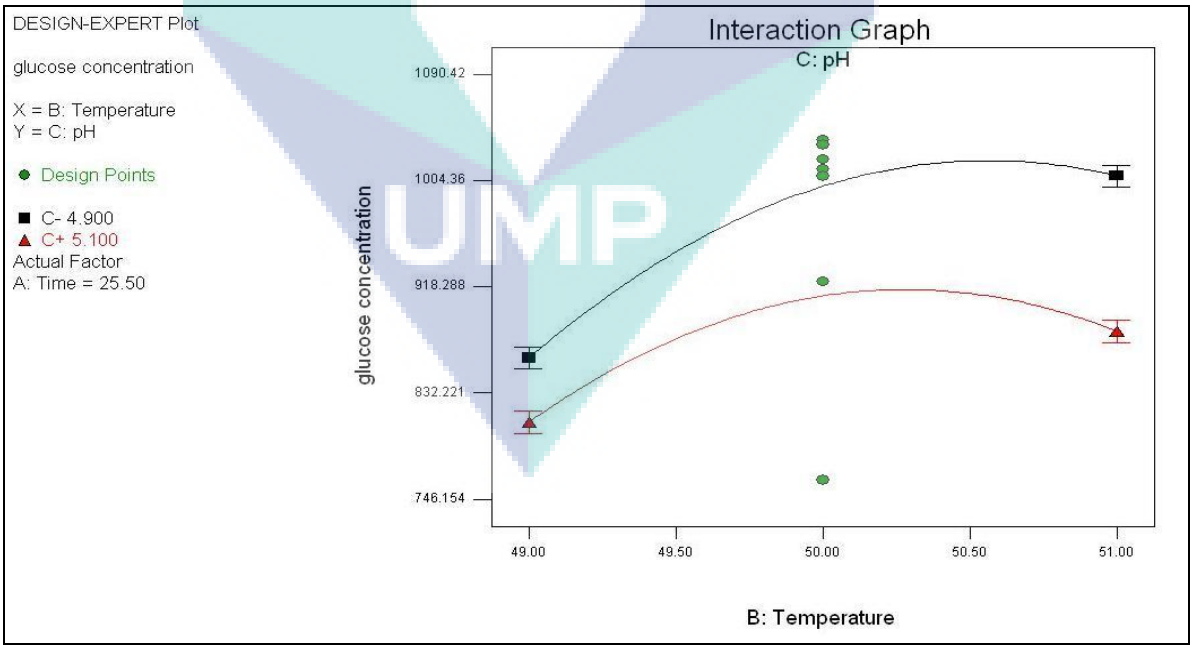


Figure 4.30: Interaction graph of glucose production from the model equation: effect of temperature and pH

The Figure 4.30 and Figure 4.32 show the graph of the interaction between temperature and pH. Based on the result (graph), the maximum glucose production of 1008.26 mg/L is obtained when pH at 4.9 and temperature set at 51^0C respectively. In addition, that the temperature affects the glucose production in a linear way as a pH, where the glucose production is found to be maximum at high level of factor. In fact, the slope of the increasing graph is dependent on temperature and pH, and appears to be gradually steep at higher level conditions. The results were found to be consistent even though the slope become quadratic's trend first and then become increase uniformly. As the results found, the slope was decreased but not affected the production of the glucose.

