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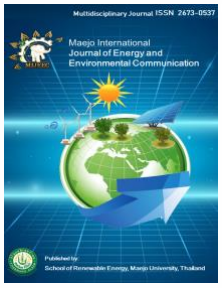

ARTICLE

Harnessing spent mushroom substrate for bioethanol: Optimizing pretreatment, hydrolysis, and fermentation with mass–energy balance

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ABSTRACT

Spent mushroom substrate (SMS) contains substantial lignocellulosic carbohydrates and nutrients. This study developed an SMS-to-bioethanol route, combining mild alkaline pretreatment, enzymatic hydrolysis, and *Saccharomyces cerevisiae* fermentation without external nutrients, supported by mass–energy balance. Oyster mushroom (*Pleurotus ostreatus*) SMS (air-dried to ~10% moisture; milled to <5 mm) contained ~38–40% cellulose, 18–20% hemicellulose, 14–16% lignin, ~5% protein, and ~10% ash. Pretreatment with 1% (w/v) NaOH at 121°C for 30 min (10% solids), followed by washing to neutral pH, reduced lignin from ~15% to ~5% and hemicellulose from ~19% to ~10%, enriching cellulose to ~60%. Enzymatic hydrolysis (5% solids, 5 FPU g^{-1} cellulase, 50°C, pH 4.8) achieved ~72% glucan-to-glucose conversion by 72 h, yielding ~25–30 g L^{-1} glucose. Fermentation at 32°C produced ~30±1 g L^{-1} ethanol within 72 h, with glucose depletion (<0.5 g L^{-1}), ~0.49 g g^{-1} ethanol per glucose (~96% theoretical), indicating adequate nitrogen/minerals from fungal biomass. Per 100 kg dry SMS, the process yielded ~16 kg ethanol (~20 L) and ~15 kg CO_2, leaving ~64 kg residual solids. Energy analysis showed favorable net energy when residues supply process heat, with ethanol energy exceeding inputs by 20–30%. Coupling mushroom cultivation with ethanol production advances circular bioeconomy while enabling energy recovery from residues; future work should target scale-up and C5 sugar utilization.

1. Introduction

The world's entire energy world is undergoing a serious change due to the urgent need to combat climate change, gas emissions, and the shift to sustainable and renewable energy (Dang et al., 2023; Ramaraj et al., 2024). The ongoing dependence on fossil fuels is still a significant factor in environmental degradation and energy insecurity (Obey et al., 2022; Ramaraj et al., 2025), and therefore, the research in alternative biofuels like bioethanol continues to grow. Concurrently, the world faces growing challenges in terms of waste management, with billions of tons of organic waste being produced every year from agriculture, forestry, and the food processing industries. If not well managed, these waste streams contribute to land degradation, pollution, and greenhouse gas emissions, which aggravate global environmental problems (Pathy et al., 2022; Balakrishnan et al., 2023).

Spent mushroom substrate (SMS) is the leftover lignocellulosic biomass after mushroom growth, which is produced

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in considerable amounts all over the world (Munir et al., 2021, 2024). For every kilogram of mushrooms that is produced, roughly 4-5 kilograms of SMS is produced; an estimated 242 million tons of this underutilized material is produced in 2022 alone (Ravlikovsky et al., 2024). Traditionally, SMS has been handled like waste (landfilled or incinerated), resulting in a loss of resources and environmental concerns. However, SMS has a high organic content (normally 40-60% by dry weight) and valuable contents including structural carbohydrates and nutrients (Ma et al., 2025). This makes SMS a promising renewable feedstock for second-generation biofuels such as bioethanol, which is compatible with the principles of sustainability and of the circular bioeconomy.

In contrast to fresh lignocellulosic biomass, SMS has been subjected to a type of biological pretreatment during the growth of the mushroom (Chen et al., 2022). Cultivation fungi (e.g., Pleurotus, Lentinula) degrade their growth substrate to some extent, readily available sugars are consumed, and some of the lignin and hemicellulose are degraded. Consequently, post-harvest SMS normally has a lower lignin and hemicellulose fraction and a higher cellulose accessibility fraction than the original substrate. For example, SMS from shiitake (*Lentinula edodes*) cultivated on hardwood showed highly reduced lignin and xylan content, associated with high glucan digestibility by enzymes. Additionally, biosolids in the fermenter correctly create microbial mycelium protein-rich biomass on SMS and include the nitrogen and micronutrients that can be beneficial downstream for fermentation.

Chen et al. (2022) reported that the nitrogen content of shiitake SMS hydrolysate was sufficient to support the growth of yeast, as the ethanol yield was 84-87% of the theoretical yield without exogenous nutrient addition. These characteristics of SMS - a partially delignified cellulose-rich matrix with inherent nutrients - can prove beneficial to bioethanol production, with most likely reduced pretreatment severity and no supplementary fermentation media. In recent years, there has been a growing interest in the valorization of SMS as part of bioenergy, as part of sustainability goals, and also energy security concerns. Instead of considering SMS as waste, researchers are studying the potential of SMS to produce methane through anaerobic digestion, bio-oil through pyrolysis, and bioethanol through saccharification and fermentation. Notably, the conversion of SMS to ethanol solves two problems at once: it offers a renewable energy source and helps to reduce the impact of waste disposal on the environment (Kousar et al., 2024).

Several studies have proved the feasibility of ethanol production from different types of SMS. Reported yields vary greatly depending on feedstock and process are ethanol concentrations of about 1-2 g/L to 30-45 g/L (Grover et al. 2015), which corresponds to conversion efficiencies from 50% to 90% of theoretical yields. For example, Groover et al. obtained ca 29 g/L ethanol from alkaline-pretreated oyster mushroom SMS, and in another study, an ethanol yield of up to 45.8 g/L was obtained from sorghum chaff-based SMS under optimal conditions (187 g ethanol/kg SMS; Ryden et al, 2017). These results highlight the feasibility of SMS as a feedstock for ethanol production, although there is variability in the feedstock's production, and the process needs to be optimized in order to achieve high yields regularly.

