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AGRICULTURAL WASTE AFTER CULTIVATION OF *PLEUROTUS OSTREATUS* AS A POTENTIAL BIOMASS RESOURCE FOR ETHANOL PRODUCTION

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ABSTRACT

Spent mushroom substrate (SMS) gathered after *Pleurotus ostreatus* cultivation was investigated as a potential biomass resource for ethanol production. The chemical composition, morphological characteristics, total saccharized sugars obtained from enzyme hydrolysis and ethanol production from SMS were compared with those from mushroom substrate (MS). The experiments were conducted at Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom province, Thailand from 2016-2017. SMS had lower lignin content than MS, but higher cellulose and hemicellulose (58.15%). Analysis through scanning electron microscope (SEM) showed that the SMS had a large numbers of pores than MS, leading to an increased availability of surface area for pre-treatment and enzymatic activity. These results are related to findings indicating that SMS requires milder pre-treatment conditions and easier saccharification through enzymatic hydrolysis than MS. When SMS was used as a substrate for the simultaneous saccharification and fermentation (SSF) process, the maximum ethanol concentration produced from SMS hydrolysate by *Saccharomyces cerevisiae* ATKU132 was 10.00 g/L, while the corresponding value for MS hydrolysate was 4.12 g/L. These results indicated that SMS, available from agricultural waste all year round, can be used as a potential source of raw material for bioethanol production.

Key words: spent mushroom substrate, agricultural waste, lignocellulosic biomass

INTRODUCTION

The global energy crisis has increased the need for biofuel production using renewable raw material. Lignocellulosic biomass is an attractive source of hexose and pentose sugar substrates, making it a potential source for ethanol production. Agronomic and forestry residues can provide promising feedstock sources for lignocellulosic ethanol production, since they are renewable, abundant, cheap, and do not compete with food sources (FitzPatrick et al. 2010). However, lignin-carbohydrate cross-linkages and the crystalline structure of cellulose hinder the successful implementation of a cellulosic ethanol-based biorefinery (Castoldi et al. 2014).

Pleurotus ostreatus is an edible mushroom cultivated globally, including in Thailand, and is a good source of carbohydrates, essential amino acids, minerals, and vitamins (Cohen et al. 2002). This saprophytic fungus can form colonies on numerous forms of lignocellulosic wastes (Elisashvili et al. 2008, Luz et al. 2012, Fernandes et al. 2015). In Thailand, *P. ostreatus* is cultivated commercially using rubber wood (*Hevea brasiliensis*) sawdust supplemented with rice bran and other additives (gypsum, lime, calcium sulphate, or magnesium sulphate) (Kwon and Thatithatgoon 2004).

The genus *Pleurotus* belongs to the group of white-rot fungi, which are known for their ability to synthesize extracellular lignocellulosic enzymes. Enzymes secreted from *Pleurotus* digest lignocellulosic substrates for fruiting body production (Taniguchi et al. 2005). Lignin degradation enzymes (laccases and peroxidases) and hydrolytic enzymes (cellulases and xylanases) are secreted from *P. ostreatus* during cultivation (Ghosh et al.1998, Elisashvili et al. 2008, Castoldi et al. 2014). The removal of lignins and/or hemicelluloses, which impede enzymatic hydrolysis, can substantially reduce the recalcitrance of biomass into chemical or enzymatic hydrolysis products. The exposed complex of celluloses and hemicelluloses are then easily hydrolyzed into simple sugars (Taniguchi et al. 2005, Zhu et al. 2013). Spent mushroom substrate (SMS), an organic waste by-product of mushroom cultivation, is composed of fungal mycelia and unutilized substrates, with partial degradation products of lignocellulose by extracellular lignocellulosic enzymes produced by mushroom (Phan and Sabaratnam 2012). Generally, SMS is disposed by spreading it on land, burying, composting with household waste, or through burning in open fields. Therefore, this agricultural waste was investigated for its potential production of valuable substances. In the present study, SMS from *P. ostreatus* cultivation was examined as a biomass resource for the production of ethanol during the simultaneous saccharification and fermentation (SSF) process. The chemical composition of SMS and total saccharized sugars of the SMS hydrolysate were also investigated and compared with mushroom substrate (MS).

