Bioethanol from Spent Mushroom Sawdust Waste by Perchloric Acid: Easy Sugars Separation and Hydrolysis Without Charring

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ABSTRACT

Spent mushroom sawdust waste (SMSS), a waste product from a local, *Pleurotus* spp. mushroom-growing company, was appraised for its potential as a source for the production of ethanol. The successive growth of mushrooms weakens the ligno-cellulosic structure of the wood based substrate, thus making it more amenable to acid hydrolysis. Perchloric acid (70 %) was used to hydrolyze the cellulose into a glucose solution suitable for fermentation. This rather unusual catalyst bears the advantageous easy removal of neutralization products. The poor water soluble potassium perchlorate separates in crystalline form and enables the hydrolysis without external heating. A time course investigation indicated a hydrolysis treatment for 10 min at about 50°C as optimum conditions. Fermentation of the sugar solution with baker’s yeast, *Saccharomyces cerevisiae*, yielded up to 4 g ethanol/100 g ligno-cellulose. This translates to 40 L of biofuel per ton of dried substrate.

Keywords: aqueous perchloric acid, acid hydrolysis, spent mushroom sawdust substrate, glucose fermentation, ethanol

1. INTRODUCTION

Energy issues continue to beset the world with far-reaching impact on global warming, geopolitical imbalance and the current economic scenario. Although an enormous amount of effort has been made to wean away global addiction on fossil fuels through the development of alternative energy sources, breakthrough discoveries remain elusive [1]. Many national biofuel programs set ambitious targets to produce biofuels, but these are often based on food sources, such as sugar-cane, corn, cassava, palm and rapeseed oil, leading to the conversion of vast area of land for the planting of energy crops. Food-sourced biofuels are regarded as socially unacceptable in a world with a population of 7.0 billion people [2], many of whom have yet to be adequately fed. In order to address criticisms on food-for-fuel programs, much research has shifted to the use of cellulosic biomass for the production of ethanol [3]. The US government has mandated the use of 100 million gallons of cellulosic ethanol by 2010, a goal that is not likely to be met because there
is no unsubsidized commercially viable cellulosic ethanol plant in operation yet in 2012 [4, 5].

An obstacle to the exploitation of lignocellulosic biomass lies in the difficulty of hydrolyzing the cellulosic materials to sugars due to the molecular architecture of cellulose, hemicellulose and lignin that renders it intractable to mild chemical and enzymatic treatment [6, 7]. Waste cellulosic biomass is a worthy source for conversion to bioethanol, in particular spent mushroom sawdust substrate (SMSS), as many tons of the material require daily disposal. Recently, corncob-based spent mushroom substrate [8] and mushroom spent from rice straw [9] have been reported as important ligno-cellulosic biomass for bioethanol production and ethanol-based biorefinery after chemical pretreatment and then followed by enzymatic hydrolysis. The most common chemical media for hydrolysis are the mineral acids. Reactions were conducted at high temperatures and pressures [10, 11]. The neutralization of the acid yields salts that are difficult to separate and require expensive disposal procedures. Here we report an investigation on the use of aqueous perchloric acid for the hydrolysis [12] of SMSS. Perchloric acid has hitherto been avoided as a medium for the hydrolysis of cellulosic materials due to its reputation as being shock sensitive and unpredictably explosive [13]. However, aqueous perchloric acid has certain redeeming features, as it hydrolyzes cellulosic biomass with less destruction of the sugars formed, requires no heating and the neutralization salt product, potassium perchlorate, is easily separated.

2. MATERIALS AND METHODS

2.1 Materials

Spent mushroom sawdust substrate (SMSS) from *Pleurotus* spp. mushrooms was obtained from Ganofarm Sdn. Bhd., Tanjung Sepat, Selangor, Malaysia. The collected SMSS was already in ground form of mesh size 0.5 - 1.0 mm. Common baker’s yeast, *Saccharomyces cerevisiae*, of local food grade (Mauri-Pan, Malaysia) was applied for the fermentation. Standards such as glucose, cellulose, hemicellulose and lignin, and chemicals such as perchloric acid, potassium hydroxide and ethanol were purchased from Sigma-Aldrich. The HPLC system consisted of Perkin Elmer Series 200 pump with Agilent RI detector and Zorbax Carbohydrate Analysis column, 4.6mm IDX 150mm (5μm). Gas chromatography investigations applied a capillary column 80 Porapak Supelco 1230-8 on a Shimadzu GC with a flame ionization detector. Nitrogen was used as the mobile phase at a temperature of 170°C and a pressure of 100 kPa.

