CHAPTER FOUR

RESULTS AND DISCUSSION

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

Water content in SMSS and GS was determined by the drying method as shown in section 3.4.1. Phosphorus pentoxide was used as a drying agent. The percentage of water in SMSS is 65.6 ± 0.9% whereas in GS is 55.64 ± 0.09%. The results showed that SMSS has a higher percentage of water and hence a less close-packed structure compared to GS.

TGA was carried out on lignin, hemicellulose, cellulose, GS, SMSS and DSMSS in order to study their structures and characteristics (Section 3.4.2). At the temperature where the weight change is most apparent is considered as the decomposition temperature.

Table 4.1: Decomposition temperatures of GS, SMSS and DSMSS

Substrates	Decompostition Temperature/ °C
GS	361
SMSS	358
DSMSS	339

The TGA was performed by Perkin-Elmer Pyris Diamond thermogravimetric analyzer.

The following figures show the decomposition temperatures of lignin, hemicellulose, cellulose, GS, SMSS and DSMSS. From the results obtained, DSMSS has the lowest decomposition temperature, and it shows that it has the most loosened structure.

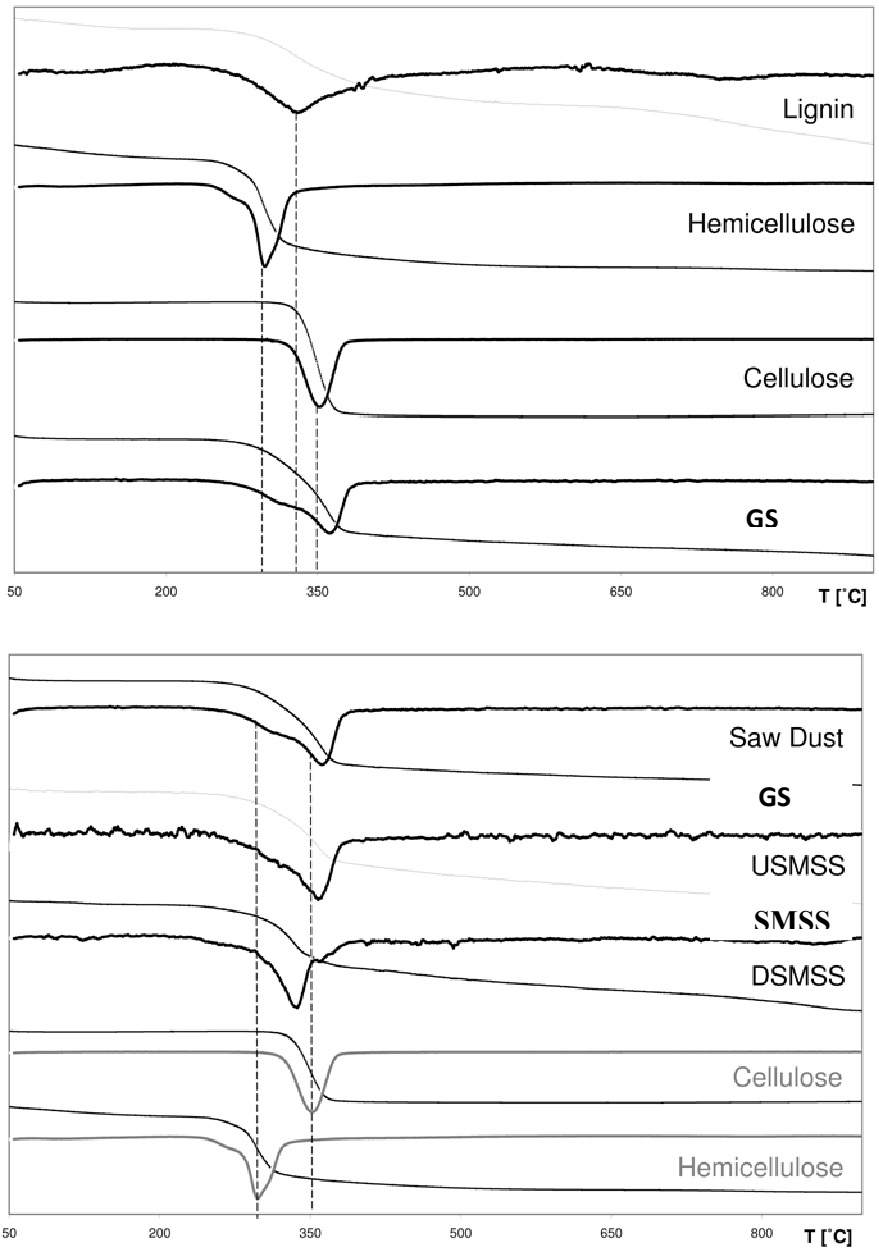


Figure 4.1: Decomposition of lignin, hemicellulose, cellulose, GS, SMSS and DSMSS

The green sawdust which was obtained from rubber wood (*Hevea brasiliensis*) was used as a substrate bed for growing mushrooms. Rice bran and water were mixed to the green sawdust and later steamed. The resulting mixture serves as an excellent bed for growing mushrooms. After a few rounds of harvest, the used sawdust is called spent mushroom sawdust substrate (SMSS). According to Minowa, rubber wood consists of about 46 % cellulose, 16 % hemicellulose and 21 % lignin (Minowa *et al*., 1998).