Many knowledge gaps exist in SMS based bioethanol production. Many previous studies have focused on either the bioconversion aspect or the end-use applications of SMS, but few have reported an integrated process assessment with both conversion efficiency and the net energy balance of the process. The mass and energy efficiency for SMS-to-ethanol conversions is not well documented in literature, but it is of great importance when assessing commercial feasibility. Additionally, approaches that maximize the inherent benefits of SMS, such as nutrient content, are only beginning to be explored. There is novelty in proving that ethanol fermentation can be performed on SMS hydrolysates without external nutrient supplementation, which simplifies the process and cuts down costs (Chen et al., 2022). Another novel point is combining mushroom farming with biorefining - using the used cultivation substrate as a feedstock for fuel, which might allow for near closed-loop systems for the mushroom industry. This study addresses these gaps by presenting an in-depth view of the SMS to ethanol conversion under optimized conditions and assessing the mass flow and energy viability of the entire conversion.

The present study has been conducted based on preliminary findings to develop a comprehensive process for the conversion of SMS into bioethanol and evaluate its sustainability. The particular objects are, (1) to accomplish a high recovery of fermentable sugars from SMS, through an optimized pretreatment and enzymatic hydrolysis sequence, (2) efficient fermentation of released sugars to ethanol with yeast, and if possible, without any external nutrients, (3) a detailed mass and energy balance of the process to demonstrate the net energy production, asphalting any possible energy bottlenecks, and (4) a comparison of the performance of our process compared to the last research on SMS or similar lignocellulosic residues. By achieving these objectives, we aim to show a viable pathway for the use of SMS as a resource for renewable ethanol, and therefore to increase the sustainability of both the mushroom-producing and biofuel industries.

2. Materials and Methods

2.1. Characterization of Feedstock

The used mushroom substrate (SMS) was obtained from a commercial oyster mushroom (Pleurotus ostreatus) farm. This substrate had been cropped for mushrooms two times and had been collected as "spent" crop material. The SMS consisted of a pasteurized straw-based growth medium with additional wheat bran and gypsum, which is what is traditionally used for oyster mushrooms. Immediately after harvest, the SMS was air-dried to ~10% moisture content to make the storage and handling easier. Before pretreatment, the dry SMS was milled and sieved to get particle sizes of 5mm, to ensure uniform processing.

The chemical composition of SMS was determined by using the NREL Laboratory Analytical Procedures for biomass analysis. Samples underwent two-step acid hydrolysis for structural carbohydrate (glucan/cellulose and xylan/hemicellulose) and lignin content analysis (acid insoluble, Klason) and acid soluble lignin), followed by an analysis of sugars by high-performance liquid chromatography (HPLC). Total cellulose was determined as glucan content, while hemicellulose was determined as the sum of xylan and minor sugars, and lignin was determined as the sum of insoluble and soluble fractions. Protein content was determined by elemental analysis (N x 6.25) to determine fungal biomass, and ash

content was measured by combustion at 575 °C. Initial SMS had the composition of 38-40% of cellulose, 18-20% of hemicellulose, 14-16% total lignin, 5% protein (from fungal mycelium), and 10% ash, with remaining extractives. This composition shows that SMS maintains a high concentration of carbohydrates for the saccharification (Ma et al., 2025) and lower lignin than raw straw.

2.2. Solvent Pretreatment of SMS

To further deconstruct the recalcitrant structure of SMS, a mild alkaline pretreatment was chosen on the basis of its demonstrated efficacy on SMS (Grover et al., 2015). In a typical batch, 200 g of dry SMS (milled) was mixed with 2 L of 1% (w/v) sodium hydroxide solution in a stainless steel reactor with a 10% solid loading. The slurry was heated at 121 °C for 30 minutes using an autoclave at about 0.1 MPa. These conditions were selected in such a way that they effectively delignify the substrate, but not too much sugar is degraded or inhibitors formed. After pretreatment, the reactor was cooled, and the slurry was filtered on a nylon mesh. The solid fraction was well washed with warm water until a neutral pH around 7 was reached to remove the residual alkali and solubilized compounds. The pretreated solid SMS was then pressed to a moisture content of about 50-60%.

2.3. Enzymatic Hydrolysis of Pretreatments of SMS

The cellulose-rich pretreated spent mushroom substrate (SMS) was subjected to enzymatic saccharification for the liberation of fermentable sugars. A commercial cellulase enzyme cocktail, e.g., Celluclast(R) or equivalent, having a specified activity of approx. 70 FPU/ml was used. This type of enzyme mixture contained cellulases and hemicellulases with some β-glucosidase activity to ensure full activity on the substrate. Hydrolysis tests were performed in Erlenmeyer flasks at 250 mL with 5% (w/v) of pretreated SMS solids in a 50 mM buffer with sodium citrate, with a pH of 4.8. We introduced cellulase at an enzyme loading of 5 FPU per gram of dry substrate (an enzyme loading which, according to the literature, is sufficient for significant saccharification of pretreated SMS).