MATERIALS AND METHODS

Biomass material and compositional analysis

Rubber tree sawdust (without additives), MS, and SMS were obtained after 4-5 months of *P. ostreatus* cultivation from a local mushroom farm located in Nonthaburi province, Thailand. After which, the materials were oven-dried at 60 °C for 48-60 h prior to chemical analysis. The dried material was analyzed for extractives, lignin, and alpha-cellulose according to the Technical Association of the Pulp and Paper Industry (TAPPI) methods of solvent extractives in wood and pulp (TAPPI-T204-cm-97 1997), acid-insoluble lignin in wood and pulp (TAPPI-T222-om-02 2002), and alpha-, beta- and gamma-cellulose in pulp (TAPPI-T203-cm-99 1999). Holocellulose was analyzed by Browning's method (Browning 1967).

Pre-treatment and enzyme hydrolysis

SMS and MS were subjected to ammonia pre-treatment, either with or without heat. Oven-dried samples were soaked in 3% (v/v) NH_4OH, with a solid to liquid proportion of 1:10 (w/v) at ambient temperature for 1 h with shaking at 100 rpm, and then heated at 121 °C for 30 min. The pre-treatment condition without heat was performed by soaking the biomass in 3% (v/v) NH_4OH at ambient temperature for 24 h, with shaking at 100 rpm. The mixture was filtered through cheesecloth to separate the solid residue. The solid material was washed with water until it reached a neutral pH and then dried at 55 °C for 48 h.

The hydrolysis mixtures contained 1 g of dry weight ammonia pre-treated with SMS or MS, as well as 15 filter paper unit (FPU)/g of substrate of commercial cellulase enzyme (Accellerase 1500, Genencor, Suzhou, China) in 50 mL of 50 mM sodium citrate buffer (pH 4.8). The mixtures were incubated at 50 °C, and the hydrolysate samples were taken every 24 h to analyze their saccharized sugar concentrations. Total sugar yield was calculated as the sum of glucose and xylose present in the hydrolysate.

Simultaneous saccharification and fermentation (SSF)

The SSF experiments were carried out in an Erlenmeyer flask placed in a shaking incubator at a speed of 100 rpm. SSF reaction mixtures containing 15 g of ammonia-pretreated material at a solid loading of 10% (w/v), 50 mM citrate buffer (pH 4.8), and fermentation nutrients (peptone 1 g /L, yeast extract 5 g/L, KH_2PO_4 5 g/L, $(NH_4)_2SO_4$ 2 g/L, and $MgSO_4.7H_2O$ 0.2 g/L) were sterilized in an autoclave at 121 °C for 15 min, and then Accellerase 15 FPU/g of substrate was added to the mixture.

After pre-saccharification at 50 °C for 16 h at 100 rpm, *Saccharomyces cerevisiae* ATKU132 (Thammasittirong et al. 2012) was inoculated into the medium to a final concentration of 5×10^5 cells/mL. SSF was conducted at 40 °C for 48 h. Fermentation samples were taken every 12 h to determine sugar and ethanol concentrations using high performance chromatography (HPLC).

Scanning electron microscope (SEM)

SMS and MS morphology were observed using a scanning electron microscope (Quanta 400, FEI, Brno, Czech Republic) with an operating voltage of 20 kV.

Analytical method

Sugars, glucose and xylose, and ethanol concentrations were analyzed using HPLC (Waters 600E, Waters Inc., Milford, USA), with a refractive index detector and a Sugar Pak I column at 85 °C. The mobile phase was deionized water at a flow rate of 0.5 mL/min (Senatham et al. 2016). HPLC analysis was performed at Faculty of Liberal Arts and Science, Kasetsart University, Kamphaeng Saen Campus, Thailand.

Statistical analysis

Statistical analysis analysis was performed using SPSS 16.0 statistical package (SPSS Inc., Chicago, USA). Significance was defined as $p < 0.05$. Data are represented as mean ± standard deviation.

