

CHAPTER THREE

EXPERIMENTAL

3.1 Materials and Chemicals

Green sawdust (GS) and spent mushroom sawdust substrate (SMSS) used in this study were contributed by Ganofarm Sdn. Bhd., GS is the green sawdust or virgin sawdust that has not been used after growing mushroom, SMSS is the spent mushroom sawdust substrate that has been used after growing mushroom. Delignified spent mushroom sawdust substrate (DSMSS) was obtained by pre-treating the SMSS by 2M potassium hydroxide solution.

Phosphorus(V)oxide (extrapure) used for drying was purchased from HmbG. Cellulose (reagent grade), hemicelluloses (reagent grade) and lignin (reagent grade) used as the standards in TGA were purchased from Sigma-Aldrich. Potassium hydroxide (reagent grade) used in the pretreatment and in neutralization of the perchloric acid was purchased from R&M. Hydrochloric acid (fuming 37%) used to neutralized the KOH in the pretreatment was purchased from R&M. Perchloric acid (reagent grade, 70%) used in the hydrolysis was purchased from R&M. The fructose (99% purity) used as a standard in HPLC was purchased Sigma-Aldrich. The glucose (99.5% purity) used as a standard in HPLC was purchased from Sigma-Aldrich. The sucrose (100% purity) used as a standard in HPLC was purchased from Riedel-de Haen. The lactose (100% purity) used as a standard in HPLC was purchased from Sigma-Aldrich. The maltose (99% purity) used as a standard in HPLC was purchased from Sigma-Aldrich. The maltotriose (95% purity) used

as a standard was purchased from Sigma-Aldrich. The ultrapure water, Atrium 611UV used to prepare the standard solutions in HPLC and GC was purchased Sartorius. The methanol (analytical reagent, 99.8%) used as a standard in GC was purchased from Merck. The ethanol (analytical reagent, 99.9%) used as a standard in GC was purchased from BHD. The propanol (ACS, 100%) used as a standard in GC was purchased from Merck. The isopropanol (ACS, 99.5%) used as a standard was purchased from Merck. The butanol (analytical reagent, 100%) used as a standard in GC was purchased from Merck. The Sodium thiosulfate pentahydrate (chempure) used in the Fehling's test was purchased from HmbG. Copper(II)sulfate pentahydrate (analytical reagent) used in the Fehling's test was purchased from HmbG. Sodium hydroxide (reagent grade) used in the Fehling's test was purchased from R&M. Potassium sodium tartrate tetrahydrate (reagentplus) used in the Fehling's test was purchased from Sigma-Aldrich. Potassium iodide (analytical reagent) used in the Fehling's test was purchased from HmbG. Sulphuric acid (95%) of analytical reagent grade was purchased from HmbG.

3.2 Analysis of Water Content and Thermogravimetric Analysis on Raw Spent Mushroom Sawdust Substrate and Green Sawdust

The loosened structure of SMSS has a lower density and hence will have a greater ability to hold water in its structure. Therefore, the study of water content can give an idea of the density of the sawdust substrate.

20g of wet SMSS was pre-dried in Memmert 40050-IP20 oven at 45°C for four days. The pre-dried SMSS was completely dried for one day in Lab-Line vacuum oven at 50°C. Phosphorus pentoxide was used as a drying agent. A constant dry mass of SMSS

was recorded. The water content in SMSS was calculated according to the following equation:

$$\text{Water content, \%} = \frac{\text{mass of wet SMSS} - \text{mass of dry SMSS}}{\text{mass of wet SMSS}} \times 100$$

(Equation 3.1)

The experiment was repeated by using green sawdust (GS). Five replicates were produced for each substrate.

TGA is an analysis performed on samples to determine changes in weight with increasing temperature. Analysis is carried out by increasing the temperature of the sample gradually and a graph of weight percent against temperature is plotted (Rennekar *et al.*, 2004). The temperature at which the decomposition rate is the highest is called the decomposition temperature. Decomposition temperatures of GS, SMSS and delignified spent mushroom sawdust substrate (DSMSS) are compared.