In this study, thermogravimetric analysis was done to determine the thermostability and decomposition pattern of different sawdust substrates and pure cellulose. This technique measures the weight change of sawdust as a function of temperature. From the results shown in section 4.2 and 4.3, GS has the highest decomposition temperature, at 361ºC, followed by SMSS at 358ºC and lastly by DSMSS at 339ºC.

The difference between the decomposition temperatures of GS and SMSS indicates that the glycosidic linkages between cellulose, hemicelluloses and lignin have been altered. It is a well known fact that polysaccharides in wood fuction as structural components and protective substances, in the form of fibres and matrices. The structure of these carbohydrate altered by the growth of mushrooms, which through their mycelia will have released a variety hydrolytic and oxidative enzymes that weakens the integral structure of GS (Imamura *et al*., 1984). It is presumed that the cellulose structure will have also been hydrolysed by mushroom mycelia to give oligosaccharides. Thus GS had a stronger lignocellulosic structure that prevented the easy extraction of lignin.

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

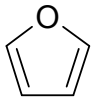
The dry mass of SMSS and GS is determined after pretreatment by the method shown in section 3.5. The sawdust substrate was soaked in 2M KOH for two days and washed with distilled water, dilute HCl and distilled water again and then dried. The percentage of the dry mass of SMSS is 51.2 ± 0.8% whereas the GS is 89.1 ± 0.7%. The dry mass of SMSS obtained after the pretreatment is close to the cellulose content in the rubber wood determined previously which is 46% (Tomoaki et al., 1998), the slight difference might be due to the incomplete removal of hemicellulose in SMSS by pretreatment. From the result, the dry mass of GS obtained is 89.17%, it shows that pretreatment does not completely remove all the lignin and hemicellulose in the GS that has a strong hemicelluloses and lignin matrix. Therefore the dry mass of GS is extraordinary high.

The pretreatment on GS led to a 10% reduction in mass whereas the pretreatment of SMSS led to a 50% reduction. This result shows that the lignin and hemicelluloses in the SMSS can be removed more easily than that of GS. The incomplete extraction is due to limited accessibility of the pretreatment agent as the lignin lies deep inside the lignocellulosic composite structure. After several rounds of mushroom harvest, the lignocellulosic structure is greatly weakened and becomes more easily digested (Imamura *et al*. 1984). This corresponds with a significantly increased water-content in SMSS, which appears more sponge-like than compact in structure. With a 50 % weight loss, the reduction of dry mass upon alkali treatment for SMSS exceeds the lignin content of the

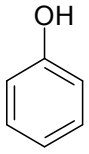
rubber wood by far. Besides a change in composition due to the degradation, it is assumed that some of the cellulose and hemicellulose materials dissolve due to the reduction in molecular size of the cellulose chain is initiated by the mushroom.

Mushrooms feed on sawdust's cellulose and hemicellulose by using enzymes such as cellulase and peroxidase that are secreted through their mycelia onto the lignocellulosic mass. The process causes the breakdown of cellulose to oligosaccharides, disaccharide and monosaccharides such as glucose that are eventually absorbed by the mushroom. The effect of the mushroom growth on the biomaterial can easily be seen in the results of the alkali based extraction of lignin from SMSS and its starting material, GS. SMSS has a lower thermostability than GS as the mycelium of mushroom secreted enzymes that loosen up the packed lignocellulosic structure. Pretreatment further reduces the crystallinity in DSMSS and led to a greater reduction in mass compared to that of SMSS.

In the pretreatment process, lignin can be removed by solubilisation in NaOH (Balat, 2011). However, compounds formed in the alkali pretreatment such as phenols, furans, carboxylic acids and inorganic salts might inhibit downstream process including fermentation (McIntosch and Vancov, 2010). It was also recorded that alkali pretreatment can cause a loss of polysaccharides due to hydrolytic reaction and decompostion reaction (Hendriks and Zeeman, 2009) leading to a lower yield for DSMSS compared to SMSS.