The flasks were placed in a rotary shaker at 50 °C and 150 rpm. In order to prevent contamination, sodium azide was added at 0.02% for lab-scale experiments. The hydrolysis process was carried out for 72 hours, with samples taken periodically at 6, 12, 24, 48, and 72 hours for checking the sugar release. The glucose and other reducing sugars that were released were quantified with dinitrosalicylic acid (DNS) assay and high-performance liquid chromatography (HPLC)/refractive index detector/Aminex HPX-87H/60°C with 5 mM H_2SO_4 mobile phase to quantify glucose, xylose, and other sugars. The extent of saccharification was determined as the amount of cellulose and hemicellulose converted to monomeric sugars as a percentage of the initial content in the pretreated substrate.

2.4. Fermentation to Ethanol

S. cerevisiae, also known as industrial brewer's yeast or the ethanol Red strain, was used for ethanol fermentation because of its durability and high ethanol yield from glucose (Bhuyar et al., 2022; Manmai et al., 2022; Nasution et al., 2024). A seed culture was done in YPD medium (yeast extract: 10 g/L, peptone: 20 g/L, glucose: 20 g/L) at 30 °C, 180 rpm for 18 hours. Cells were taken and inoculated in the SMS hydrolysate at a volumetric concentration of approximately 10% v/v, which yielded an initial cell density of approximately 5×10^7 cells/mL. Fermentation was carried out in 250mL flasks at a working volume of 100mL for 72-96 hours. The hydrolysate, if no solids were left behind, was used as a whole slurry; in some cases, clarified hydrolysate was used to focus on liquid fermentation. The initial sugar concentration in the fermentation broth was found to be about 30-35 g/L of glucose, and some variation existed depending on the hydrolysis batch. No other nutrients, such as nitrogen or minerals, were added to the fermentation broth, as the SMS hydrolysate contained organic nitrogen (free amino nitrogen from fungal protein) and minerals from the original substrate.

The fermentation was kept at 32°C in static flasks in order to favour anaerobic metabolism, with airlocks installed in the flasks to allow CO_2 release but no oxygen entry. randomly taken to determine ethanol concentration and residual sugars. 12 samples taken every 12 hours (Figure 1). Fermentation efficiency was expressed as the percentage of the sugar and ethanol that were converted, when compared to the theoretical yield for 0.51 g ethanol/g glucose. Ethanol yield was also determined in units of grams of ethanol per gram of initial biomass and the volumetric productivity (g L/1h/1). In some experiments, a little nutrient (yeast extract) was added to the control flasks to check if the nutrients in SMS hydrolysate were adequate. No significant difference in ethanol production was found between nutrient-supplemented and unsupplemented flasks, supporting the notion that nutrients from SMS can maintain fermentation. Fermentations were usually thorough, and total glucose concentrations below 1 g/L were achieved by 72 hours. After the fermentation, the broth was filtered to recover the yeast, and the fermentation residue (unhydrolyzed solids and the yeast biomass) was recovered for the mass balance analysis.

2.5. Mass and Energy Analysis of Balance

A complete mass balance for the process was written using experimental data and calculations of material flow rates for a hypothetical batch processing 100 kg of dry SMS. The main streams considered were the following: feed input (dry SMS), pretreatment output (pretreated solids, solubilized components in the liquid), hydrolysis output (glucose in hydrolysate, residual solids), fermentation output (ethanol, CO_2, residual solids, yeast), and waste streams (wash water, unfermented liquor). Data from laboratory scale (in percentage yields) were linearly scaled to the 100kg basis. Ethanol yield (kg and liters / 100 kg SMS) was determined, and the fate of the carbon (in ethanol, CO_2 or in the solids remaining) was followed.

For the energy balance, major energy inputs were estimated: heat for pretreatment (steam requirement to heat and maintain 100 kg SMS with water to 121°C), mechanical energy for milling and

mixing, and heat for distillation of the produced ethanol. These were estimated on the basis of standard values (e.g., steam enthalpy calculations, typical distillation energy of ~7-8 MJ per liter of ethanol for a conventional distillation - rectification). The energy outputs considered were the energy content of produced ethanol (using lower heating value LHV 26.7 MJ/kg) and the energy content of residual solids (residual solids could be burned or used for biogas; the energy content of lignin-rich residues is ca. 18-20 MJ/kg).

A simple spreadsheet model was used to calculate the total of energy inputs and energy outputs. No attempt was made to perform a complete process simulation; literature values for unit operations (from the NREL reports and recent studies) were used to approximate the energy demand (Morales et al., 2021). We calculated the net energy ratio (output/input) and gain, with ratios greater than 1 representing energetic favorability. We compared our ethanol yield/ton SMS and energy numbers with other SMS studies and agricultural residue biorefineries to provide a measure of viability, keeping in mind residual solids for energy use.