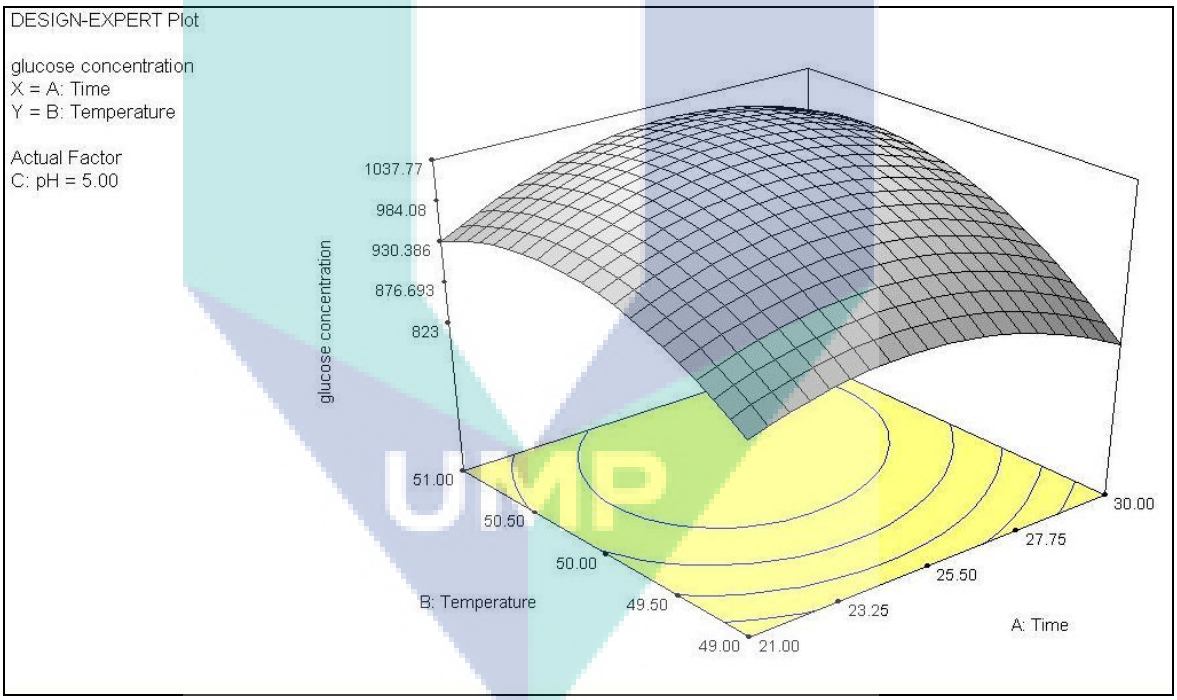


Figure 4.31: Three dimensional (3D) graph of glucose production from the model equation: effect of time and temperature

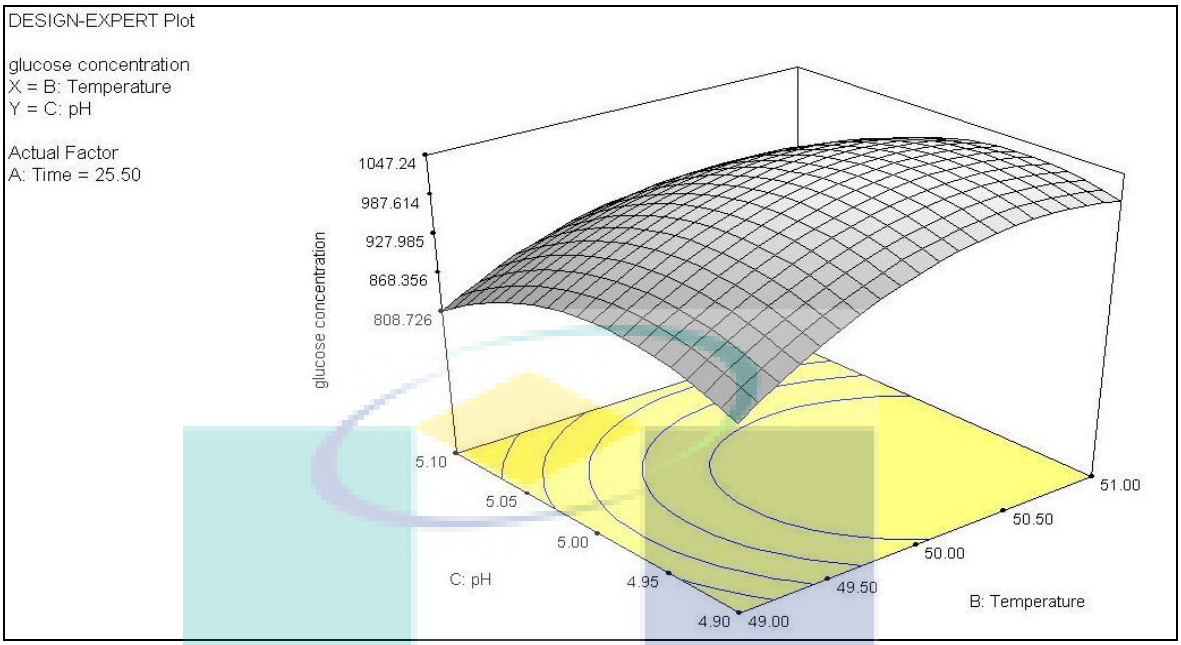


Figure 4.32: Three dimensional (3D) graph of glucose production from the model equation: effect of temperature and pH

4.3.8 ENZYMATIC HYDROLYSIS OF PRETREATED MERANTI WOOD SAWDUST; VALIDATION OF EMPIRICAL MODEL ADEQUACY

Adequacy of the developed empirical models needs to be verified or validated in order to confirm the prediction accuracy, which is generated by the regression equation in predicting the glucose production at any particular time, temperature and pH within the range of level defined previously. Experimental rechecking was performed using conditions that were previously used and combined with the additional experiments which have not been tried before but was within the limits tested previously. Table 4.15 shows the results of operating conditions with experimental design in confirmation run. The obtained actual values and its associated predicted values from the selected experiments were compared for further residual and percentage error of analysis.