furan



phenol

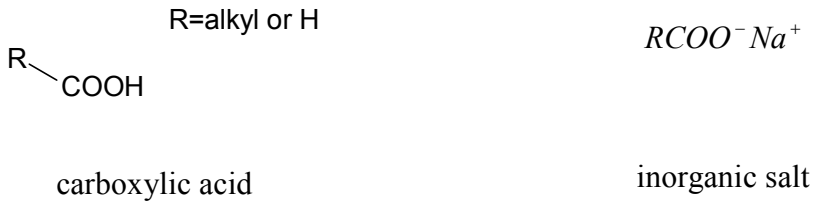


Figure 4.2: By-products produced in pretreatment

The pretreatment process is labelled as the most important process in the enzymatic hydrolysis and it is to increase the accessibility of enzymes towards the lignocellulosic structure. In this research, we found that pretreatment prior to acid hyrolysis is not necessary as the yield of SMSS is higher that that of the DSMSS and that lignin appears to be non toxic to the yeast, *Saccharomyces cerevisiae.* In most cases of enzymatic hydrolysis, pretreatment is crucial because it reduces the crysallinity of lignocellulosic material and increase the accessibility of enzyme towards cellulose. In our study, perchloric acid is capable to break down the lignocellulosic structure and then hydrolyses the cellulose to yield glucose without the need of pretreatment. Moreover, the lignocellulosic structure had been broken down partially by the mycelia of mushrooms prior to the hydrolysis, hence facilitating hydrolysis. Results showed that DSMSS gave a lower yield of ethanol compared to SMSS, this might be due to the conditions of alkali pretreatment that favor the degradation of glucose and the production of by-products such as furfural, organic acids, and phenols that are produced during the pretreatment might act as inhibitors during fermentation (Rogalinski *et al.*, 2008).

4.3 Optimization of Hydrolysis Parameters

In order to increase the yield of glucose, the conditions of hydrolysis need to be optimized. The two important parameters in the hydrolysis are the concentration of perchloric acid and the hydrolysis time. The concentration of perchloric acid must not be be too low to break down the lignocellulosic biomass. Anhydrous perchloric acid should not be used due to the risk of its explosive nature.

4.3.1 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 hydrolysis, the hydrolysis of 50g of DSMSS was carried out as shown in method 3.13. The following table shows the percentage of mass reduction of SMSS by 70%, 60% and 50% of perchloric acid. Results showed that 70% perchloric acid is the most efficient concentration for hydrolysis.

Table 4.2: Percentage of mass reduction of DSMSS by different concentrations of perchloric acid

% of Perchloric Acid	Percentage of Mass Reduction/ %	Percent Yield of Glucose/ %
70	77.1 $\pm$ 0.2	13.9 ± 0.2
60	59.5 $\pm$ 0.3	8.0 ± 0.8
50	19.5 $\pm$ 0.3	1.8 ± 0.2

50g of SMSS was hydrolysed by different concentrations of perchloric acid. The mass of the residue obtained after hydrolysis was recorded. The percentage of mass reduction shows the efficiency of hydrolysis. The greater the reduction in mass, the higher the efficiency of hydrolysis.

4.3.2 Hydrolysis of Spent Mushroom Sawdust Substrate with Different Hydrolysis Times

In order to find out the effect of hydrolysis times on the yield of glucose, DSMSS samples were hydrolysed by 70% perchloric acid with different hydrolysis times as shown in section 3.14. The following table shows the yield of glucose produced after 60 mins, 30 mins and 10 mins hydrolysis. Results show that 10 mins hydrolysis gave a highest yield of glucose.

Table 4.3: Mass of glucose produced from 50g SMSS with different hydrolysis time

Hydrolysis Time/ min	Mass of Glucose/ g
60	2.6 ± 0.7
30	4.7 ± 0.2
10	8.1 ± 0.6

50g of SMSS was digested with 70% perchloric acid for different period of time. The sugar obtained was analysed by HPLC. The mass of glucose produced was calculated.

4.4 Hydrolysis at Optimum Conditions

After the optimization of hydrolysis parameters, we found out that the optimum conditions of hydrolysis are 70% perchloric acid and 10 minute hydrolysis time. SMSS, DSMSS and cellulose are hydrolysed under these conditions.

4.4.1 Analysis on the Constituents of the Hydrolysates

Generally, the main constituents of the hydrolysates are glucose and xylose. The presence of xylose is due to the presence of hemicellulose. In order to find out the most accurate and time- efficient analysis method, we compare the results of analysis for three possible methods, namely HPLC, glucometer and Fehling's test.

4.4.1.1 Analysis of Glucose and Xylose by High Performance Liquid Chromatography

The percent yield of glucose from DSMSS, SMSS and cellulose can be calculated after hydrolysis (Equation 3.3). The following table shows the results obtained from three of the substrates. The percent yield of SMSS and DSMSS have large standard deviations, this might be due to different degrees of sugar degradation that occur during hydrolysis.