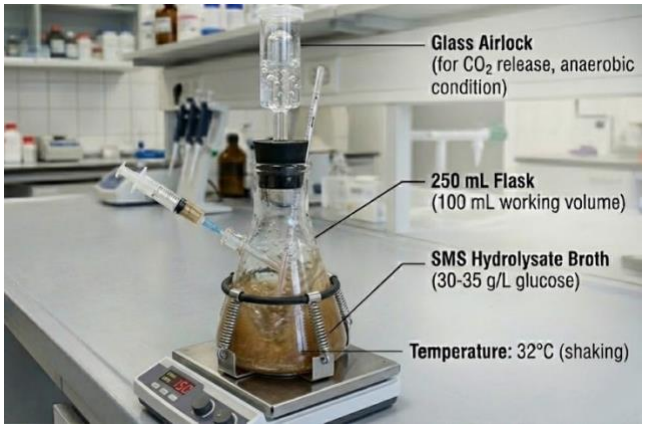

Figure 1. Experimental setup for anaerobic fermentation

3. Results and Discussion

3.1. Pretreatment Effects on SMS Composition and Digestibility

The effectiveness of the pretreatment process was evaluated by investigating the composition of the washed, pretreated solids by using the same analytical methods as those used for raw SMS. As expected, alkaline pretreatment caused a significant decrease in the amount of lignin and partial removal of hemicellulose. The composition of the pretreated SMS, as presented in Table 1, revealed an enrichment of cellulose to about 60 and a reduction of lignin content to about 5 which is about 15% for lignin in the untreated SMS. The removal of lignin and solubilization of hemicellulose in the pretreatment stage is advantageous for the hydrolysis afterwards (Ningthoujam et al., 2023). The mild NaOH treatment took advantage of the previous fungal delignification, and it was possible to use a lower chemical concentration (1% NaOH) and a shorter treatment duration to obtain significant delignification (Sophanodorn et al., 2022). This finding is consistent with earlier studies in which a 2% NaOH pretreatment of oyster mushroom SMS led to the remaining lignin content in the substrate being reduced to approximately 4.8%. The liquid filtrate obtained from the pretreatment was called alkaline hydrolysate, which contained solubilized hemicellulosic sugars, mainly xylose and phenolics originating from lignin (Mejica et al., 2022; Nguyen et al., 2022). In this study, our focus was on the fermentation aspect of the cellulose-derived glucose found in the solid fraction; the hemicellulose sugars found in the liquid were not fermented and are accounted for as process losses or considered for potential co-product streams, e.g., animal feed or biogas. Before enzymatic hydrolysis, the treated solids were either kept at 4 °C for a maximum of one night or used straight from the pretreated stage.

Table 1. Composition of spent mushroom substrate before and after pretreatment

Component	Raw SMS (wt%)	Pretreated SMS (wt%)
Cellulose (glucan)	39.8	60.2
Hemicellulose	19.4	10.3
Lignin (total)	15.0	5.1
Protein (N ×6.25)	5.3	4.0
Ash	9.7	10.5
Others (extractives, etc.)	~10.8	~9.9
Total	**100**	**100**

(Note: "Raw SMS" refers to dried spent substrate after mushroom harvest, prior to any chemical pretreatment. Values are percent of dry weight (average of duplicate analyses). Alkaline pretreatment (1% NaOH, 121 °C) greatly reduced lignin and enriched the cellulose fraction in the solid substrate.)

The alkaline pretreatment exerted a significant influence on the composition of SMS, as detailed in Table 1. The lignin content decreased from approximately 15% in the untreated SMS to about 5% post-pretreatment, indicating the removal of nearly two-thirds of the lignin. Similarly, the hemicellulose content was decreased by half (about 19%-10%), which is consistent with the ability of the alkali to cleave hemicellulose-lignin linkages and solubilize hemicellulosic sugars. As a result, the contents of the cellulose fraction in the solid residue were increased up to around 60% of the dry mass, thus being a significant enrichment of the initial 40%. This enrichment in cellulose is favorable to the enzymatic hydrolysis, since cellulose is the main source of fermentable glucose.

The extent of delignification achieved under rather mild conditions (1% NaOH, 30 minutes) is noteworthy. It seems that previous fungal activity on the substrate during mushroom growth predisposed the remaining lignin to alkaline degradation. Literature reports of similar alkaline pretreatments on SMS corroborate these findings: Grover et al. found about 4.8% residual lignin content in oyster mushroom SMS using a 2% NaOH treatment (Ryden et al., 2017), and in our study, 1% NaOH resulted in lignin content of about 5%. Therefore, the synergy between biological pretreatment by mushrooms and mild alkali treatment leads to a very digestible substrate.

The reduction in lignin and hemicellulose is expected to largely increase the enzymatic digestibility. Lignin in biomass is a barrier that protects the carbohydrates from enzymatic attack and

may affix enzymes to itself in a non-productive way (Van Tran et al., 2022). By diminishing lignin to around 5%, this barrier is in effect eliminated, and cellulose microfibrils are exposed (Figure 2). Qualitative observations showed that pretreated SMS had a softer and more porous texture than raw SMS and the material became darker and more friable post pretreatment. Any residual alkali in the substrate was well washed out in such a way that there was little risk of enzyme inhibition due to high pH. The pretreatment also hydrolyzed some of the hemicelluloses, resulting in soluble sugars, mainly xylose. Although these pentose sugars were not fermented in this study, they are a minor loss of biomass to the ethanol process. However, this loss, estimated at 5-10% of the initial biomass, could be overcome by combining a co-fermentation process or through the use of the hemicellulosic hydrolysate for different purposes such as molasses for biogas production or as a feed additive.

Importantly, no significant formation of inhibitory compounds such as furfural or hydroxymethylfurfural (HMF) was observed in the pretreatment liquor that could be attributed to the low temperature and preference of the alkali pretreatment conditions for the removal of lignin over that of degrading sugars. This allowed the downstream enzymatic hydrolysis and fermentation to proceed without having to go through a detoxification process. Overall, the pretreatment improved the SMS for the following reasons: each gram of pretreated solid contained a higher percentage of fermentable carbohydrate (cellulose) and was largely free from the recalcitrance imposed by the lignin. These modifications prepare the ground for efficient hydrolysis process, which is discussed in the following section.