The percentage error between actual and predicted value of response over a selected range of operating levels are calculated based on equation 4.3 and 4.4. Results of

Table 4.15 have shown that the percentage errors are ranging from 0.50% to 4.54% for glucose production. Thus implied that the empirical model developed were considerably accurate for responding term (glucose production) as the percentage error between the actual and the predicted values were well within the value of 5.0%, suggesting that the model adequacy is reasonably within the 95.0% of prediction interval. By this means further analysis with regards to ideal operational process for optimal production of glucose would be based on this developed model.

Table 4.15: Results of operating conditions with experimental design in confirmation run.

No	Run factor			Glucose Production (mg/L)			
	Time(hr)	Temp (^{0}C)	pH	Predicted	Actual	Residual	Error (%)
1	29.8	49.3	4.97	894.54	923.27	28.73	3.11%
2	26.8	49.6	5.09	896.81	932.31	35.50	3.81%
3	22.0	50.8	5.01	969.30	933.04	-36.26	-3.89%
4	25.9	50.9	4.94	1035.22	1043.04	7.82	0.75%
5	25.2	50.7	5.03	1006.81	1001.81	-5.00	-0.50%
6	29.6	50.8	4.96	1009.13	976.58	-32.55	-3.33%
7	22.3	50.4	4.92	995.61	1004.81	9.20	0.92%
8	27.5	49.1	4.98	902.84	872.81	-30.04	-3.44%
9	28.2	50.3	5.00	1020.05	1000.62	-19.43	-1.94%
10	26.6	49.4	5.06	906.70	949.81	43.11	4.54%

4.3.9 ENZYMATIC HYDROLYSIS OF PRETREATED MERANTI WOOD SAWDUST; PROCESS OPTIMIZATION

Considering the previous discussion, main effect of time, temperature and pH were found more dominant than their interaction effects with respect to glucose production. Nevertheless one cannot simply neglect the importance of various interaction effects, for that interaction among them had only little influence on responses because any individual factor that involved in interactions have to be considered jointly. In general, for any given hydrolysis, there is usually a trade-off relationship of between production and catalyst of the reaction. Hence a desirable combination of time,

temperature and pH is required in order to achieve the optimum operational conditions for glucose production. Utilizing the optimization mode capability of the software enables the prediction of response complete with 95.0% prediction interval. Optimization procedure has been conducted for the pretreated meranti wood sawdust and the prediction results of the empirical model are tabulated in Table 4.16. The temperature, time and pH were set to range within the levels defined. Results have shown the optimum pH, time and temperature for optimal glucose production of pretreated meranti wood sawdust. The result was determined to be 25.4 hour, 50.5^{0}C and pH 4.95 respectively. Under these proposed optimized conditions, the maximumvalue of glucose production from the model was 1045.67mg/L. In order to confirm the predicted optimization conditions, experimental confirmation runs were performed by employing the suggested model conditions. Apparently the optimal value of glucose production was 1028.35mg/L. It is worth to note results of the experimental varied out here adequately implied that the proposed mathematical models suggested are reasonably accurate and reliable as most of the actual values for the confirmation runs are well within the 95.0% prediction interval, hence it could be reliably be employed for the prediction of optimum sugar alcohol production process .

Table 4.16: Results of optimum operational conditions for *meranti* wood sawdust

Factor	Proposed optimal conditions	Glucose Production	
		Predicted (%)	Actual (%)
time (hour)	25.4	1045.67	1028.35 (1.68%)
Temp (^{0}C)	50.5		
pH	4.95		

4.4 FERMENTATION PROCESS OF GLUCOSE TO PRODUCE SUGAR ALCOHOL

Fermentation process was the last phase of this research work after pretreatment and enzymatic hydrolysis process. In this phase, *lactobacillus plantarum*, NCIMB8826, lactic acid bacteria was used as a media to convert glucose to produce sugar alcohol especially sorbitol. This experiment was done using anaerobic reactor fermentation and the Erlenmeyer flask 250mL was designed as a reactor. The production of sugar alcohol was studied based on the effect of temperature and incubation reaction time.

4.4.1 STUDY THE PROFILE GROWTH OF THE *LACTOBACILLUS PLANTARUM*

Figure 4.33 shows the calibration curve of the sugar alcohol (sorbitol) production using UV-VIS as a method of the analysis. The wavelength used was 600nm because the optical density of the *lactobacillus plantarum* NCIMB 8826 was at 600nm. In profile growth of a study, the UV-VIS was used because the reduction of the sugar and substrates was used this method for the amount of calculation. From the Figure 4.33, the equation of the calibration curve indicating that the correlation coefficient was 0.9991. The x-value of the equation was concentration of sugar production/reduced and y-value of the equation was absorbance at 600nm respectively.