The TGA was performed by Perkin-Elmer Pyris Diamond thermogravimetric analyzer. Nitrogen gas with a flow rate of $2 \text{ cm}^3 \text{ s}^{-1}$ was used as the atmosphere. The samples, weighing between 8 mg to 12 mg, were heated from $50 \text{ }^\circ\text{C}$ to $900 \text{ }^\circ\text{C}$ at a scanning rate of $10 \text{ }^\circ\text{C min}^{-1}$ (Fu *et al.*, 2008). The analyzed samples include SMSS, DSMSS and GS, as well as commercial reference compounds, cellulose, hemicellulose and lignin purchased from Sigma-Aldrich.

3.3 Pretreatment of Spent Mushroom Sawdust Substrate and Green Sawdust by Potassium Hydroxide

Pretreatment was carried out to loosen up the closely packed lignocellulosic structure by dissolving the lignin and hemicellulose that are held strongly with the cellulose (Section 1.2). Aqueous alkali pretreatment is more efficient than acid pretreatment as less degradation of sugar occurs in alkali pretreatment (Carrillo *et al.*, 2005; Guo *et al.*, 2009).

10 g of wet sawdust substrate were treated with 20 ml of 2M potassium hydroxide (KOH), and the mixture was left undisturbed for two days at under room temperature (Hendriks and Zeeman, 2009). The mixture was filtered through a sintered glass crucible (P2) by suction. The solid obtained was rinsed with distilled water for a few times then followed by dilute HCl (aq) to remove the remaining KOH. Lastly the solid was rinsed by distilled water again to remove the excess HCl. Drying of the resulting solid was done according to section 3.4.1.

The percentage of dry mass in SMSS was calculated according to the following equation:

* mass of water is predetermined in section 3.5

$$\% \text{ of dry mass,} = \frac{\text{dry mass}}{\text{mass of wet sawdust substrate} - * \text{mass of water}} \times 100$$

(Equation 3.2)

3.4 Optimization of Hydrolysis Parameters

In order to improve the production of bioethanol in terms of the yield of ethanol, the cost of production and the time consumed by the whole production, the concentration of perchloric acid used and the hydrolysis time are studied.

3.4.1 Hydrolysis of Spent Mushroom Sawdust Substrate at Different Concentrations of Perchloric Acid

In order to find out the effect of concentration of perchloric acid on the efficiency of hydrolysis, 50g of SMSS were hydrolysed by 70%, 60% and 50% (v/v) of perchloric acid. 50g of dry SMSS was added portion by portion into 100 cm³ of 70% HClO₄ (aq) with continuous stirring. The mixture was then placed in a water bath at 80°C with constant stirring for 10 minutes. 100 cm³ distilled water was added into the hydrolysate, and the residue was filtered by a sintered glass filter. The residue was washed by distilled water through the sintered glass filter for a few times. The washed residue was dried in the air (*not* in oven, as there might be traces of residual perchloric acid.). The dry mass of the residue was recorded. The percentage of mass reduction can be calculated by the following equation:-

$$\text{Percentage of mass reduction, \%} = \frac{\text{mass of DSMSS} - \text{mass of residue}}{\text{mass of SMSS}} \times 100$$

(Equation 3.3)

3.4.2 Hydrolysis of Spent Mushroom Sawdust Substrate with Different Hydrolysis Times

In order to find out the effect of length of hydrolysis time on the hydrolysis, SMSS samples were hydrolysed with different lengths of time. 50g of SMSS were added into 100 cm^3 70% perchloric acid at 80°C with constant stirring for 10 minutes. The mixture was left undisturbed for another 50 minutes, making up the total hydrolysis time 60 mins. The above procedures were repeated for 50g of SMSS with 10 mins stirring and 20 minutes residence time and 50g of SMSS with 10 minutes stirring and zero residence time. The mass of glucose produced can be calculated by the following equation:-

$$\text{Mass of glucose, g} = \frac{\% \text{ of glucose in hydrolysate} \times \text{volume of hydrolysate}(cm^3)}{100}$$

(Equation 3.4)

3.5 Hydrolysis at Optimum Conditions

Hydrolysis was carried out to break the long polymeric cellulose chain into its monomer which is glucose. During the hydrolysis, perchloric acid helps to hydrolyse the β -1,4-glycosidic bonds in cellulose.