2.2 Determination of Water Content

20 g of wet sawdust or mushroom waste was pre-dried in a conventional oven at 45°C for four days before finally being subjected to high vacuum at 50°C overnight. Remaining water was trapped by phosphorous pentoxide. The loss in weight was recorded on an analytical balance in five replicates.

2.3 Delignification of Mushroom Waste

10 g wet SMSS was treated with 20 mL 2 M NaOH [14] and the mixture was left for two days at room temperature before being diluted with water. The remaining solid was filtered through a sintered glass (P3) and rinsed with dilute HCl and water to remove the remaining alkali. Drying of the resulting solid followed the procedure indicated above.

2.4 Thermogravimetric Analyses (TGA)

The TGA was performed on a Perkin-Elmer Pyris Diamond thermogravimetric analyzer. Nitrogen gas with a flow of 2 cm³ s⁻¹ was used as the atmosphere. The
samples, weighing between 8 mg and 12 mg, were heated from 50°C to 900°C at a scanning rate of 10°C min⁻¹. Analyzed samples included SMSS, delignified SMSS (DSMSS) and green sawdust (GS) [15], as well as commercial reference compounds, i.e. cellulose, hemicellulose and lignin.

2.5 Hydrolysis Time of Cellulose, GS and SMSS

10 mL of 70% HClO₄ (lower concentrations of HClO₄ was not able to hydrolyze at 50°C, as indicated by the failure of turning a white cellulose slurry into a clear golden solution) was slowly added with continuous stirring to 5 g of dry cellulose in a fume cupboard. The temperature of the mixture rose to 47°C and 20 mL of distilled water was added to the mixture after a variable time ranging from 1 to 16 min. A series of nine experiments in triplicates was performed for each of the substrates. Remaining biopolymer was determined after vacuum-drying at 60°C overnight.

2.6 Hydrolysis of Cellulose, SMSS and Delignified SMSS (DSMSS)

1.2 L of 70% HClO₄ was slowly added with continuous stirring to 600 g substrate, cellulose or dry mushroom waste in a fume cupboard. The temperature of the mixture rose to 50°C and a dark-colored solution was obtained. 1.2 L distilled water was added to dilute the hydrolyzate after 10 min. The acidic mixture was neutralized with ice-chilled 10 M KOH in a fume cupboard. Continuous stirring and cooling were maintained during the neutralization process. Upon neutralization, potassium perchlorate precipitated and was filtered off. The filtrate obtained was goldcolored in case of DSMSS and cellulose, but darker colored for SMSS due to lignin content. It was then concentrated to 900 ml. All experiments were performed in triple replicates. The concentration of glucose was determined by HPLC.

2.7 Fermentation of the Cellulosic Hydrolyzates

Hydrolyzates of cellulose, SMSS and DSMSS were fermented (not conducted for GS) at room temperature (~30°C) for a period of 7 days using 1 % (w/w) of Saccharomyces cerevisiae without any additional nutrients in a 1 L flask and a rubber balloon was attached as an indicator for the CO₂ released. The concentration of the ethanol produced was determined by GC after distillation.

3. RESULTS AND DISCUSSION

3.1 Substrate Comparison: SMSS vs. GS

SMSS was supplied by Ganofarm Sdn. Bhd., a local mushroom farm that cultivates edible oyster mushrooms such as Pleurotus spp. (both grey and white strains). The farm discards up to 10 tons of SMSS per day. The mushrooms feed on cellulose, hemicellulose and lignin from rubberwood sawdust, thereby weakening the compact ligno-cellulosic structure of the rubberwood. The effect of the mushroom growth on the biomaterial can easily be seen in the results of the alkali based extraction of lignin [14] from SMSS and its starting material, green sawdust (GS), as shown in Table 1. According to Tomoaki et al. [16], rubberwood (Hevea brasiliensis) consists of about 46 % cellulose, 16 % hemicellulose and 21 % lignin. However, the mass reduction upon alkali treatment of dried GS only accounts for 10 %, which is significantly below the reported lignin content. This incomplete extraction is due to limited accessibility of the lignin inside the ligno-cellulosic composite. After several rounds of mushroom harvest, the ligno-cellulosic structure is greatly
weakened and becomes more easily digested [17]. This corresponds with a significantly increased water-content for SMSS, which reflects more a sponge-like than a compact structure. With 50% weight loss, the reduction of dry mass upon alkali treatment for SMSS exceeds the lignin content of the base material by far. Besides a change in composition due to the degradation, it is assumed that some of the cellulose and hemicellulose material dissolve due to the reduction in molecular size initiated by the mushroom.