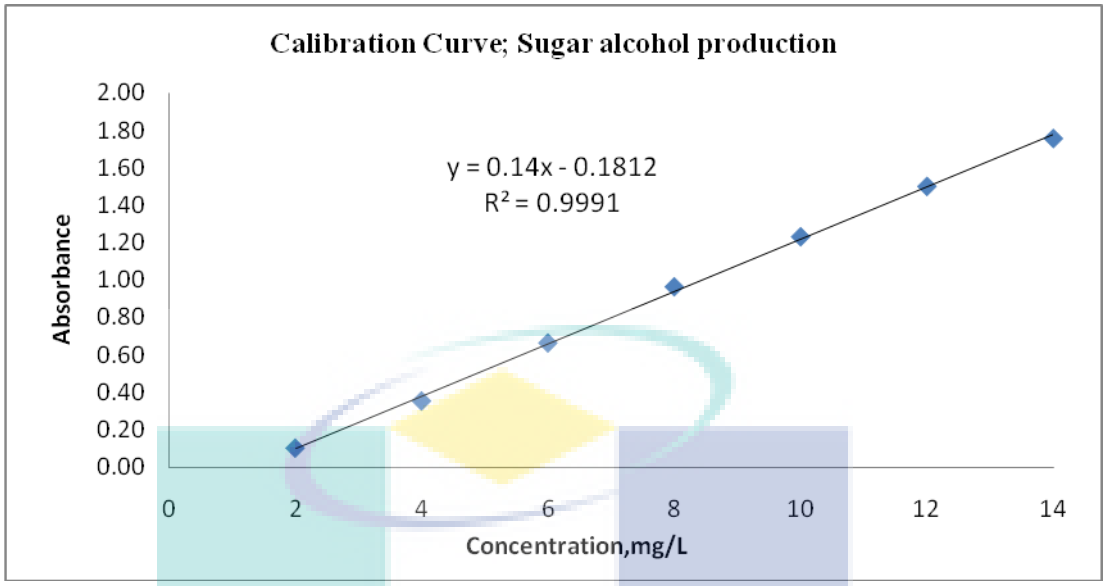


Figure 4.33: Calibration curve of the sugar alcohol production, at 600nm

Figure 4.34, Figure 4.35 and Figure 4.36 shows the growth analysis of the *lactobacillus plantarum* NCIMB 8826 based on the sugar alcohol produced, substrate remaining and the dry weight of cell respectively. From Figure 4.34, the production yield was slowly incrased at 6hours and then linearly increased starting from 12hours. This is because, the bacteria already started to interact with substrate with maximum interaction . From the graph trend, the production of sugar alcohol slowly constant with the bacteria activity. This is because, the bacteria already optimum interacted with substrate at 72 hours with sugar alcohol production, 7.88mg/l respectively. After this hour, the production of the sugar alcohol still at the same yield because the bacteria was not active after the optimal time.

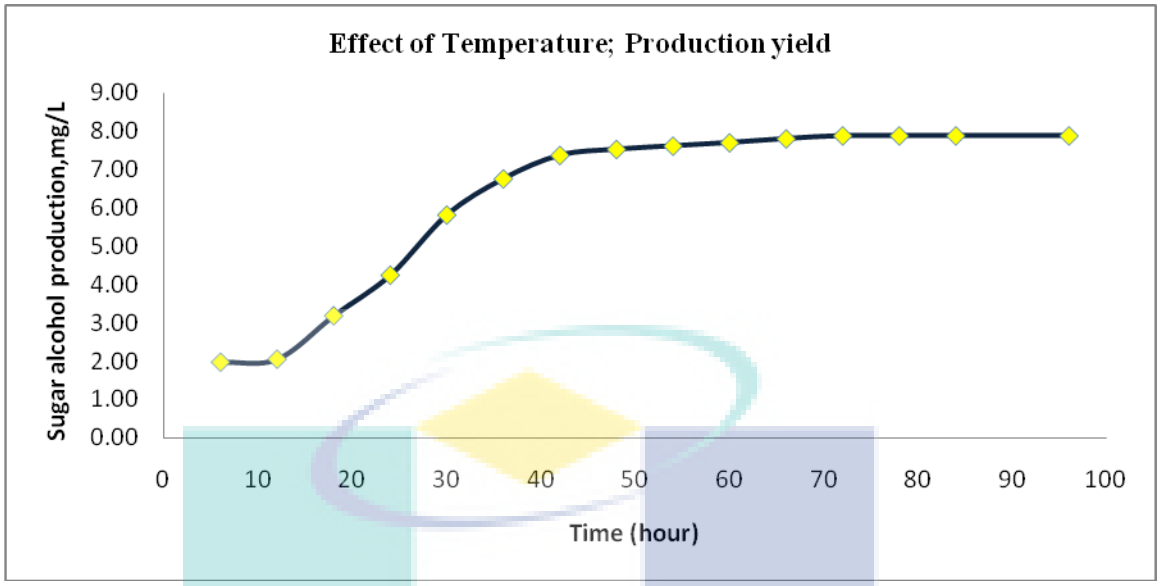


Figure 4.34: Growth analysis of Lactobacillus Plantarum NCIMB 8826 in batch cultures on MRS (30^0C); based on production yield

