Influence of Different Pretreatment Methods and Conditions on the Anaerobic Digestion Efficiency of Spent Mushroom Substrate

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Abstract: Consumption of mushrooms has recently increased due to their health benefits; consequently, increased mushroom cultivation generates large volumes of spent mushroom substrate (SMS) and effective methods for SMS valorization are thus required. Anaerobic digestion (AD) processes SMS with minimal energy and reduces the amount of waste generated; moreover, it contributes to energy generation through biogas production. To improve the energy efficiency of AD and promote sufficient biomass pretreatment, thermal pretreatment conditions require further investigation. Here, we evaluated the pretreatment efficiency and biogas production of the SMS thermal pretreatment process, studying different pretreatment temperatures to understand the formation of SMS degradation products and the changes in AD efficiency. Particularly, mechanical and hydrothermal pretreatment (HTP) methods were employed to improve SMS biodegradability. By increasing the substrate solubilization efficiency, HTP increased the biogas yield more effectively than mechanical pretreatment. Additionally, HTP improved the substrate’s physicochemical properties and increased the reactive surface area for microorganisms by changing the substrate morphology. Further, the biodegradability of the hydrothermally pretreated SMS was higher (87.46%) than that of the mechanically pretreated SMS (61.37%). Thus, SMS could be employed in biogas production and HTP play a key role in improving the biogas yield during SMS processing.

Keywords: anaerobic digestion efficiency; spent mushroom substrate; hydrothermal treatment; mechanical pretreatment method; biogas production

1. Introduction

Spent mushroom substrate (SMS) is solid residual material having depleted nutrients that is produced during mushroom cultivation. The production of 1 kg of mushrooms generates approximately 5 kg of SMS [1]. The characteristics of SMS vary depending on the raw materials, mixing ratio, and mushroom cultivation methods [2]. In particular, different materials and mixing ratios of SMS are used to cultivate different mushroom species, consequently resulting in the generation of SMSs with numerous properties. Sawdust, corncob, cottonseed meal, and beet pulp are the main raw materials of SMS [3]. However, when the generated SMS is left unattended under natural environmental conditions it may cause water pollution and soil contamination. Thus, SMS should be appropriately managed and disposed of. Recently, utilization of SMS through different methods has been actively studied. SMS valorization methods include vermicomposting or composting in horticulture [4]. SMS generated using wheat straw, rice straw, or sugarcane bagasse, having high lignocellulosic content as the raw materials, can be used as alternative feeds for ruminants [5]. Furthermore, lignocellulosic enzymes and other secondary metabolites...
of microorganisms in SMS can be extracted and utilized to produce industrial enzymes (e.g., amylase, cellulase, protease, and pectinase), antibiotics (e.g., penicillin), and organic acids (e.g., citric acid and fumaric acid) [6,7]. Moreover, several studies have investigated the applications of SMS in soil amendment (e.g., soil management, soil structure improvement, prevention of soil erosion, and improvement in soil moisture retention) [8,9] and water pollution remediation [10]. Recently, the consumption of mushrooms has increased because of their health benefits; consequently, mushroom production has increased at an industrial scale using advanced automated facilities. Year-round mushroom cultivation and harvesting through greenhouse farming continuously generates a large amount of SMS. Therefore, such an increase in the SMS volume necessitates the development of effective methods for its valorization.

In addition to the reuse and upcycling of SMS or extraction of useful components from SMS as described above, the value of SMS as feedstock for renewable energy generation has been gaining increasing interest [11]. Particularly, a large amount of carbon-based organic matter containing SMS can be converted into gas and thermal energy to generate electricity after combustion through drying and forming processes in the heat recovery system [12]. Additionally, under the hydrothermal carbonization of SMS, the carbon-rich material in SMS is converted to fixed carbon to produce char [13], thus demonstrating the applicability of SMS as a solid biochar fuel that has improved energy density compared to the energy acquired after the combustion of SMS through simple drying. However, energy input requirements for SMS carbonization act as a limitation, and thus, the effectiveness of energy input and recovery should be evaluated to determine its applicability [14]. Conversely, anaerobic digestion (AD) based on biochemical reactions allows the processing of SMS with minimal energy input; moreover, it reduces the amount of waste generated for final disposal and contributes to energy generation through biogas production [15]. Nevertheless, during SMS processing and biogas production through anaerobic digestion (AD), the low biodegradability of the lignocellulosic components in the raw materials of SMS may hinder the process efficiency [16]. Therefore, research should be conducted to address this issue in order to improve the process efficiency.