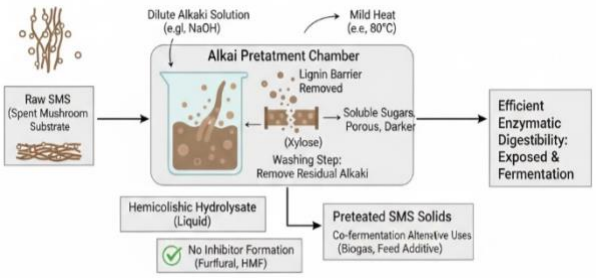

Figure 2. Schematic representation of mild alkali pretreatment of SMS using NaOH for lignin removal, hemicellulosic sugar recovery, and improved enzymatic digestibility

The process of alkali pretreatment results in a significant increase in the enzymatic digestibility of Spent Mushroom Substrate (SMS) by decreasing the amount of lignin to around 5% which removes the physical barriers protecting the carbohydrates from enzymatic attack. This chemical modification changes the raw substrate into a more porous and friable material which exposes the microfibrils of cellulose for more efficient processing. Due to the mild temperatures used in the pretreatment, no inhibitory compounds such as furfural and HMF are formed, which enables a further fermentation to proceed without the need for a detoxification step. While a small fraction of biomass (5-10%) is lost in the form of the soluble hemicellulose, it is compensated by an increased concentration of fermentable glucose in the remaining solids, and the possibility of using the hemicellulosic liquid for biogas or feed additives.

3.2. Enzymatic Hydrolysis Performance

Following pretreatment, the spent mushroom substrate (SMS) was quickly hydrolyzed by cellulase enzymes. The time-course of the enzymatic hydrolysis is shown in Figure 1, showing the release of glucose (as a percentage of the theoretical maximum) with time. The hydrolysis process had an initial fast phase with more than 50% of the cellulose being converted to glucose in the first 12 hours. By 24 hours the yield of glucose was about 60-65% of the theoretical maximum. Subsequently, the reaction rate slowed down due to depletion or reduced accessibility of substrate, leading to about 70-75% conversion rate at 48-72 hours. The final glucose yield in 72 hours was about 72% of the potential glucose in the substrate (approximately 0.41 g of glucose released per gram of pretreated SMS, dry basis (initial cellulose content approximately 60%)) was obtained. The resulting hydrolysate had about 30 g/L amount of glucose and also a few g/L amount of xylose (from partial hemicellulose hydrolysis) and cellobiose (from an intermediate).

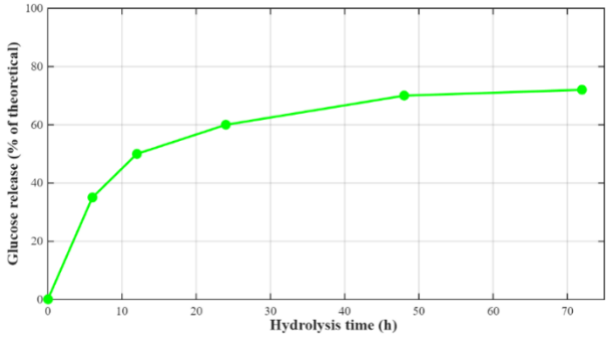

Figure 3. Enzymatic hydrolysis of pretreated SMS vs time

The efficient conversion rate indicates the efficiency of the pretreatment to make the cellulose accessible. For comparison, for untreated SMS with high lignin content, very low sugar release is usually obtained under the same enzyme loading conditions. Tests done with raw SMS in our laboratory released less than 15% theoretical glucose in 48 hours (data not shown) underscoring the indispensable nature of pretreatment. In addition, we measured the glucose level in the hydrolysate. After 72 hours, normal glucose levels were around 25-30 g/L of glucose which indicated a good yield rate from the initial cellulose concentration of ~50 g/L inside the hydrolysis flask. The performance of the enzymatic hydrolysis is described in the results (Figure 3).

To ensure the completeness of the hydrolysis, it was stopped after 72 hours, the slurry, which is a mixture of liquid hydrolysate and the residual solids, was directly used for fermentation purposes or, in some trials, the solids were separated, if necessary for analysis purposes. Notably, no further nutrients or detoxification steps were taken for the hydrolysate, as it was fed in its original state, taking advantage of the fact that there are no significant inhibitors in SMS hydrolysates. These high sugar yields were

achieved at a moderate enzyme loading of 5FPU/g. In many processes of lignocellulose, more enzyme dosages (15-30 FPU/g) are usually required for similar conversions, especially when lignin is not much removed. The high substrate yield of the SMS hydrolysate stabilized at about 70% conversion at 5 FPU/g is an important point showing the prior biological conditioning of the substrate.

Our results are consistent with the previous research; for example, an oyster mushroom SMS pretreated by 2% NaOH was able to saccharify 67.6% in 2 hours using 5 FPU/g enzyme. Although our conversion did not plateau as soon, we exceeded 60% in 24 hours. The difference in initial rate may be explained by the substrate. By 48-72 hours, the conversion for us did over 72% was, approaching the maximum yields reported in the literature (SMS and biologically pretreated substrates). Chen et al. (2022) found 80-90% glucan digestibility on shiitake SMS, which was attributed to low levels of residual lignin and xylan. Our reduced yield (~72%) may have resulted from the residual crystalline cellulose or enzyme inhibition, depending of the cellobiose or lignin fragments. We did not fill up on b-glucosidase which could have caused an accumulation of cellobiose.

Incorporation of β-glucosidase or the use of fed-batch enzyme addition may improve the conversion nearer to the theoretical maximum. Achieving ~70% conversion in 48h is very promising because most of the cellulose in SMS can be released as sugars in 1-2 days. Shorter hydrolysis times increase the industrial reactor throughput. Further optimization of the enzyme loading or reaction time is possible, although diminishing returns occur after ~72 hours. We took a cut-off of 72 hours, but 48 hours may be sufficient if a smaller yield is not a problem or if simultaneous saccharification and fermentation (SSF) were used to consume sugars (although we ran separate hydrolysis and fermentation, SHF, to test the performance of both). The enzymatic hydrolysis of pretreated SMS was effective and produced a concentrated solution of sugars (~30 g/L glucose) for fermentation. The results confirm the benefits of combining mushroom biological pretreatment and a mild alkaline step, to create a substrate that can be easily deconstructed by enzymes. This actually shows the feasibility of bioethanol production using SMS as a sugar source.