Table 1. Chemical composition of GS and SMSS based on alkali extraction.

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<th>GS</th>
<th>SMSS</th>
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<tr>
<td>[%]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellulose &amp; Hemicellulose</td>
<td>90 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>50 ± 1</td>
</tr>
<tr>
<td>Lignin</td>
<td>10 ± 1</td>
<td>50 ± 1</td>
</tr>
<tr>
<td>Water</td>
<td>55.5 ± 0.1</td>
<td>65.6 ± 0.9</td>
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<sup>a</sup> The high cellulose content of GS demonstrates resistance of the composite towards alkali treatment. All values are presented as mean ± standard error.

Figure 1 shows a comparison of the TGA spectra of sawdust (GS) and its contents, i.e. cellulose, hemicellulose and lignin. Unlike for cellulose and hemicellulose, the degradation temperature of lignin is not sharp. A minimum of the broad peak for the derivative of GS cannot be easily located due to the overlay of several components. The peaks reflecting cellulose and hemicellulose are shifted slightly to a higher temperature. This reflects a stabilization due to the ligno-cellulosic composite in GS.

**Figure 1.** TGA spectra of sawdust and its major components; in order to demonstrate differences more clearly, the derivatives are plotted on top of the spectra.
The TGA spectra in Figure 2 demonstrate the effect of mushroom harvest and delignification. The spectrum of SMSS resembles the one of GS. However, the peak for the cellulose is slightly shifted towards lower temperature, thus approaching the degradation temperature of pure cellulose. It reflects the weakening of the lignocellulosic composite. The removal of lignin by alkaline treatment leads to a sharper profile in DSMSS and reduces the thermostability significantly. The shift towards a lower temperature compared to pure cellulose might be due to a reduced molecular weight. This suggests a partial degradation of cellulosic material.

![Figure 2. Comparison of TGA spectra and respective derivatives for sawdust (GS) and mushroom treated sawdust wastes, as well as for the main components of the material.](image)

The conversion of cellulosic biomass to ethanol often applies an alkaline extraction [14] to remove lignin. This process, aiming to protect expensive enzymes utilized for the hydrolysis of cellulose, is costly. Alternatively, a treatment of the ligno-cellulosic substrate with mushrooms, e.g. *Shitake* or the Chinese black forest mushroom *Entinus edodes*, can remove up to 65% of the lignin content [17]. However, in case of a chemical, acid catalyzed hydrolysis neither of these pre-treatments is required. Moreover, common baker’s yeast, *Saccharomyces cerevisiae*, appears to be moderately affected by lignin contents, so that even the subsequent fermentation can be operated without additional purification steps.

3.2 Optimum Hydrolysis Time of Cellulose, GS and SMSS

In order to determine the optimum time for the hydrolysis treatment of cellulose, a time course investigation was performed. Figure 3 shows the result for pure cellulose. The reaction follows an initial linear correlation of the logarithmic substrate amount against the time. The deviation from a linear trend for reaction times above 6-7 min originates from degradation of cellulose due to side reactions, leading to carbonized insoluble material, which affect the determination of remaining starting material.
A comparison of the hydrolysis of cellulose, GS and SMSS is shown in Figure 4. All curves indicate practically complete conversion within 10 min. Both GS and SMSS reach the plateau earlier than pure cellulose. The curve for cellulose indicates carbonation in the range of 13 %, while the higher leftover material for GS and SMSS may reflect additional non-soluble products from lignin. GS exhibits with about 35 % the by far highest content of solid waste. This can be related to incomplete hydrolysis of cellulose, due to the compact ligno-cellulosic structure, which resists penetration of the acidic catalyst.