Several pretreatment technologies (e.g., chemical, mechanical, biological, thermal, and their combinations) have been introduced to the application of AD using biomass for enhancing the biogas conversion efficiency by increasing the biodegradability of biomass [17]. A chemical biomass pretreatment method using acid or alkali substances can improve the biomass biodegradation rate in the AD process by increasing the hydrolysis rate; however, acid/alkali chemical reagents contained in the biomass after pretreatment should be washed off carefully [18]. This is because chemical reagents in the biomass affect the pH stability of the AD process and cause problems such as corrosion of the anaerobic reactors [19]. Several physical pretreatment methods, such as an increase in the surface area of the substrate by mechanical comminution of biomass or biological pretreatment methods that use hydrolytic enzymes, exhibit limitations such as lower pretreatment efficiency and slower biodegradation rate than the chemical pretreatment methods [15]. Under high temperatures, thermal pretreatment methods effectively remove hemicellulose from lignocellulosic components in the biomass that have low biodegradability. This increases the surface area of the substrate, thus promoting a direct reaction of the substrate with and utilization by microorganisms during the AD process and thereby achieving high efficiency of conversion from biomass to the final product (i.e., biogas) [20]. However, the high energy input needed to sustain the high-temperature pretreatment condition is a drawback of the thermal pretreatment method. Moreover, potential inhibitors, such as furan derivatives, are generated as intermediates during the pretreatment of substrates that have high lignin content, such as SMS [21]. These inhibitors may consequently influence the subsequent AD process [22]. Therefore, to improve the energy efficiency of the AD process, the thermal pretreatment conditions that promote both sufficient pretreatment of the biomass and improve AD efficiency due to the generation of inhibitory intermediates require further investigation.
In this study, the applicability and value of SMS as feedstock for biogas production were evaluated through the biochemical methane potential (BMP) test. To this end, by comparing the level of substrate pretreatment and the improvement in the biogas production yield through mechanical and thermal pretreatment methods, an optimal SMS pretreatment method that can be associated with the AD process was investigated. In particular, the pretreatment efficiency and biogas production of the thermal pretreatment process of SMS were comprehensively evaluated by varying the pretreatment temperature to better understand the formation of SMS degradation products formed during thermal pretreatment and understand the changes in AD efficiency. Furthermore, we aimed to propose optimal conditions for the process design of the thermal pretreatment method that can increase the yield of biogas production and reduce the formation of inhibitory SMS degradation intermediates, which may adversely affect the efficiency of the AD process.

2. Materials and Methods

2.1. Test Material (SMS) and Inoculum for the BMP Test

The SMS used in this study was collected from a king oyster mushroom farm in Hadong-gun, Gyeongsangnam-do in South Korea. Due to the use of continuous water supply for mushroom cultivation, the moisture content of the SMS was high (71.3%), while the carbon, oxygen, and nitrogen contents were 41.0%, 43.1%, and 2.7%, respectively; moreover, its C/N ratio was 15.4 (Table 1).

![Table 1. Characteristics of raw spent mushroom substrate (SMS).](image)

Furthermore, the digested sewage sludge generated at the sewage treatment plant was collected and used as an inoculum for the BMP test. Impurities such as sand granules were removed from the collected sludge using a sieve with a mesh size of 0.5 mm. The resultant sludge was refrigerated at 4 °C until further use.

2.2. Pretreatment of SMS

Before initiating the pretreatment process of the prepared SMS, the SMS was washed with distilled water to remove impurities and was subsequently dried at 70 °C for 24 h to remove moisture and achieve a constant dry weight. The dried SMS underwent mechanical comminution to a size of approximately 10.0 mm to facilitate easy handling of the sample during the pretreatment process.

A thermal pretreatment method was applied, hydrothermal pretreatment (HTP), which can promote biomass hydrolysis at a relatively low temperature. The laboratory-scale HTP reactor (volume = 1 L) was filled with 300 mg of SMS and 300 mL of distilled water, and the headspace was set to approximately 400 mL. Furthermore, to establish anaerobic conditions for the HTP reaction, the reactor was purged with nitrogen gas and the HTP reaction temperature was set to 150 °C, 180 °C, and 210 °C. The SMS was allowed to react with subcritical water for 30 min; subsequently, the effect of increased hydrolysis and production of intermediates on the biogas production yield was compared, studying different
pretreatment temperatures. The mixture of SMS and distilled water was stirred continuously using a mechanical stirring system attached to the reactor (stirring speed = 200 rpm). After HTP, the samples were centrifuged and filtered through a membrane with a pore size of 0.45 µm to obtain a soluble fraction. A soluble fraction of a sample without HTP (i.e., raw) was obtained according to the same procedure. The soluble chemical oxygen demand (SCOD) of raw and HTP samples was determined using a digestion test kit containing dichromate (LCK 514, HACH Co., Loveland, Colorado, USA); the method involved simple digestion and the changes in color were measured using a UV spectrophotometer (DR 2800, HACH Co., Loveland, Colorado, USA).