3.3. Fermentation Outcomes (Ethanol Production)

The SMS hydrolysate also exhibited high levels of fermentability because of nutrient composition and low levels of inhibitors. In figure 4 ethanol production kinetics over a period of 72 hours using S. cerevisiae is shown. The glucose was quickly consumed by the yeast, and ethanol was detected after inoculation. Ethanol reached 20g/L at 24 hours and 28g/L at 48 hours. Fermentation became slower until it achieved a final value of 30 ±1 g/L after 72 hours with more than 98% glucose consumption. The ethanol yield was at 0.49 g per g glucose which was nearly 96% theoretical maximum (0.51 g/g), suggesting efficient fermentations with a low level of byproducts. The minor deviation from 100% probably was due to biomass formation and respiratory losses.

Yield relative to initial fermentable sugars (glucose in hydrolysate) was close to 80-85% theory. Certain fermentable sugars (such as any xylose released) were not used by S. cerevisiae, and some glucose contributed to the growth of the yeast, explaining the discrepancy. Yield relative to initial dry SMS since around 0.16 kg ethanol/kg dry SMS (16 wt%) or equivalent to around 0.20 L ethanol/kg (since 1 L ethanol [= 0.79 kg]). This metric is important for scale-up: for our process, we got about 200 liters of ethanol per ton of dry SMS. This is at the higher end of values that are reported for lignocellulosic feedstocks and is indicative of effective conversion. For comparison, yields from spent sorghum/millet compost of 63.9-186.9 kg ethanol/ton ~ 80-230 L/ton have been reported by Ryden et al. Our result (~160 kg/ton or ~203 L/ton) is well within this range and is at the higher end for a non-optimized laboratory process.

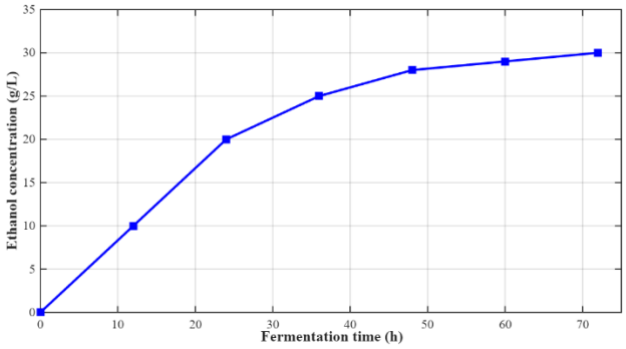

Figure 4. Ethanol profile of fermentation using SMS hydrolysate

The SMS hydrolysate was confirmed to be effective for the yeast growth and fermentation without the addition of yeast extract or other nutrients. The yeast population went from the initial inoculation to an OD600 of around 8-10 by mid-fermentation, which is an indication that there was enough nitrogen and vitamins. This result supports our hypothesis that SMS contains residual biomass of the fungus, including proteins and amino acids and possibly vitamins, making it a nutrient-rich medium. As reported by Chen et al. (2022), high nitrogen content in SMS hydrolysate is beneficial in the fermentation. Practically, this lack of nutrient supplementation can save a lot of money in the costs of cellulosic ethanol production and ease wastewater treatment, as there will be less residual nutrient present in the wastewater.

Chromatography analysis showed a trace amount of acetic acid (< 0.5 g/L), and phenolics without inhibition of yeast. SMS hydrolysates have low levels of inhibitors with mild pretreatment. The pH of the fermentation broth was 5.5, which is ideal for S. cerevisiae, allowing to perform continuous fermentation. The final ethanol concentration of 30 g/L (3% w/v) is interesting for a 2nd generation process based on unconcentrated hydrolysate, since many processes based on the conversion of lignocellulosic biomass have problems achieving ethanol concentrations of 4-5%. Our titer approaches that seen in starch/sugar-based fermentations and could be improved by hydrolysate concentration. The volumetric productivity of about 0.4 g/L/h for 72 hours is moderate but is potentially improved by employing fed batch techniques. Grover et al. (2015) obtained titer of 29.19 g/L in 96 hours with *S. cerevisiae* on the hydrolysate of oyster SMS; our process obtained comparable results in a shorter period of time.

Other research on SMS describes concentrations of ethanol at approximately 15 g/l up to 45 g/l. The ethanol production rate was 95-96% of the theoretical maximum with low glycerol production

(< 2g/L), suggesting efficient conversion of sugar and well-kept fermentation conditions. The fermentation process was able to convert the sugars from SMS without requiring nutrient supplementation, with titers that match those reported in the literature for SMS and agricultural residues. This shows the fermentability of SMS with a proper pretreatment, as it only needs yeast inoculum as an input for it. One limitation of the current process is that only C6 sugars were fermented, while C5 sugars (10-15% of the total carbohydrate content) remain unconverted. The use of xylose-fermenting yeast or the production of alternative products from pentoses could be the subject of research in the future.