Table 4.4: Percent yield of glucose and the mass of xylose obtained from DSMSS, SMSS and cellulose

Substrates	Percent Yield of Glucose, %	Mass of Xylose
Cellulose	24.8 ± 3.7	Not detected
SMSS	13.9 ± 1.2	3.9 ± 0.7
DSMSS	8.0 ± 0.7	1 ± 1

DSMSS, SMSS and cellulose were hydrolysed by 70% aqueous perchloric acid. The sugary solution was separated from the unhydrolysed residue by filtration. The perchloric acid remained in the sugary solution was removed by neutralization with 10M KOH. The product of neutralization, potassium perchlorate was separated by filtration. Xylose was found in the hydrolysate of SMSS and DSMSS due to the presence of hemicellulose. DSMSS has lower xylose content compared to SMSS due to the partial removal of hemicellulose.

4.4.1.2 Anaysis of Glucose by Glucometer

100g of cellulose was hydrolysed according to section 3.6.1, the hydrolysates obtained were analysed by a glucometer for the percentage of glucose. Results obtained by the two methods, HPLC and glucometer were compared. Glucometer gave a much lower glucose content compared to HPLC. This is attributed to the lack of sensitivity of the glucometer for high concentration of glucose in the hydrolysate.

Table 4.5: Percentage of glucose in the hydrolysates of cellulose determined by HPLC and glucometer

Cellulose	HPLC	Glucometer
Replicate 1	6.98%	0.27%
Replicate 2	4.89%	0.30%
Replicate 3	6.21%	0.34%

100g of cellulose is hydrolysed by 70% perchloric acid. The hydrolysate is analysed by HPLC and glucometer to determine its glucose content. Glucometer gives an inaccurate result.

4.4.1.3 Analysis of Reducing Sugar by Fehling's Test

In order to estimate the glucose content in the hydrolysate samples by Fehling's test, a calibration curve is obtained by using standard glucose solutions with 0.5%, 1.0% and 1.5% glucose solutions. The followong table shows the percentage of glucose in the hydrolysates, the results are obtained by comparing the titre volumes of SMSS samples with the calibration curve. Results show that Fehling's test is less accurate compared to HPLC.

Table 4.6: Percentage of glucose determined by Fehling's test

DSMSS	HPLC	Fehling's Test
Replicate 1	0.41%	0.27%
Replicate 2	0.76%	0.50%
Replicate 3	1.32%	1.22%

The glucose content in DSMSS hydrolysate approximates the reducing sugar content as other reducing sugars are being removed in the pretreatment. 5ml of hydrolysate sample is added into 10ml of solution A and solution B. KI is added to convert the unreacted Cu^{2+} ions into I_2. The I_2 is then back-titrated with $Na_2S_2O_3$ solution.

4.4.1.4 Determination of Methanol Content in Hydrolysates by Gas Chromathography

In order to find out the origin of methanol in the ethanol produced from SMSS, the distillate obtained from the hydrolysates after the hydrolysis (section 3.6.1) were analysed by GC as shown in section 3.6.2.4. Results show that methanol originates from the hydrolysis.

Table 4.7: Methanol content in the hydrolysates of DSMSS, SMSS and cellulose

Substrates	Mass of methanol/ g
DSMSS	0.41 ± 0.03
SMSS	0.52 ± 0.03
Cellulose	Not detected

Methanol was found in DSMSS and SMSS but not cellulose. The methanol was originated from the hemicelluloses or lignin that found in the DSMSS and SMSS.

Theoretically, 100g of cellulose yield 110g of glucose according to the following equation:-

$$(C_6H_{10}O_5)_n + nH_2O \longrightarrow nC_6H_{12}O_6$$

(Equation 4.1)

The average glucose yield (determined by HPLC) from pure cellulose was 24.8%; from SMSS was 13.9%; and from DSMSS was 8.0%. These results suggested that DSMSS gave a lower yield of glucose and ethanol compared to that of SMSS. This showed that alkali pretreatment is not required in chemical hydrolysis of SMSS, as pretreatment increases the cost of bioethanol production as pretreatment is very costly and time consuming.

The glucose content in the hydrolysate was determined by HPLC with a time delay, resulting in an underestimation of glucose. This might be due to the degradation of the

glucose that occurred before the samples were analysed by HPLC. It was noticed that the determination of sugar content in SMSS and DSMSS were less accurate as the yield of ethanol was greater than 100% of the theoretical yield based on the glucose content determined by HPLC, we can therefore conclude that the glucose content was underestimated. In contrast, determination of glucose from hydrolysis of cellulose was more reliable and the yield of ethanol was below the theoretical yield based on the glucose content in the hydrolysate. The glucose content in SMSS and DSMSS were underestimated because the sawdust hydrolysate may contain by-products that might quicken the degradation of sugars. Glucose produced might be destroyed before the HPLC analysis was performed. However, we can predict the minimum amount of glucose produced in the hydrolysis from the amount of ethanol produced. Pure cellulose produced only glucose in the hydrolysis, the sample is less complex and less degradation had occurred.