Figure 4. The weight of remaining substrate versus time plot for the pure cellulose, GS and SMSS.
3.3 Hydrolysis of SMSS and Fermentation

An initial test on pure cellulose as a reference indicates an efficiency of the perchloric acid hydrolysis of up to $22 \pm 2$ g glucose/100 g cellulose. This refers to a conversion yield of only 24% although most of the polymer visibly dissolved. The degradation processes of the sugar led to a significant coloring of the reaction mixture. Besides, the viscosity of the reaction mixture suggests incomplete hydrolysis based on the presence of soluble oligomer and polymer fragments in solution. Common baker’s yeast, *Saccharomyces cerevisiae*, was applied for the fermentation, because of easy access and its robustness to withstand the myriad of solutes formed from the hydrolysis of SMSS by aqueous perchloric acid. In this study, 0.5 - 1 g of a locally available commercial dry baker’s yeast *Saccharomyces cerevisiae*, was used for 100 g of sugar hydrolysate (cal. 0.5 - 1%) with the cost of between USD 0.01 ~ 0.02 (RM 0.03 ~ 0.06) which is negligible to affect on the ethanol production cost to ensure that this technology is practical. After fermentation for 48 hours, as suggested for reducing sugars [14], an ethanol yield of 8 g per 100 g initial cellulose was obtained, referring to an overall conversion yield of 14%, according to the process sketched in Figure 5.

![Chemical reaction scheme for the conversion of cellulose into ethanol.](image)

For SMSS an overall conversion of 5 g glucose/100 g ligno-cellulose was found and as expected a higher glucose content of 9 g glucose/100 g substrate was obtained in DSMSS indicating that delignification process is necessary to double the glucose yield, as shown in Table 2. GC determination indicated an ethanol-content after fermentation of about 4 g/100 g substrate, thereby exceeding the corresponding glucose content. A reason for this mismatch may be found in glucose oligomers that could also be fermented to ethanol. We note that since SMSS is derived from GS which contains higher lignin, thus the ethanol yield for GS is expected to be less than 4g/100g substrate. In addition, as shown in Table 2, the higher bioethanol yield in DSMSS illustrated that higher lignin amount in SMSS has inhibited the fermentation process per se. Considering the 50% lignin content of SMSS, which does not give rise to the production of ethanol, this refers to a conversion yield of 14%, thus equivalent to those obtained from pure cellulose. The alcohol content after fermentation remained with nearly 3% significantly below the maximum capacity of *Saccharomyces cerevisiae*, thus indicating an up-scaling option based on increased concentrations, if the heat for the neutralization of the perchloric acid solution can be handled without previous dilution.
Table 2. Experimental results for the hydrolysis and subsequent fermentation of the sugars from cellulose and mushroom waste.

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<thead>
<tr>
<th>Sample</th>
<th>Hydrolysis (glucose yield)</th>
<th>Fermentation (overall ethanol yield)</th>
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<tr>
<td></td>
<td>HPLC</td>
<td>GC</td>
</tr>
<tr>
<td></td>
<td>g/100 g substrate&lt;sup&gt;a&lt;/sup&gt;</td>
<td>g/100g substrate&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cellulose</td>
<td>22 ± 2</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>SMSS</td>
<td>5 ± 1</td>
<td>3.7 ± 0.1</td>
</tr>
<tr>
<td>DSMSS</td>
<td>9 ± 3</td>
<td>5 ± 1</td>
</tr>
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</table>

<sup>a</sup> Yields are based on dry substrate. All data are presented as mean ± standard error.

Comparing the efficiency for the hydrolysis of cellulose in SMSS with those previously reported for native rubberwood sawdust [18] shows a considerable improvement. While both processes furnished glucose contents of 5% (w/w substrate), the cellulose content in SMSS is only half of that in GS; therefore the efficiency for the hydrolysis is doubled. Despite the comparable ethanol conversion of cellulose and SMSS, the compositions of the distillates are significantly different. While pure cellulose almost exclusively led to ethanol, SMSS produced a mixture of ethyl and methyl alcohol in a molar ratio of about 9:1 by GC determination.