Figure 4.35 shows the graph of growth analysis of *lactobacillus plantarum* NCIMB 8826 based on substrate remaining. From the graph plotted, the substrate, glucose was still not degrade smoothly and the bacterial activity still in pre-reacted with the surrounding. When the reaction at 18hours, the graph trend was decreased linearly. Hence this occurred because the bacterial activity in maximum actively and when the reaction achieved the optimal time, 72hours, the activity of the bacterial slowly down and then constant at that time and future. This condition was held because the growth of the bacterial was not in active mode and all optimal substrate with optimal bacterial activity was constant.

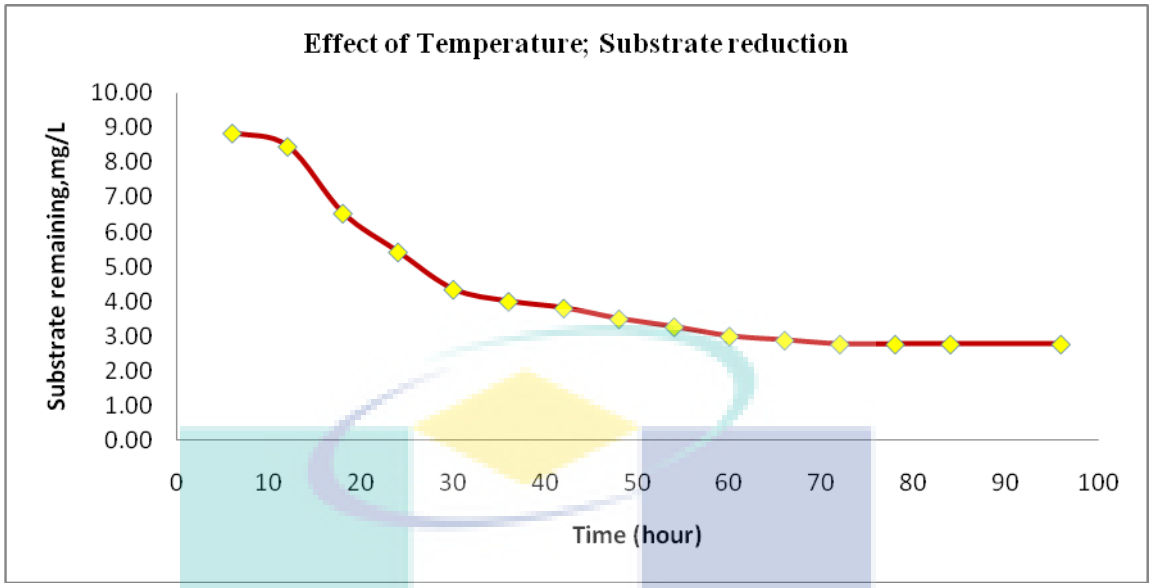


Figure 4.35: Growth analysis of Lactobacillus Plantarum NCIMB 8826 in batch cultures on MRS (30^0C); based on substrate remained

Figure 4.36 shows the graph of the growth analysis of lactobacillus plantarum NCIMB 8826 in batch cultures on MRS medium, based on the dry weight of the cell calculation. As combined all the three graph from figure 4.34, figure 4.35 and figure 4.36, the dry cell weight production was parallel with production yield. This condition means, when the production was at optimum point, the cell starting to decrease.