In this study, the sugar content of DSMSS was also determined by Fehling's test. However, the results obtained were less than that determined by HPLC. This might be due to some sugars in the hydrolysate being oxidized by the side-products produced during hydrolysis and during heating in the Fehling's reaction thus causing less Cu^{2+} used. The advantages of using Fehling's test are that it is cheaper and more convenient. However, it is less accurate for the hydrolysate samples due to the presence of many side products. Like Fehling's test, the glucose content could not be properly determined by the glucometer as well. We have also chosen the glucometer for glucose determination because the glucose oxidase enzyme immobilised glucometer is specific to the presence of glucose and avoids the overestimation of glucose concentration that often accompany other

methods such as the Fehling's test and dinitrosalicylic acid method. However, a comparison of glucometer and HPLC results indicated a significant and systematic underestimation of the sugar content by the assays. The glucometer used in this experiment has a measuring range of between 20-600mg/dL (0.02-0.6%). The results obtained by the glucometer differed much from the results obtained by HPLC as the glucose percentage in the hydrolysate range from 1-6% which is out of the range that can be determined accurately by the glucometer.

The Figure 5.2 below shows the hydrolysis of cellulose by the action of perchloric acid to yield glucose.

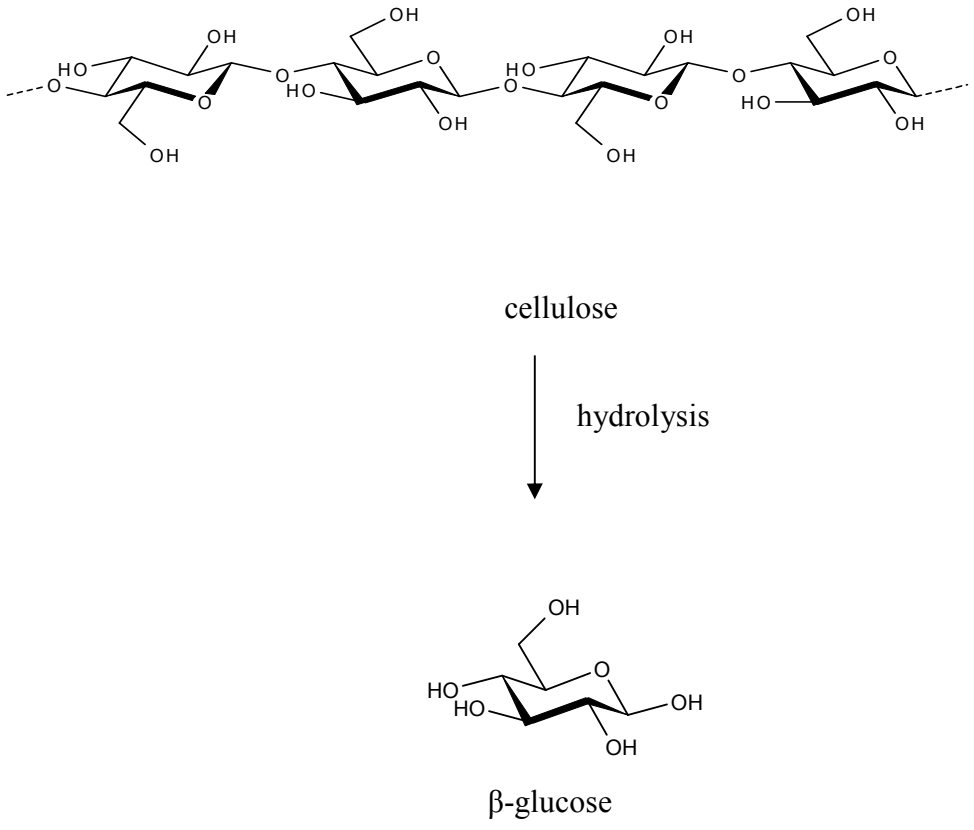


Figure 4.3: Hydrolysis of cellulose

The hydrolysis of SMSS was also done at different concentrations of perchloric acid (70%, 60%, 50%). The results showed that the efficiency of the hydrolysis decreased as the concentration of perchloric acid decreased. The sawdust became less soluble as the concentration of perchloric acid decreased. It was also found that the longer the hydrolysis time, the lower the glucose yield; this is due to the degradation of glucose being more severe when it is exposed to harsh acidic condition and high temperatures (hydrolysis is exothermic) for a longer time.

Methanol was found in the hydrolysate of SMSS and DSMSS samples, but not cellulose samples. Sawdust samples contain cellulose, hemicellulose and lignin. It was believed that the methanol did not come from hydrolysis of cellulose as cellulose samples did not give methanol. Methanol might be produced from hemicelluloses or pectin as a by-product in hydrolysis (Xiao *et al*., 2001). However, the percentage of methanol is ten times lower than the percentage of ethanol. A possible explanation for the methanol would be the hydrolysis of glucuronic methyl ester group in hemicelluloses or pectin that present in the sawdust (Bruice and Fife., 1962). Methanol is considered an unacceptable contamination for food stock due to its toxicity, in this context, methanol can contribute to the calorific content in biofuel, thus adding value rather than causing a problem. Figure 5.3 shows the possible mechanism of the formation of methanol.