3.5.1 Hydrolysis of Spent Mushroom Sawdust Substrate, Delignified Spent Mushroom Sawdust Substrate and Cellulose by Perchloric Acid

100g of dry DSMSS obtained from section 3.5 was added portion by portion into 200 cm^3 of 70% (v/v) perchloric acid with continuous stirring. The mixture was then placed in a water bath at 80°C with constant stirring for 10 minutes (Camacho *et al.*, 1996;

Grohmann *et al.*, 1984). 200 cm³ of distilled water were added to dilute the hydrolysate. The residue was filtered through a sintered glass crucible (P2). 100g of ice was added into the acidic filtrate and then followed by neutralization with chilled 10M KOH (aq) with constant stirring. Ice was constantly added during the neutralisation in order to control the temperature of the reacting mixture thus minimizing the degradation of sugars. Upon complete neutralization, potassium perchlorate precipitated and it was filtered off by a sintered glass crucible. The filtrate obtained was a dark brown sugary solution.

The above procedures were repeated for SMSS and cellulose and the yield of glucose and xylose were compared. Due to the reactive nature of perchloric acid, great care has to be taken to ensure that perchloric acid used does not exceed 70% concentration in view of its reputation to explode unpredictably when the more concentrated forms get into contact with cellulosic materials. The proper handling of perchloric acid is rigorously adhered to (Material Safety Data Sheet (MSDS)-Perchloric acid).

Theoretically, 100g of cellulose can yield 110g of glucose due to the addition of water to the β-1,4-glycosidic bond (Jakobsson, 2002). The percent yield of glucose can be calculated from the following equation:-

$$\text{percent yield of glucose, \%} = \frac{\text{mass of glucose}}{\text{mass of cellulose}} \times \frac{100}{110} \times 100$$

(Equation 3.5)

The mass of xylose produced can be calculated by the following equation:-

$$\text{mass of xylose, g} = \frac{\% \text{ of xylose in hydrolysate (w/v)}}{100} \times \text{volume of hydrolysate}$$

(Equation 3.6)

3.5.2 Analysing on the Constituents of the Hydrolysates

Glucose is the most important sugar that can be converted into ethanol in fermentation. The amount of glucose produced in the hydrolysis is crucial as it directly determines the yield of ethanol in fermentation. Currently, the methods used to determine the glucose content are high performance liquid chromatography (HPLC), Fehling's test and dinitrosalicylic acid test. Among all the methods, HPLC is the most accurate one, as it is designed to determine the concentration of merely glucose, rather than concentration of all reducing sugars. In Fehling's or dinitrosalicylic acid test, an oxidising agent is used to oxidise all the reducing sugars in a sample, the amount of reducing sugar can be calculated by a back titration of the remaining oxidising agent. The latter methods are more convenient and cost efficient but less accurate.

Methanol is a found in a trace amount in the ethanol produced from lignocellulosic biomass. In order to find out the source of the methanol, whether it is a product of hydrolysis or fermentation, we analysed the hydrolysate by GC to detect the presence of methanol.

3.5.2.1 Analysis of Glucose and Xylose by High Performance Liquid Chromatography

The solution obtained after the hydrolysis (Section 3.6.1) was analysed by High Performance Liquid Chromatography (HPLC) in order to determine its glucose and xylose content (Foyle *et al.*, 2007; Vidal-Valverde *et al.*, 1984). HPLC is a separating technique in which the mobile phase is a liquid. A HPLC system consists of solvent reservoirs

containing the mobile phase, a high-pressure pump for pumping the mobile phase through a column, an injection device to introduce the sample, a column where the stationary phase is packed, a detector that generates electrical signals and a data system that displays the chromatogram (Hung *et al.*, 1988).