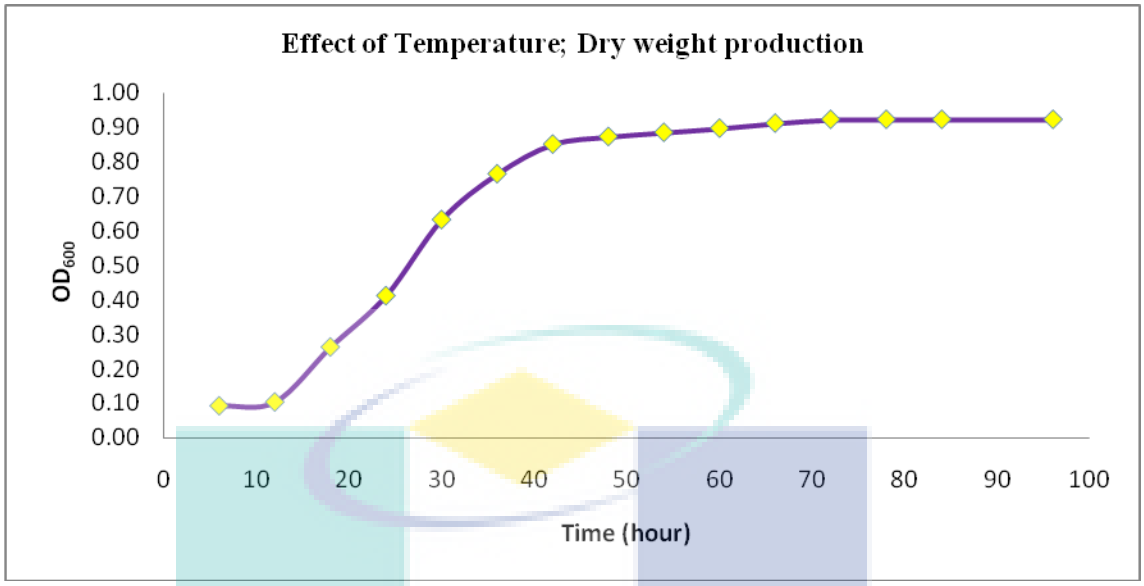


Figure 4.36: Growth analysis of Lactobacillus Plantarum NCIMB 8826 in batch cultures on MRS (30^0C); based on dry weight, OD_{600}

4.4.2 PRODUCTION OF SUGAR ALCOHOL (SORBITOL) FROM FERMENTATION PROCESS

After the trend of the profile growth were studied and plotted, then the production of sugar alcohol (sorbitol) was occurred using the optimal condition that achieved from the graph of the profile growth. Figure 4.37 shows the calibration curve that plotted from the data collected based on the fermentation process at 30^0C for 72hours and so on. From the calibration graph, the equation was created from the linear line and the correlation coefficient of the graph was 0.9997. This data was collected from HPLC analysis using RI detector. The x-value and y-value were concentration and area respectively.

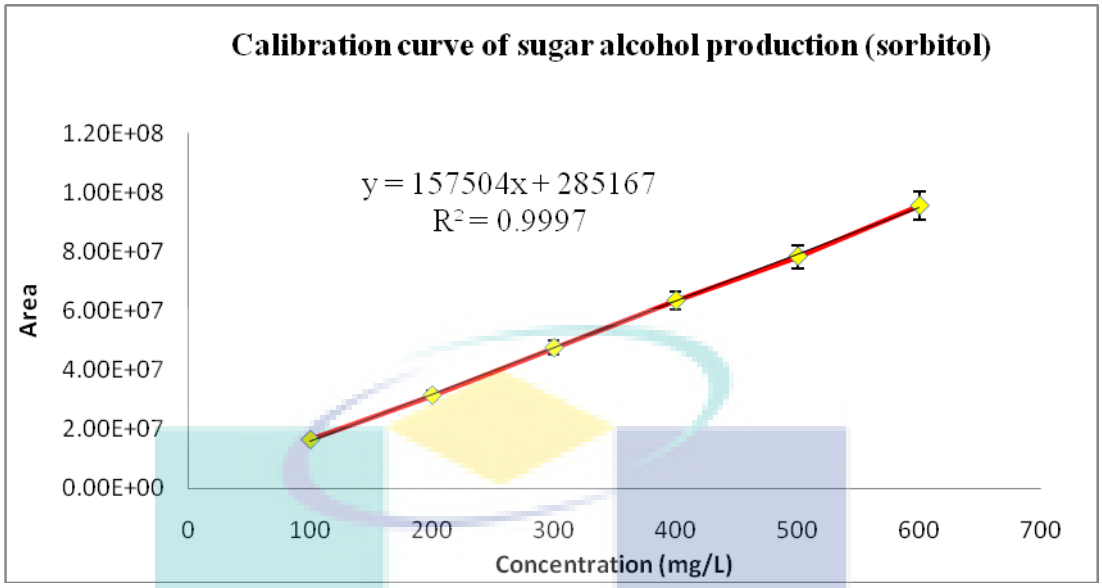


Figure 4.37: Calibration graph of the sugar alcohol (sorbitol) production, based on HPLC analysis

Figure 4.38 shows the production of sugar alcohol from fermentation process by *lactobacillus plantarum* bacteria, NCIMB 8826. From the graph plotted, the production of sugar alcohol was slowly at the starting point and then increased smoothly proportional with bacterial growth with the optimum time was 72hours with sugar alcohol production was 61.66mg/L. The percentage of the conversion glucose to sugar alcohol was about 63.3% based on the bacterial growth. The effect of the temperature to produce sugar alcohol was not studied here because, when the temperature used was high than 30^0C but not higher than 60^0C such 37^0C, the bacterial growth was increased and actively react with substrate compared at 30^0C. The time taken to produce sugar alcohol also will short than time taken to produce sugar alcohol at 30^0C. Here, the studies still at 30^0C and 72hours because if we do the cost analysis comparison between them, the temperature at 30^0C still in lower energy consumption.