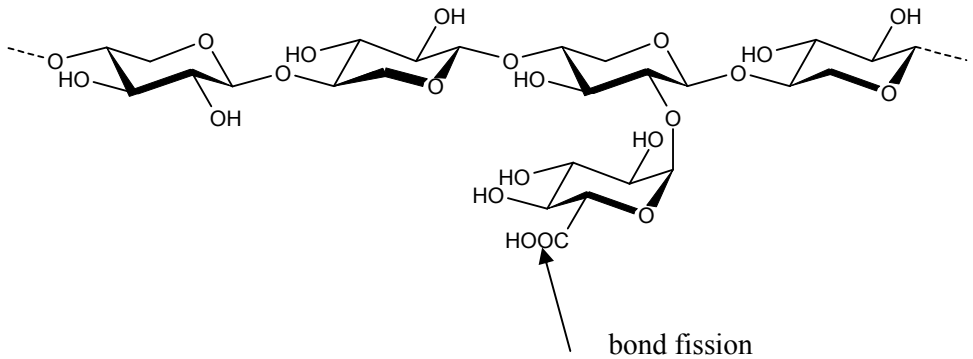


Figure 4.4: Production of Methanol from D-glucuronic acid methyl ester group in hemicellulose

Initial tests on pure cellulose as a reference indicate an efficiency of the perchloric acid hydrolysis of only 20-30 %, although most of the polymer visibly dissolved. This is partially due to the degradation of the sugar, which leads to a significant brown coloring of the reaction mixture. Besides, the viscosity of the reaction mixture suggests an incomplete hydrolysis based on the presence of soluble oligomeric and polymeric fragments in solution. Surprising results were obtained for DSMSS. The pretreatment of SMSS was expected to facilitate the hydrolysis of cellulose based on a spongelike structure. However, the glucose contents of DSMSS and SMSS hydrolyzates are practically the same. More surprisingly, the ethanol conversion is substantially lower than that for SMSS (Table 4.9). It clearly demonstrates that alkaline delignification of SMSS is not only uneconomical but counter-productive.

The percent yield of glucose from SMSS based on the cellulose content in SMSS was between 7.5-14.7%, determined by HPLC. These results represent a substantial

increase compared to the previously reported 5% glucose in rubber wood sawdust hydrolyzate (Tomimura, 1993). However, the glucose content for SMSS appeared to be significantly lower compared to cellulose.

The yield of ethanol based on the glucose content determined by HPLC is greater than the theoretical ethanol yield. In fact, the ethanol production exceeds the theoretical yield based on the measured glucose content considerably because the glucose content in the hydrolysate was underestimated. The reason may be due to degradation processes based on the unidentified contents of the mushroom waste, since the HPLC samples were measured with a time delay, whereas the fermentation was started immediately after hydrolysis. However, the overall yield of ethanol after fermentation matched the cellulose content perfectly (based on correction with respect to the lignin content).

The choice of SMSS as our substrate for the production of ethanol was prompted by the need to dispose several tons of the waste daily. Mushroom cultivation on rubber wood sawdust has reduced the cellulose content of SMSS, but much cellulose still remains to be converted to ethanol. Present approaches in the digestive conversion of cellulosic biomass take the form of chemical and biological processes, although the latter is presently far from having commercial applications. An example of the chemical hydrolysis is the Arkenol process (Farone and Cuzens, 1997) which uses approximately 70 % sulfuric acid to hydrolyze cellulose to sugars. The reaction requires heating to 100 ºC which results in charring of the sugars formed. Application of dilute sulfuric acid (0.5 to 15 %) helps to avoid the problem of charring, but the reaction still requires heating from 90 to 600 ºC, and thus constitutes a highly energy-intensive process. Sugars can be separated from the acid by chromatographic columns (Farone and Cuzens, 1997) but this method is time-

consuming and costly. The sulfuric acid is neutralized by lime water and yields a mushy precipitate of $CaSO_4.2H_2O$, which requires pressing in order to release the remaining sugar solution.