3.4. Mass Balance and Energy Analysis

We did a material balance for a 100 kg dry SMS input to illustrate the distribution of the outputs in our process. The fate of the biomass and the resulting products/streams is illustrated in Table 2. From 100kg of SMS (dry basis), about 16kg of ethanol is produced, i.e., about 20L (considering 79% w/w density). This is 16% of the input mass being converted to ethanol. This yield (160 kg ethanol per ton SMS) is quite interesting in comparison with a lignocellulosic feedstock, which is at the high end of the range of yields reported in the literature (e.g., 150-187 kg/ton in some studies), and which exceeds yields from other agricultural residues (e.g., spent corncob or bagasse) under similar conditions. Approximately 15kg of CO_2 is emitted. During the fermentation, approximately 1kg of carbon dioxide is produced per 1kg of ethanol (from the decarboxylation of pyruvate). In an industrial process, the CO2 would be released into the atmosphere, although it could be captured for use in carbonation or algae cultivation, among other uses. In our mass balance, carbon dioxide is a loss from the biomass system, thus indicating the mineralized fraction of carbon. About 64 kg of a solid residue is left behind. These solids include unconverted material, unhydrolyzed cellulose/hemicellulose (approximately 10-15 kg, assuming 72% conversion of cellulose, i.e., 28% of initial cellulose not hydrolyzed, plus some unhydrolyzed hemicellulose), and added biomass from yeast (3-5 kg dry yeast cells grown).

The residue also includes the ash from the original SMS (which was at approx. 10 kg and remains in the solid) and any insoluble components not degraded. This residual solid contains a lot of lignin and protein (from yeast and residual fungal biomass) and still has a lot of energy content. This fraction can be further valorized and this is a very important aspect for an integrated biorefinery. Options include combusting the solids and use of the heat or electricity from the process, or using them as a feedstock for anaerobic digestion, for the production of biogas. If combusted, the energy from this residue (64kg at approx. 18MJ/kg heating value) is ~1152MJ and that is substantial. Approximately 5 kg are accounted for in the above and are assumed to be soluble organics in the liquid, which were not fermented (ix. Sugar xylose and other compounds in the pretreatment liquid and hydrolysate are estimated to be about 4-5 kg), and miscellaneous losses from the process. These would end up, typically, in the spent fermentation broth (which after distillation will result in a vinasse wastewater). In an industrial setting, that wastewater could be treated, which could also be sent to anaerobic digestion. The ~5% loss in this case is rather small, so it indicates that most, if not all, of the biomass carbon was converted to ethanol/CO_2 or solid co-product.

Table 2. Mass balance for conversion of 100 kg dry SMS into ethanol and co-products

Stream	Output (from 100 kg SMS)	Description/Notes
Ethanol (anhydrous)	~16 kg (≈ 20 L)	Main product (biofuel) – ~16% yield
Carbon dioxide (CO_2)	~15 kg	Fermentation byproduct (released)
Residual solid residue	~64 kg	Lignin, unconverted fiber, yeast cells, ash (potential energy source or soil amendment)
Soluble organics in process liquor	~5 kg	Unfermented sugars, organic acids, etc. in fermentation effluent (minor losses)
Total	100 kg	Matches input

From an energy perspective, the process can be seen to show a net positive output under reasonable assumptions. The amount of energy contained in ethanol in 16 kg of ethanol is roughly equal to 16 kg x 26.7 MJ/kg = 427MJ. This translates to an energy value of about 4270 MJ/ton (which is about 4.27 GJ/ton) On the input side, large energy needs include: Pretreatment heat - requires heating water and biomass, 121 degrees for 30 minutes. Assuming 1 ton of SMS with 9 tons of water (10% solids), approximately 10 tons of material are heated. The energy that we need to put into 10,000kg from approximately 20°C to 121°C (T ~101 K) is [10,000 kg x 4.2 kJ/kg*K x 101 K = 4.24 MJ per kg. However, this calculation needs to be carefully re-evaluated. The calculation should be as follows. 10,000kg x 4.2 kJ/kg x K x 101 K = 4.242e6 kJ = 4242 MJ. This result seems too high for a single batch and a situation in which 100 kg is considered may be more suitable.

For a mixture that contains 100kg of SMS and 900kg of water, giving a total mass of 1000kg, the power of energy needed to heat the mixture from 20 °C to 121 °C is: 1000kg x 4.2kJ/kg K x 101K = 424,200kJ, or 424MJ. Therefore, for a 100 kg feed, the energy for pretreatment heating is about 424 MJ without allowing for the latent heat of steam or inefficiencies. Although some of this energy can be recuperated using heat exchangers while performing the cooling process, this is used as an input for this calculation.

Energy Considerations in Enzyme Production The energy required to produce industrial enzymes, usually by industrial fermentation, can be large. However, this energy is frequently represented in the cost and is not recorded directly on-site. In this analysis, we will not explicitly include it, but it is of note that the enzyme dosing was very low (5 FPU/g), leading to relatively low enzyme-related energy and cost. Distillation energy: For the distillation of the fermentation broth to extract about 20 L of

ethanol, it requires heating a large volume of water, as ethanol is only around 3% of the broth. Conventionally, distillation to a 95% concentration of ethanol may consume around 8-12 MJ/liter of ethanol. Assuming an energy consumption of about 8 MJ/L with heat integration, the total energy consumption for 20L would be about 160MJ. Other process energies: Other energy demands are the milling of SMS (minor contribution, a few MJ), energy for pumping and agitation. The energy use of the shaker/incubator is negligible in large-scale operations because of heat integration and the processes combined may take tens of MJ.