HPLC is a popular chromatographic separation method as it can separate complex mixtures in a very short time. Due to the high pressure of the HPLC system, a loop injector is used to inject sample to the flowing mobile phase. It is a two-position valve that directs the flow of the mobile phase in different paths in the load position and the inject position. In the inject position, the sample is carried by the flowing mobile phase into the column; in the load position, the mobile phase is directed to flow into the column while the sample is loaded to the sample loop at the atmospheric pressure.

In HPLC, components can be separated by different types of columns such as normal phase column, reverse phase column, adsorption column, ion exchange column and size exclusion column. Normal phase or reverse phase column separates components by partition, it utilizes a liquid stationary phase that is chemically bonded to the solid support in the column. In a normal phase column, the stationary phase is polar and the mobile phase is non polar and vice versa for the reverse phase column. In the adsorption column, the solid stationary phase is exposed to the mobile phase directly for instance, the polar silica or the aluminium oxide. Ion exchange stationary phase consists of solid charged resin particles at which ions are exchanged with the mobile phase. Size exclusion column separates mixtures by different pore sizes on the surface of porous polymeric particles.

The common detectors used in HPLC are UV absorption detector, diode array detector, fluorescence detector, refractive index detector electrochemical detector and

amperometric detector. In this study, the detector used in HPLC to detect the simple sugars was refractive index detector. The refractive index of a liquid is defined as the ratio of the speed of light in the vacuum relative to the speed of light in the liquid. The refractive index of the mobile phase acts as a reference. A light beam is passed through two cells; one containing pure mobile phase, another one containing effluent. A photosensitive surface detects the changes of the light focused at different points and causing the recorder pen to deflect and give a peak on the chromatogram (Snyder *et al.*, 1997; Snyder *et al.*, 2010; Dong and Wiley, 2006).

In this study, the hydrolysates obtained from DSMSS, SMSS and cellulose (Section 3.6.1) were analysed by HPLC (Perkin Elmer Series 200) with a refractive index detector (Agilent). Water and acetonitrile in a ratio of 1:3 served as a mobile phase with a flow rate of 1.4ml/min at 30°C. The column used was Zorbax Carbohydrate Analysis 4.6mm IDX 150mm (5µm) (Tomás-Pejó *et al.*, 2009).

3.5.2.2 Analysis of Glucose by Glucometer

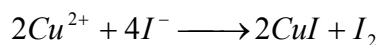
Glucometer is a medical device used to determine the concentration of glucose in blood. A glucometer is used together with a test strip that contains glucose oxidase. Glucose oxidase oxidises glucose to gluconolactone. A mediator reagent such as ferricyanide ion is used to reoxidise the enzyme. The mediator reagent will then be oxidised at an electrode, and hence generates an electric current. The charge produced at the electrode is proportional to the concentration of glucose in the blood sample.

In this method, the concentration of the glucose in the hydrolysate was determined by placing a drop of hydrolysate on the test strip of a glucometer. The glucometer used is Ascensia Elite glucometer developed by Bayer. The reading shown on the glucometer was recorded.

3.5.2.3 Analysis of Reducing Sugar by Fehling's Test

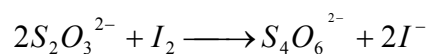
In Fehling's test, Cu^{2+} ions in the Fehling's reagent are reduced by the reducing sugars such as glucose in the hydrolysate to Cu^+ ions in an alkaline condition. The concentration of the unreacted Cu^{2+} ions can be determined by a back titration method. Therefore, the content of the reducing sugar can be calculated.