In another example, 70 % phosphoric acid was used to phosphorylate the cellulose to transform it into a starchy gel-containing sugar solution (Harvey, 1972). However, the separation of sugars from phosphoric acid remains difficult. With respect to separation problems for sulfuric and phosphoric acid indicated above, we searched for an alternative acid for the hydrolysis of cellulose. Because hydrochloric acid and nitric acid are not particularly efficient, we applied perchloric acid. Aqueous perchloric acid appears to be less dehydrating compared to concentrated sulfuric acid, as less charring of the sugars was observed. Another advantage is that the neutralization salt, $KClO_4$, is insoluble and can easily be separated from the sugar solution by filtration. The reaction can be represented by the following equation:-

$$KOH_{(aq)} + HClO_{4(aq)} \rightarrow KClO_{4(s)} + H_2O_{(l)}$$

(Equation 4.2)

In order to save costs and materials, $KClO_4$ can be converted back to perchloric acid by reaction with sulfuric acid and distillation of the aqueous solution under vacuum (Zinov'ev, 1963). The final product is K_2SO_4, a salt that could be used as a fertilizer. The reaction can be represented by the following equation:-

$$2KClO_4 + H_2SO_4 \rightarrow K_2SO_4 + 2HClO_4$$

(Equation 4.3)

Another possibility is to recycle the perchloric acid by using hydrolychloric acid according to the following equation:-

$$KClO + HCl \rightarrow KCl + HClO_4$$

(Equation 4.4)

4.5 Production of Bioethanol by Fermentation of Hydrolysates

The fermentation of hydrolysates was catalysed by enzyme produced by *Saccharomyces cerevisiae* as shown in section 3.7.1. The following table shows the percent yield of ethanol from 100g of SMSS, DSMSS and cellulose. The ethanol yield can be calculated according to Equation 3.7. Interesting results show that SMSS gave a higher percent yield of ethanol than that of DSMSS.

4.5.1 Determination of Alcohol Content

In this study, we compare the accuracy of two possible methods ofanalysis of alcohol, namely GC and hand-held refractometer. GC is capable of detecting methanol and gives a more acccurate result, however it is time consuming.

4.5.1.1 Analysis of Ethanol and Methanol by Gas Chromatography

Table 4.8: Percent yield of ethanol from 100g of cellulose, SMSS and DSMSS

Substrate	Percent yield of Ethanol/ %	Mass of Methanol/ g
Cellulose	13.5 ± 2.3	Not detected
SMSS	12.3 ± 2.3	0.4 ± 0.3
DSMSS	9.2 ± 4.1	0.3 ± 0.3

100g of cellulose, SMSS and DSMSS was hydrolysed by 70% perchloric acid. The sugar solution is neutralised and then fermented by *Saccharomyces cerevisiae.* Ethanol produced from the fermentation is distilled and analysed by GC. Percent yield of ethanol based on the mass of cellulose can be calculated.

4.5.1.2 Analysis of Ethanol by Hand-held Refractometer

The ethanol content produced after fermentation can be determined by GC and refractometer as shown in section 3.11. The following table shows the percentage of ethanol in the distillate of the fermentation product, determined by GC and refractometer. Results show that the percentage of ethanol determined by both methods are comparable.

Table 4.9: Percentage of ethanol in distillates determined by refractometer and GC

Cellulose	Refractometer	GC
1	4.2 %	2.63 %
2	17 %	15.65 %
3	4.2 %	4.97 %

Percentage of ethanol in the distillate obtained after fermentation is determined by refractometer and GC. The refractometer enables a quick determination of ethanol with comparable accuracy to GC.

4.5.2 Fermentation of Standard Xylose

Section 3.12.1 was carried out to study the effect of *Saccharomyces cerevisiae* on xylose. From the results obtained, *Saccharomyces cerevisiae* is incapable to ferment xylose, hence it can be concluded that methanol was not produced by the fermentation.

Table 4.10: Effect of *Saccharomyces cerevisiae* on xylose solutions

Xylose Solutions	Mass of Methanol Produced/ g
Replicate 1	Not detected
Replicate 2	Not detected
Replicate 3	Not detected

4.5.3 Variaiton of Ethanol Yield Against Time of Fermentation

In order to find out the variation of ethanol yield of fermentation against time, the ethanol produced daily by 50g of SMSS was recorded as shown in section 3.15. The following table shows the ethanol produced against time. Results show that the mass of ethanol increases with time and the increase slowed down from 4^{th} to 5^{th} day.

Table 4.11: Ethanol produced by hydrolysate against time

Day	Ethanol Produced/ g
1	1.16
2	1.32
3	1.50
4	1.55
5	1.57

50g of SMSS was hydrolysed by 70% aqueous perchloric acid. The acidic sugar solution was neutralised. The insoluble potassium perchlorate was filtered, and the total volume of the sugar solution was made up to $500\,cm^3$. The sugar solution was divided into five portions and fermented. Ethanol produced daily was recorded for five days.