Some of the dried SMS did not undergo the HTP process; it was milled into fine particles using a laboratory-scale ball mill to examine the level of improvement in biogas production yield of SMS through mechanical pretreatments. The fine crushed particles were sieved and classified into three levels according to the particle sizes (<1.0, 1.0–2.0, and 2.0–10.0 mm). Sieving was conducted following the method for determining the particle size distribution as specified by the British Standards Institution [23]. Subsequently, the biogas production efficiency was compared, examining the different crushed particle sizes.

2.3. BMP Test

The BMP test was conducted by referring to and modifying the method first proposed by Owen et al. (1979) [24]. During the test, the value of SMS as feedstock for biogas production and the improvement in the biogas yield were evaluated with respect to the pretreatment method. A serum bottle (volume = 250 mL) was used as the test bottle and seeding sludge (10 mL) and nutrient medium (90 mL) were injected into the bottle to make up the liquid volume to 100 mL. The nutrient medium was prepared according to the method proposed by Shelton and Tiedje (1984) [25] and the prepared medium facilitated sufficient anaerobic bacterial growth and activity. Each test bottle was injected with mechanically or hydrothermally pretreated SMS at an organic loading rate of 1 g VS/L. Tests were performed using triplicates of the samples for each experimental condition to reduce experimental errors. After injecting the seeding sludge, nutrient medium, and SMS (raw or pretreated), the test bottle was purged with nitrogen gas for approximately 5 min to ensure an anaerobic condition. Subsequently, the test bottle was closed with a butyl rubber stopper and sealed with aluminum foil. All test bottles were stirred at 150 rpm in a temperature-controlled shaking incubator maintained at a constant temperature of 35 ± 1 °C, and the anaerobic digestion was run for 41 d in total. The simple flowchart of the total experimental procedure is shown in Figure 1.

![Figure 1. Flowchart of total experimental procedure.](image)

2.4. Analytical Methods

Proximate analyses of SMS (raw and pretreated), inoculum, and digestate were performed according to the standard test methods E871-82, E872-82, and E1755-01 of the American Society for Testing and Materials (ASTM), respectively [26–28].

To evaluate the effects of lignocellulosic components on the production of furan derivatives and AD efficiency during the HTP process, the fiber composition (neutral detergent fiber, NDF; acid detergent fiber, ADF; and acid digestible lignin, ADL) of the SMS (raw and pretreated) was analyzed according to the methods specified in the Association of Official Analytical Chemists’ official methods 973.18 and 2002.04 [29]. The lignin content
was determined through the ADL gravimetric method, while hemicellulose and cellulose contents were calculated based on the differences between the values of NDF and ADF and ADF and ADL, respectively.

The amount of biogas produced from the SMS (raw and pretreated) during the BMP test was monitored and measured using the flow meter scale by connecting the t-valve installed on top of the serum bottle to the gas flow meter. The biogas composition (i.e., CH\(_4\), CO\(_2\), and N\(_2\)) and volatile fatty acids (VFAs) in the digestate were determined using a thermal conductivity detector (TCD) and a flame ionization detector (FID) attached to a gas chromatography (GC) meter (ACME 6100, Younglin, Republic of Korea), respectively. While analyzing the biogas composition, helium was used as a carrier gas and the temperatures of the TCD, injector, and oven were set to 120 °C, 120 °C, and 35 °C, respectively. The measured amounts of biogas and methane production were converted to standard conditions (273 K, 101.325 kPa). The FID and injector temperatures of GC for VFA analysis were set to 250 °C and 240 °C, respectively, and the oven temperature was maintained at 100 °C for 2 min and then increased to 190 °C (heating rate = 10 °C/min).

All analyses were conducted in triplicate and the corresponding mean value was calculated. Analysis of variance was conducted for evaluating the significant differences, and the differences were determined to be significant at \( p < 0.05 \).

3. Results and Discussion
3.1. Effects of Pretreatment on SMS Characteristics

Table 2 outlines the characteristics of raw SMS, hydrothermally pretreated SMS, and mechanically pretreated SMS.