This can be done by reacting the unreacted Cu(II) ions with KI and the following reaction occurred:-



(Equation 3.7)

The brown iodine solution was then titrated with potassium thiosulphate solution as followed:



(Equation 3.8)

34.64g of reagent grade copper sulphate pentahydrate was dissolved in 300ml distilled water and diluted to 500ml to prepare Fehling's solution 'A'. 173g of reagent grade potassium sodium tartrate tetrahydrate and 50g reagent grade sodium hydroxide was dissolved in 300ml distilled water and diluted to 500 cm^3 to prepare Fehling's solution 'B'. The solution was left to stand overnight. Any precipitate formed was filtered through glass wool prior for use. A 30% potassium iodide solution was prepared by dissolving 150g of reagent grade potassium iodide in distilled water and diluted to 500 cm^3 volume. The prepared solution was kept in a dark cupboard to avoid exposure to sunlight. A 28% sulphuric acid solution was prepared by adding 145 cm^3 of 96% concentrated sulphuric acid to 400ml of distilled water with stirring and cooled to room temperature and diluted to 500 cm^3 . 0.1M sodium thiosulphate solution was prepared by dissolving 124.09g of sodium thiosulphate pentahydrate in 300ml of distilled water and diluted to 500 cm^3 .

10 cm^3 of solution 'A', 10 cm^3 of solution 'B' and 5 cm^3 of the DSMSS hydrolysate from section 3.6.1 were transferred into a 250 cm^3 conical flask. A few anti-bumping granules were added into the solution and heated in a water bath at 90°C for 15mins. 10ml of potassium iodide solution were added into the conical flask to liberate I_2 and titrated the liberated I_2 with sodium thiosulphate solution immediately. The percentage of reducing sugar can be determined by comparing the titre volume with the calibration curve. The experiment was done in three replicates.

3.5.2.4 Determination of Methanol Content in Hydrolysates by Gas Chromatography

The hydrolysates of DSMSS, SMSS and cellulose obtained from section 3.6.1 were analysed by gas chromatography (GC). The hydrolysates were distilled after the hydrolysis and the distillates were analysed by gas chromatography (Shimadzu) with a flame ionization detector. The column used was 80 Porapak Supelco 1230-8 and nitrogen was used as the mobile phase at a temperature of 170°C and a pressure of 100kPa. The mass of methanol can be calculated according to Equation:

$$\text{mass of methanol, g} = \frac{\% \text{ of methanol in distillate (w/v)}}{100} \times \text{volume of distillate (cm}^3\text{)}$$

(Equation 3.9)

3.6 Production of Ethanol by Fermentation of Hydrolysates

Fermentation is a process that produces ethanol from glucose. The yeast, *Saccharomyces cerevisiae* produces zymase enzyme to catalyse the conversion of glucose obtained from the hydrolysis, into ethanol in an anaerobic condition according to Equation 2.2. This process is similar to the production of beer or wine, however the bioethanol can not be consumed because it contains many other toxic side-products such as methanol.

3.6.1 Fermentation of Hydrolysates of Spent Mushroom Sawdust Substrates, Delignified Spent Mushroom Sawdust Substrate and Cellulose by *Saccharomyces cerevisiae*

Hydrolysates of SMSS, DSMSS and cellulose (section 3.6.1) were fermented at room temperature ($\pm 30^{\circ}\text{C}$) by using 1% (w/v) of *Saccharomyces cerevisiae*, a common baker's yeast. Ethanol produced by yeast can be separated from the solution by distillation. The distillation was carried out by using rotary evaporator at 70°C until the ethanol content of the distillate dropped to zero. The percentage of ethanol in the distillate was determined by GC and by hand-held alcohol refractometer. GC provides more detailed information on the composition of the distillate and enables the quantification of side products such as methanol.

The percent yield of ethanol can be calculated by the following equation:-

$$\text{percent yield of ethanol, \%} = \frac{\text{mass of ethanol} \times 100 \times 100}{\text{mass of cellulose} \times 110 \times 0.51}$$

(Equation 3.10)

3.6.2 Determination of Alcohol Content

Ethanol was obtained from the distillation of the fermented hydrolysates. The concentration of the ethanol solution obtained after the first round of distillation is normally less than 5%. The concentration of ethanol can be determined accurately by GC, alternatively, it can also be determined by hand-held refractometer that is more convenient to operate.