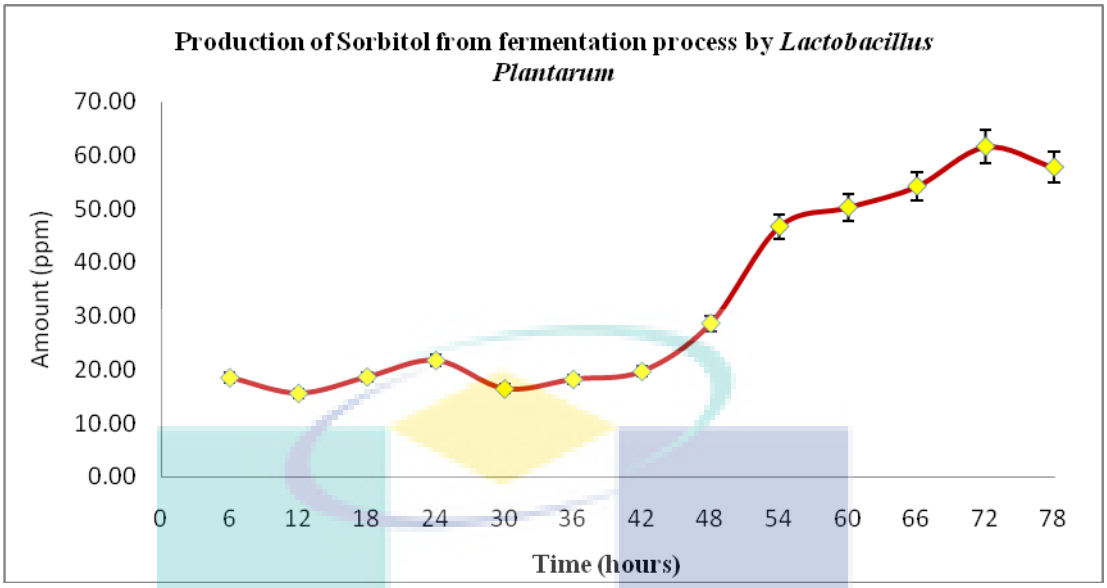


Figure 4.38: Production of sugar alcohol (sorbitol) of *lactobacillus plantarum* affected by time

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 GENERAL CONCLUSIONS

The chemical composition of *meranti* wood sawdust was being determined and showed the percentage of cellulose was higher than other biomass fibers. The two stages of pretreatment started with pre-delignification using NaOH (sodium hydroxide), then followed by the first stage of pretreatment using PAA (peracetic acid) and then followed by two stages of pretreatment using diluted (H_2SO_4) sulfuric acid have been applied in the first phase of the research works. In this phase, the one factor at a time (OFAT) study was applied to narrow down the upper and lower limits the variables in terms of the response. From the graphs that have been plotted, the range of the limits getting smaller and can proceed to the RSM application. After achieved the OFAT study target, the response surface methodology (RSM) was applied and achieved the optimal condition of the three variables: time, temperature and concentration of acid. Besides, the characterization of *meranti* wood sawdust before and after treatment also investigated and satisfied with lignin degradation percentage when the comparison between untreated samples and treated samples were being done. The determination of lignin degradation was being calculated by using Kappa number equation.

In the second phase of the research works, the enzymatic hydrolysis was applied to degrade the polysaccharide (cellulose fibers that had been treated by pretreatment) to monosaccharide (glucose). In this phase, *Cellulase* and *β-Glucosidase* enzyme were used

as a catalyst to hydrolyze the cellulose fibers. The study of kinetic enzyme was applied here based on the effect of the temperature of the incubation shaker, pH of the buffer and also reaction time of the sugar production. From the calculation of the enzyme activity (IU), the sugar production was proportional with enzyme activity and unproportional with reaction time. The kinetic study shows the enzymes (*Cellulase* and *β-Glucosidase*) were acidic enzyme and need to be reacted in acidic condition. The stability of enzyme also was studied and hence that enzyme was denatured at temperature higher than 60^0C and pH more than pH8, respectively. DNS method was used to analyze the glucose production. In this phase also, the response surface methodology (RSM) was applied to get the optimum value of the glucose production based on the variables: reaction time, temperature and pH and after the optimization process done, the maximum value of the glucose production was achieved, based upon the regression analysis of total sugar yield.