**Table 2. Changing characteristics of SMS by hydrothermal and mechanical pretreatment.**

|     | Hydrothermal (°C) | Mechanical (mm) |
|-----|-------------------|-----------------|
|     | 150 | 180 | 210 | <1 | 1–2 | >2 |
| Proximate analysis (% w/w) | Moisture \( ^a \) | 84.8 | 85.0 | 85.3 | 44.8 | 63.6 | 65.1 |
|     | Total Solid (TS) \( ^a \) | 15.2 | 15.0 | 14.7 | 55.2 | 36.4 | 34.9 |
|     | Volatile Solid (VS) \( ^b \) | 89.6 | 89.0 | 87.6 | 83.4 | 87.6 | 88.7 |
| Elemental analysis (% w/w) | C \( ^b \) | 39.0 | 39.7 | 44.7 | 41.0 | 41.0 | 41.0 |
|     | H \( ^b \) | 5.12 | 4.81 | 4.70 | 5.33 | 5.33 | 5.33 |
|     | O \( ^b \) | 45.5 | 43.9 | 37.8 | 43.1 | 43.1 | 43.1 |
|     | N \( ^b \) | 2.28 | 2.95 | 3.13 | 2.66 | 2.66 | 2.66 |
|     | S \( ^b \) | 0.220 | 0.240 | 0.270 | 0.200 | 0.200 | 0.200 |
|     | C/N | 17.1 | 13.5 | 14.3 | 15.4 | 15.4 | 15.4 |

\( ^a \) % by wet weight, \( ^b \) % by dry weight.

As the HTP process of SMS requires large amounts of water, the moisture content of the SMS sample was high; however, when compared with the untreated SMS, no significant changes in the physicochemical properties of the solids were observed for either HTP or mechanical pretreatment methods. However, in terms of substrate solubilization through SMS pretreatment, the potential of increasing biogas production through different pretreatments was confirmed (Figure 2).

The SCOD concentration representing solubilization in the mechanically pretreated SMS and untreated CMS did not vary considerably; however, the SCOD concentration of the hydrothermally pretreated SMS increased with the pretreatment temperature. This indicated that the SMS was crushed into smaller particles during mechanical pretreatment, leading to changes in the morphology and an increase in the surface area that can directly benefit microorganisms in the future AD process [30]; however, the solubilization effect of the mechanical pretreatment was not significant. Conversely, during the HTP process, the biomass swelled during the hot water reaction with SMS, which in turn increased
the availability of active substrate components in a soluble state from the previous solid state. Previous studies have reported an improvement in biomass solubility after HTP. For example, Zhang et al. (2018) [31] and Phuttaro et al. (2019) [32] conducted biomass pretreatment using a hot water reaction under conditions similar to those of HTP conducted in this study and examined the changes in the fiber composition. The results of the present study and previous studies indicated that during biogas production using SMS, the HTP method, similar to the mechanical pretreatment method, changes the substrate morphology and increases its surface area. Furthermore, by improving the physicochemical properties of SMS, the HTP method is expected to increase the potential of the microorganisms in the AD process to directly utilize the substrate, thus increasing biogas production.

VFAs were produced as a result of the HTP process, and those concentrations increased with increasing HTP temperature (Figure 3). Acetic acid was the dominant species of VFA with a maximum concentration of 1003.7 ± 13.8 mg/L. The organic acids formed (including acetic acid) are known to be associated with the degradation of the acetyl group in hemicellulose [33]. The high temperature and pressure during HTP could boost generation of hydrogen ions through the autohydrolysis of water (the dominant feature of the pretreatment method), and the H+ ions act as a catalyst for destroying the hemicellulose structure [34]. Moreover, the VFA production and its accumulation could lower pH and thereby might result in the formation of acidic conditions.

![Figure 2](image2.png)

**Figure 2.** Changes in soluble chemical oxygen demand (SCOD) concentrations according to different pretreatment conditions.

![Figure 3](image3.png)

**Figure 3.** VFA concentrations after HTP in hydrolysate.
3.2. Effects of Pretreatment on AD

Cumulative methane production (CMP) for raw SMS and hydrothermally and mechanically pretreated SMS is shown in Figure 4.