3.6.2.1 Analysis of Ethanol and Methanol by Gas Chromatography

Gas chromatography (GC) is a separating method to separate gas mixtures or volatile liquids. In GC, the mobile phase is an inert gas such as helium or nitrogen and the stationary phase is either a liquid that is adsorbed to a solid support or a solid. Compressed gas cylinder supplies the carrier gas which acts as a mobile phase through a column that contains the stationary phase via a flow controller. The analyte is injected into the flowing carrier gas ahead of the column at the injection port. The injection port is heated to a high temperature to flash vaporize a very small liquid sample (0.1-3 μ L) rapidly so that it can be carried by the carrier gas through the column.

The column used in GC is usually long, unlike other types of chromatography. The column is coiled into the column oven and kept at an elevated temperature to prevent the condensation of the sample. For liquid stationary phase, the components are separated by partition, the component with high vapor pressure and low solubility in the stationary phase will have short retention time. For solid stationary phase, the components are separated by adsorption, the component that has strong interaction with the stationary phase will have long retention time. Finally, components with different retention times will elute at the end of the column, a detector is required to generate an electrical signal that is fed into the data system, and a chromatogram will be obtained. Some of the common detectors are thermal conductivity detector, flame ionization detector and electron capture detector. In this study, flame ionization detector was used. In this type of detection, the effluent from the column is swept into a hydrogen flame, during the process, the effluent molecules will be fragmented and the resulting positive ions will be attracted to the negative collector while the electron will be attracted to the positive burner head. The

collector and the burner head are part of the electrical circuit, when this process occurs, there is a change in current, this current change is then amplified and seen as a peak on the chromatogram (Grob *et al.*, 1985; McNair *et al.*, 1969; Skoog and West, 1985).

In this research, the ethanol and methanol distilled from the solution after fermentation can be analysed by gas chromatography (Shimadzu) with a flame ionization detector. The column used was 80 Porapak Supelco 1230-8 and nitrogen was used as the mobile phase at a temperature of 170°C and a pressure of 100kPa. The results obtained are compared with that obtained from a hand held refractometer (Tomás-Pejó *et al.*, 2009; Hari Krishna *et al.*, 2001; Sukumaran *et al.*, 2009).

3.6.2.2 Analysis of Ethanol by Hand-held Refractometer

A refractometer measures the index of refraction of a liquid sample. An ethanol refractometer determines the concentration of an ethanol solution by comparing the solution's refractive index with a standard curve. In this experiment, the percentage of ethanol in the distillate can be determined by a hand-held refractometer, ATAGO (AL-21 α), Japan. A drop of ethanol solution was placed onto the sample space, and the reading shown in the refractometer was recorded.

3.6.3 Fermentation of Standard Xylose

Fermentation of pure xylose solution was carried out to find out the capability of the *Saccharomyces cerevisiae* in fermenting xylose. If *Saccharomyces cerevisiae* is unable to ferment xylose, it means that the methanol is not formed from the fermentation of

xylose. 20% (w/v) xylose solution was prepared by dissolving 20g of pure xylose in distilled water to form a 100 cm^3 solution. 1g of *Saccharomyces cerevisiae* was added into the xylose solution and left for fermentation for five days at room temperature (30°C). The product of fermentation was distilled and the distillate was analysed by GC.

3.6.4 Variation of Ethanol Yield from Fermentation against Time of Fermentation

In order to find out the optimum fermentation time (day), the mass of ethanol produced from 100 cm^3 hydrolysate (0.31-1.61% glucose) against time was studied. 50g of SMSS were hydrolysed by 100 cm^3 of 70% perchloric acid at 80°C with constant stirring for 10 mins. 100 cm^3 of distilled water were added to the hydrolysate and the mixture was neutralized by 10M KOH solution. The potassium perchlorate formed was separated by filtration. Distilled water was added to the sugar solution to obtain a total volume of 500 cm^3 . The sugar solution was divided into five portions, each portion was 100 cm^3 into five 100 cm^3 conical flasks. 1g of *saccharomyces cerevisiae* was added into each conical flask and the sugar solutions were left for fermentation. Each day, the fermented solution in each conical flask was distilled and the distillate was analysed by GC to determine its concentration of ethanol, hence the mass of ethanol produced against time was obtained.