The baker's yeast, *Saccharomyces cerevisiae*, was used for the fermentation, because it is commonly found and robust enough to withstand the myriad of solutes formed from the hydrolysis of SMSS by aqueous perchloric acid. After fermentation, ethanol yields between 44-65% based on the glucose content after the hydrolysis of pure cellulose were obtained. Around 35-56% of the glucose produced was not utilized in the fermentation, due to the yeast using up some of the glucose for their cell growth. and the inhibitory products in the hydrolysate might inhibit the activity of yeast. (Taylor, 2008)

During the fermentation, the production of ethanol will be inhibited by nutrient deficiency, high temperature, contamination, acetic acid and ethanol accumulation (Yu and Zhang, 2004). The presence of inhibitory products in the hydrolysate inhibits the activity of yeast (Taylor, 2008). The inhibitors produced during the acid hydrolysis that inhibit the production of ethanol from hydrolysate are furfural, 5-hydroxymethyl furfural (HMF), organic acids and phenol (Fig 2.1). In order to reduce the production of the inhibitors, we kept the hydrolysis temperature as low as possible by adding ice made from filtered water into the reaction mixture during hydrolysis. Acetic acid might also be produced during hydrolysis. Acetic acid can diffuse into yeast cells and affect the pH of the cell and hence affect the activity of yeast in fermentation. Glycolysis can be inhibited by furfural and 5-hydroxymethylfurfural (HMF) that are produced as a result of the oxidation of xylose and glucose respectively. In our experiment, we identified furfural by HPLC in our hydrolysate. In our experiment, caramelisation occurred during the experiment. This could be proven by the brown color of the hydrolysate as a result of browning of sugars since sugars are colourless.

The overall chemical conversion of the sugars to ethanol ranged between 12 % and 16% as shown in Appendix E. Refractometric and GC-determination of the ethanol content were in good agreement (Fig 4.10), indicating the former a quick way to evaluate the conversion process.

According to the results obtained in section 4.12, the mass of ethanol produced by the fermentation increased against time. The increase became less obvious from forth day to fifth day, this might be due to the decrease of glucose concentration and the inhibitory effect that has exceeded the tolerable level of yeast.

The yeast strain used in this experiment was not capable to ferment xylose. Xylose was found in the hydrolysate in the SMSS and GS samples only (Appendix J-iii). This might be due to hemicellulose that are present in SMSS samples. This result shows that most of the hemicellulose is removed in the pretreatment process. Hemicellulose is a natural polymer consisting of C-5 and C-6 monomers such as xylose, mannose, galactose, rhamnose, arabinose, mannuronic acid and galacturonic acid (Fig 1.7). In most cases, xylose is found in the largest quantity. In our samples, xylose was detected. In order to increase the yield of ethanol, a genetic modified yeast strain may be used to ferment xylose.

4.6 Conclusion

The impending rise in the price in crude oil and the projected depletion of fossil fuels reserve as well as peak oil scenario, have resulted in a global interest towards developing renewable sources of energy. Among all the renewable energy options, bioethanol derived from waste materials has been getting much attention, as it not only reduces the dependence on fossil fuel, but also reduces environmental pollution through the utilization of waste.

In this study, results show that SMSS can yield 12.3% ethanol (Table 4.9) after the hydrolysis and fermentation. Every day, a typically large mushroom cultivator disposes up to 10 tons of SMSS. Bioethanol production on a large scale not only reduces the cost of waste dispoal but also utilizes the spent sawdust to the maximum. Moreover, the SMSS has a loosened structure that facilitates the acid hydrolysis without prior pretreatment.

SMSS gave an ethanol yield of 12.3% (Table 4.9). The SMSS produced a higher percentage yield of ethanol compared to DSMSS. This finding helps to reduce the production cost of bioethanol as pretreatment by alkali does not give a higher yield of ethanol in this study. However, more research should be done to study the effect of other types of pretreatment on the percentage yield of ethanol from SMSS.

In this research, results showed that the optimum hydrolysis conditions for the production of ethanol from SMSS is by using 70% perchloric acid for 10 minutes at 80 ºC. These reaction conditions are intended to reduce the charring of sugar and to produce a maximum yield of glucose. One great advantage of using perchloric acid compared to sulphuric acid and phosphoric acid is that, the perchloric acid can be separated from sugar solution formed by a precipitation method. When potassium hydroxide is added into the sugar and acid mixture, an insoluble salt, potassium perchlorate will be precipitated out, and hence the perchloric acid is removed. This effect is less well known, as all potassium salts are generally regarded as soluble salts.

In order to reduce the cost of production, we suggest that the perchloric acid be recycled to from potassium perchlorate. Potassium perchlorate can react with sulphuric acid to from perchloric acid and potassium sulphate. The perchloric acid can be distilled under vacuum suction (Zinov'ev *et al*., 1963). The residue, potassium sulphate, is another useful substance which can be use as an agricultural fertilizer.

It was found that 22.4kg of SMSS can produce 1L of ethanol; 10tons of SMSS per day can be converted into 446.2L of ethanol. Our research is still on-going to increase the yield of ethanol.