The CMP yields of raw SMS and pretreated SMS were 111.64 mL/g VS and 85.71–155.08 mL/g VS, respectively. In some pretreatment conditions, the CMP yield was lower than that of the untreated SMS. Furthermore, the CMP yields of hydrothermally and mechanically pretreated SMS were 87.94–155.08 mL/g VS (Figure 4a) and 85.71–119.21 mL/g VS (Figure 4b), respectively. For the CMP from mechanically pretreated SMS, the smaller substrate size in this study showed slower and lower CMP yield. The CMP of substrate size >2 mm was higher than that of the raw; there was no significant difference. The CMP of smaller substrate sizes than >2 mm (i.e., 1–2 and <1 mm) were comparable. In general, smaller particle size could give more chances for anaerobic microorganisms to make contact with substrate based on promoting the accessible surface area of the substrate [35]. However, the contrast tendency in CMP for different substrate sizes and the observed result could be explained by the adverse effect of rapid substrate degradation. The fine particle size of the substrate is degraded too fast, the anaerobic microorganisms cannot adapt quickly enough, and methanogenesis activities might be hindered [15]. The hydrothermally pretreated SMS samples showed higher CMP yield than mechanically pretreated SMS in all conditions except 210 °C. This was because of increased substrate solubility owing to the improvement in physicochemical properties and the effect of morphological changes and increased surface area of the substrate due to the pretreatments, as described above [36].

The theoretical methane potential (TMP) of the pretreated SMS was calculated according to the method proposed by Rich (1963) and the biodegradability of the samples based on the ratio of CMP to TMP (CMP/TMP) is shown in Figure 5.

In the case of the mechanical pretreatment method with comminution, the elemental composition did not change during the pretreatment of the SMS; thus, the TMP values were the same (210.55 mL/g VS) according to the pretreatment level. Conversely, in the case of the hydrothermally pretreated SMS samples, the elemental composition (relative ratio) of the substrate changed with an increase in the pretreatment temperature, and the TMP values increased from 177.30 to 276.22 mL/g VS. Compared with the biodegradability of raw SMS (53.02%), the biodegradability of the hydrothermally and mechanically pretreated SMS was higher (87.46% and 61.37%, respectively); consequently, the HTP method showed higher pretreatment efficiency than the mechanical pretreatment method. These results...
confirmed that the HTP method was more effective in improving the solubility of SMS than the mechanical pretreatment method, similar to the results of the changes in CMP yield.

![Figure 5. Biodegradability of the raw and pretreated SMS samples.](image)

The CMP yield and biodegradability results of the HTP of SMS indicated that biogas production increased as the pretreatment temperature increased up to a certain temperature (210 °C) above which the biogas production decreased. This trend in biogas production can be explained by understanding the fragmentation process of the lignocellulosic components in SMS at high temperatures. Song et al. (2019) [37] and Ko et al. (2015) [38] reported that lignocellulosic materials generate furan derivatives, phenolic compounds, and soluble organics in the form of organic acids during hydrolysis. These compounds can inhibit the activity of microorganisms involved in the conversion of organic matter in the biogas during the AD process. Particularly, furan derivatives (e.g., 5-hydroxymethylfurfural and furfural), which are by-products of sugars produced as hydrolysates during the HTP processing of lignocellulosic materials, can directly affect the activity of methanogenic bacteria in the AD process [39,40]. Similarly, several studies have reported a decrease in the yield of biogas production when the biomass is pretreated at high temperatures [15,32,39]. In this study, the biogas production yield was affected by a temperature of approximately 200 °C, depending on the type and properties of the biomass to be pretreated. Therefore, the thermal pretreatment of SMS using HTP can improve the biogas yield by promoting hydrolysis; however, severe pretreatment conditions may negatively affect the bioconversion yield, and thus, a novel design of optimal pretreatment conditions for SMS is imperative.

4. Conclusions

In this study, the applicability of the AD process, which facilitates the final disposal of SMS and biogas production with minimal energy input, was evaluated as a method of SMS valorization. Particularly, mechanical and HTP methods were applied to improve the biodegradability of the substrate, because poor biodegradability due to SMS feedstock properties can limit AD efficiency. Moreover, conditions required for the optimal design of the HTP process were investigated. During the SMS AD process, some organic components in the substrate were converted to biogas, and the biogas production yield was increased by applying mechanical and HTP methods. Among these, the HTP method increased the biogas yield more effectively than the mechanical pretreatment method by increasing the substrate solubilization efficiency. Additionally, the HTP method improved the physicochemical properties of the substrate and increased the surface area for reaction by microorganisms by changing the morphology of the substrate during the substrate
pretreatment process. However, the results also indicated that under severe conditions, the HTP method generated degradation products during hydrolysis of the SMS biomass, thereby inhibiting the microbial activity related to the AD process. Therefore, the results confirmed the applicability of SMS in biogas production and the role of the HTP method for improving the biogas yield from SMS. However, optimal pretreatment conditions that can prevent reduction of the bioconversion yield of the biomass should be designed in the future to promote SMS valorization.

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