As expected, the delignification of SMSS (DSMSS) increases the cellulose content and leads to a facilitation of the hydrolysis of cellulose based on a sponge-like structure rendering the glucose contents of DSMSS slightly higher than that of SMSS hydrolyzates. It clearly demonstrates that alkaline delignification of SMSS does improve the hydrolysis process. The composition of the distillates for SMSS and DSMSS are almost identical. This is an indication that the methanol content is derived from the fermentation. A possible explanation could be the fermentation of pentose sugars from hemicellulose contents, leading to ethanol, methanol and carbon dioxide. *Saccharomyces cerevisiae* is commonly known to ferment hexoses only, thus the pentose fermentation must be based on other microbes, probably originating from the mushroom cultivation. An alternative explanation may refer to galacturonic methyl ester contents in hemicelluloses, although the alkali treatment for the delignification should have removed most of the methanol for DSMSS. While methanol is considered an unacceptable contamination for food stocks due to its toxicity, methanol contents can contribute to biofuel, thus adding value rather than causing a problem.

The choice of SMSS as substrate for the production of ethanol was prompted by the need to dispose tons of the waste daily. Mushroom cultivation on rubberwood 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 commercial application. An example of the chemical hydrolysis is the Arkenol process [19, 20], which uses approximately 70% sulphuric acid to hydrolyze the cellulose. The reaction requires heating to 100°C which results in charring of the sugars formed. In contrast, we have found in this study that 70% perchloric acid is able to hydrolyze cellulose into a clear golden solution within 15 min at 50°C. Application
of diluted sulphuric acid (0.5 to 15 %) helps to avoid the problem of charring, but the reaction still requires heating from 90 to 600°C, and thus constitutes a highly energy-intensive process. Sugars can be separated from the acid by chromatographic columns [19], but this method is time-consuming and costly. The sulphuric acid is neutralized by lime water and yields a mushy precipitate of CaSO₄·2 H₂O, which requires pressing in order to release the remaining sugar solution. On the other hand, the neutralization salt from perchloric acid, i.e. potassium perchlorate crystals formed is modest and can be easily filtered using a vacuum pump. In another example, 70 % phosphoric acid was used to phosphorylate the cellulose and transform it into a starchy gel-containing sugar solution [21]. However, the separation of the sugars from the phosphoric acid remains difficult. Dilute phosphoric acid had also been used to convert ligno-cellulosic biomass to sugars [22] but under the high pressure and temperature conditions which are not cost effective and not sustainable. Although the hydrolysis of ligno-cellulose using hydrochloric acid [23] and nitric acid [24] have been reported but the use of these acids should be avoided because they are extremely volatile and fuming at high temperatures and pressures. Hence, they are hazardous to the operator and the ability to hydrolysis is also reduced. The same is applied to other fuming acids such as acetic acid and acetic anhydride.

With respect to the separation problems for sulphuric and phosphoric acid indicated above, aqueous perchloric acid appears to be a favourable alternative. In order to save costs and materials, KClO₄ can even be converted back to perchloric acid by reaction with sulphuric acid and careful distillation of the aqueous solution under vacuum [25]. This way, the final waste product is K₂SO₄, a salt that can be used as fertilizer.

4. CONCLUSIONS

Our main findings suggest that perchloric acid is an effective medium for the hydrolysis of cellulosic material into glucose and oligomers, with a fast conversion without external heating and charring. The neutralized acid can be easily removed from the hydrolyzate by filter papers. Unlike pure cellulose, sawdust resists the acidic hydrolysis due to a tight ligno-cellulosic composite. This resistance, however, can be overcome by bio-induced degradation. Mushrooms, feeding on wood cellulose, have been identified as effective transformers. The hydrolysis and fermentation of dry mushroom waste can provide up to 40 L of ethyl/methyl alcohol per metric ton. Based on a local mushroom farm (~10 tons wet SMSS per day), this translates to more than 150 L of biofuel per day. Fermentations can apply common baker’s yeast, which enables the utilization of ligno-cellulosic waste without costly delignification steps. This way, conversion rates of up to 3.7 g ethanol/100 g substrate (10 % in average) can be obtained, with prospect for further improvements.

Great care has to be taken to ensure that perchloric acid and its salt, potassium perchlorate are not dehydrated at temperature more than 100°C in view of its reputation to explode unpredictably. The proper handling of perchloric acid is rigorously adhered to.

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REFERENCES