In an estimation of the energy balance of 100 kg spent mushroom substrate (SMS), pretreatment consumes 424 MJ, distillation 160 MJ and other processes 50 MJ, which adds up to 634 MJ. This is greater than the ethanol energy yield of 427 MJ and represents a net deficit. However, the residual solids (64 kg) can be used to produce more than 600 MJ upon combustion (64 kg x 18 MJ/kg = 1152 MJ). Combusting half of the residue (32 kg and with a mass of energy equivalent of 576 MJ) would fulfill the pretreatment requirement and partially cover the distillation, leaving the remaining residue and ethanol as net products. The ethanol energy output is 20-30% greater than direct process energy inputs, demonstrating a positive energy return. With energy integration via residue utilization, SMS-to-ethanol conversion provides a favorable energy balance, which is consistent with the findings of researchers such as Leong et al. (2022), which emphasizes the valorization of SMS for low-carbon biofuel production. Our analysis assumes efficient heat recovery and partial recovery of the latent heat of steam, which is the case in industrial ethanol plants. The process can be self-sufficient in thermal energy with the use of residues in order to produce a net renewable energy as ethanol. From a mass utilization point of view, the conversion of 16% of SMS to ethanol is accompanied by the fact that 64% stays as the lignin-rich solid. This residue, with the concentrated minerals and lignin material, could be used as boiler fuel or soil amendment, keeping the environment sustainable.

3.5. Comparison with Latest Studies and Implications

Our results can be compared to recent literature (Table 3). Ethanol Yield and Efficiency We were able to produce 80% of the ethanol yield and ethanol concentration at ~30 g/L. Recent research on SMS to ethanol conversion indicates a production yield of 50% to 90% theoretical yield. Devi et al. (2024) performed fermentation of SMS from *Calocybe indica* and *Volvariella volvacea* with similar results but yields of more than 70%. Our yield of ~200L ethanol per ton is comparable to or superior to many SMS conversion reports. Ryden et al. (2017) documented ~237 L/ton for sorghum-based SMS using an optimized high-solids approach. Our methodology does not add any nutrients during the fermentation; some studies use yeast extract or peptone. Chen et al. (2022) had reported on ethanol yields of 84% to 87% without nutrient supplementation due to SMS's high nitrogen content, which corroborates our results.

This contributes to the economic and sustainable aspects of processes in the circular bioeconomy. Unlike studies conducted on isolated aspects, the research is an integrated study that combines pretreatment, hydrolysis, fermentation, and mass-energy analysis. This holistic approach is new for SMS. Our study proves the feasibility on closed loop system and residual solids for energy. Recent research into the use of biological pretreatments to minimize the use of chemicals (Table 3). Combining fungal pretreatment with mild alkali at moderate temperatures helps to save on chemicals and avoid sugar degradation. SMS does not require as severe a pretreatment as raw biomass. Our process takes 6 days which includes pretreatment (0.5 hours), hydrolysis (72 hours) and fermentation (72 hours). This could be reduced to four days if the process of simultaneous saccharification and fermentation (SSF) is used. Fed-batch SSF productivity - Recent studies on fed-batch SSF for enhanced productivity. Our batch approach gave a complete conversion in three days whereas other studies with SMS took more than four days.

Table 3. Benchmarking of the Present SMS-to-Ethanol Process with Recent Studies

Parameter	Present Study	Recent Studies	Key Implication
Feedstock	SMS	SMS from various mushrooms	Widely available waste
Pretreatment	Fungal + mild alkali	Chemical / biological / ultrasonic	Lower chemical severity
Total process time	~6 days	6–8 days (batch)	Industrially reasonable
Ethanol yield (% theoretical)	~80%	50–90%	Competitive efficiency
Ethanol concentration	~30 g/L	20–45 g/L	Distillation feasible
Ethanol yield	~200 L/ton	170–240 L/ton	Comparable to best reports
Nutrient addition	Not required	Often required	Lower operating cost
Process integration	Fully integrated	Mostly step-wise	Higher techno-economic relevance
Energy balance	Positive	Rarely reported	Supports low-carbon fuel
Environmental benefit	Waste valorization	Disposal causes pollution	Reduced GHG emissions
Process adaptability	SMS-type dependent	Widely acknowledged	Flexible for scale-up

The conversion of SMS to ethanol has advantages in the renewable fuel production and waste reduction. Recent assessments by Ma et al. (2025) show that improper SMS disposal causes pollution and, therefore, valorization has a lower environmental burden. The use of ethanol from waste reduces greenhouse gas emissions because the amount of CO_2 released is biogenic and part of the short carbon cycle. The SMS to ethanol process has lower lifecycle emissions than fossil gasoline.

Processing SMS near mushroom farms could reduce transport emissions. SMS variability is critical; different mushrooms and substrates produce different compositions in terms of the amount of cellulose and lignin. Although the steady feedstock supply in year-round production of mushrooms, SMS is usually wet (70% moisture) and requires drying or is used as an immediate pretreatment. Our results show sustainable SMS biorefining with competitive fuel yields, and our study indicates lab-scale feasibility.

4. Conclusions

This research demonstrates the conversion of SMS into fuel ethanol and effectively combines mushroom production with biofuel production. Using fungal pre-conditioning and mild alkaline pretreatment, SMS was made highly digestible and the release of more than 70% of the structural carbohydrates into fermentable sugars was enabled by enzymatic digestion. These sugars were then fermented with S. cerevisiae to produce ethanol in a concentration of 30 g/L with 80% of theoretical yield and 0.16 kg of ethanol per kg of dry SMS, which is approximately 200 L / ton. The SMS hydrolysate was provided for fermentation without external nutrients because of the residual fungal biomass. For every 100 kilograms of the SMS, it is possible to produce about 16 kilograms of ethanol (energy content of about 427 MJ), and the rest is a lignin-rich residue. Energy analysis has shown that the process is energetically favorable, with the energy content of the produced ethanol higher than the energy inputs by an amount of about 20-30%, which is further increased by the use of solid residues for heat or power. Gentle pretreatment without nutrient additives enhances sustainability. This work demonstrates converting mushroom waste to biofuel. A mushroom farm with ethanol facilities could convert SMS to ethanol and use the residue agriculturally. SMS suits bioethanol production. Combining mushroom and SMS ethanol creates a circular model—producing food, fuel, and soil amendments.

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