In the last phase of the research works, the fermentation process was being occurred using *Lactobacillus plantarum* as bacteria to produce sugar alcohol from glucose as a substrate. In this phase, the profile growth of the bacteria was studied and then the effect of the temperature, incubation time and pH was achieved and the sugar alcohol that had been produced from fermentation was analyzed using HPLC method. From the graph of the profile growth, the production of sugar alcohol parallel with time and when achieved the optimal point, the production starting to decrease because of the substrate lost.

A central composite design (CCD) of response surface methodology (RSM) can be used to determine the significant variables and optimum condition for pretreatment and hydrolysis process. Experimental results show that two stages of pretreatment can be used as a way to remove the lignin from the biomass residue. Besides, the maximum value of glucose yield also can be achieved from the application of the RSM.

5.2 RECOMMENDATIONS FOR FUTURE WORK

A number of recommendations are being proposed to enhance the lignin removal, and then the production of sugar (glucose) and production of sugar alcohol from fermentation process are being listed as below:

1) Apply the response surface methodology (RSM) at the pre-delignification and at the first stage of pretreatment process.

In this study, the chemicals were used and as the government campaign, we need to reduce the usage of the chemicals. Hence, the optimization needs to be applied here to save the chemical usage in our research.

2) Additional of the parameters for the second stage of pretreatment process.

As already know, in the second stage of pretreatment, the lignin degradation was achieved but the cellulose yield content still in small percentage. So, when the CYC (cellulose yield content) was added as a variable that will apply in RSM, so that the enzymatic hydrolysis will be more valuable and more sugar production will produce based on the cellulose fibers recovery. This is because; the yield of the sugar production was proportional with substrate loading.

3) Apply the substrate of hydrolysis process as a parameters that affected the process

From the enzymatic kinetic study, the substrate plays an important role to the enzyme activity. This means the substrate will affect the enzymatic hydrolysis and because of that, why not apply it as parameter in our process for future research to make this work more valuable and more novelty.

4) Do the comparison pretreatment for the variety of biomass residue to recover the maximum of cellulose content.

In order to prove that *meranti* wood sawdust was high in cellulose recovery content, the comparison experiment can be done between other biomass residues (especially in Malaysia application industries) such sugarcane bagasse, other hardwoods like *cengal* wood, *keruin* wood etc. with the same experimental method.

5) Applying the separation process

In the enzymatic hydrolysis process, the separation need to been applied because the production of the sugar, not only glucose produced, but other sugar like xylose and monomer plus oligomer also was produced. With this application, the research will be more novelty.

6) Produce the enzymes and bacterial by own.

When talk about cost and the way on reducing of it, we need to avoid the usage from the commercial of enzyme and bacterial. Because of that, the production of enzymes needs to be produced by self (immobilized).

7) Performing additional characterization.

In order to characterize the materials, the additional characterization method such as X-ray Diffraction (XRD) and NMR to determine the crystallization of pretreatment and glucose production, could confirm the success of the treatment being applied.

LIST OF PUBLICATIONS

R. Rusmawarni and A.M. Mimi Sakinah, Enzymatic hydrolysis of pretreated *meranti* sawdust: OFAT study, International Renewable Energy & Environment Conference (IREEC 2011), Kuala Lumpur, Malaysia, 24 – 26 June 2011.

R. Rusmawarni and A. M. Mimi Sakinah, Removal of lignin and hemicellulose from *meranti* sawdust by two stages of pretreatment: OFAT study, International Conference of Chemical Innovation 2011 (ICCI 2011), Kemaman Terengganu, Malaysia, 21 -23 May 2011.

R. Rusmawarni and A.M. Mimi Sakinah, Combination effect of time and temperature on enzymatic cellulose fyber hydrolysis, Postgraduate week, Universiti Malaysia Pahang, Malaysia, 9 April 2011.

R. Rusmawarni and A.M. Mimi Sakinah , Response Surface Optimization (RSM) of Pretreatment of *Meranti* Sawdust for Higher Lignin Degradation. 1st International Conference and Exhibition of Woman Engineer (ICEWE 2011), Kuantan Pahang, Malaysia, 20-22 November 2011.

R. Rusmawarni, A. M. Mimi Sakinah, Chemical Pretreatment of *Meranti* sawdust using H_2SO_4: Optimization of Lignin Degradation. Internation Conference of Chemical Engineering and Industrial Biotechnology 2011 (SOMChe-ICCEIB 2011), Kuantan Pahang, Malaysia. 28-01 december 2011.

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