RESULTS AND DISCUSSION

Chemical composition

The chemical composition of the rubber tree wood sawdust before and after *P. ostreatus* cultivation were characterized (Table 1). The chemical components of SMS were significantly lower than those from MS. SMS had less lignin content than MS, suggesting that a portion of lignin was degraded by *P. ostreatus* during mushroom cultivation. The lignin network covering holocellulose is broken down by fungal extracellular ligninolytic enzymes, such as peroxidases and laccases (Isikhuemhen and Mikiashvilli 2009, Castoldi et al. 2014, Corrêa et al. 2016). Spent mushroom substrate also reduced cellulose and hemicellulose content, but a large amount of these polysaccharides were still present in this agricultural waste (approximately 58.15%). These results indicate that SMS has the potential as a source of fermentable sugars for ethanol production.

Table 1. Chemical composition on % dry basis of MS and SMS

Substrate	Cellulose (%)	Hemicellulose (%)	Lignin (%)
MS	40.89 ± 0.11^a	31.40 ± 0.05^a	24.63 ± 0.07^a
SMS	31.95 ± 0.08^b	26.20 ± 0.09^b	22.75 ± 0.01^b

Different superscript letters (a and b) in each column indicate significant differences between MS and SMS ($p<0.05$).
Values represent mean ± standard deviation from three independent experiments.

Morphological characteristics

Morphological changes in the cell wall structure of SMS and MS characterized through SEM contained a large numbers of holes, while MS had a flat and intact surface (Fig. 1).

Corrêa et al. (2016) found significant changes in corncob waste after *P. pulmonarius* cultivation, which corresponded to visible holes and loosening of the structure. Castoldi et al. (2014) also reported similar morphological changes for *Eucalyptus grandis* sawdust treated with *P.*

pulmonarius, where the porosity of the lignocellulosic material resulted from the removal of lignin and weakening of the compact lignocellulosic structure.

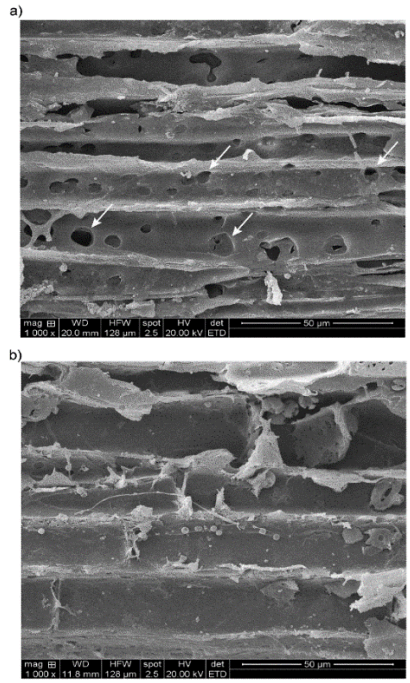

Fig. 1. Scanning electron microscope of rubber tree cell walls of sawdust after *P. ostreatus* cultivation, SMS (a) and before *P. ostreatus* cultivation, MS (b). Arrows indicate the holes.

Pre-treatment and enzyme hydrolysis

Alkaline pre-treatments using ammonia with or without heat and/or pressure have a high delignification efficiency (Aita et al. 2011, Zhu et al. 2013). In this study, SMS and MS samples were treated with aqueous ammonia at 121 °C for 30 min or at ambient temperature for 24 h. The major sugars generally obtained during enzyme hydrolysis were glucose and xylose (Senatham et. al. 2016, Visser et. al. 2015). The results of sugar analysis revealed that pre-treated SMS released 11.50-24.00 g/L and 5.00-8.35 g/L of glucose and xylose, respectively, while pre-treated MS released 4.50-12.50 g/L and 0.50-1.00 g/L of glucose and xylose, respectively. Total sugars yields based on the dry weight of the pre-treated biomass after enzymatic hydrolysis are shown in Fig. 2. There was a clear improvement in total released sugars from SMS pre-treated with ammonia at both temperatures compared with the untreated control. The cleavage of the lignin-carbohydrate complex after ammonia pre-treatment can result in pore formation and swelling of the biomass, thus increasing the surface area and subsequently improving the digestibility of the biomass by cellulase enzymes (Lin et al. 2010, Zhu et al. 2013). The significant increase in total sugars released from the pre-treated SMS and MS in this study can be attributed to the depolymerization of lignin-carbohydrate complex.

Pre-treatment of SMS with ammonia at 121 °C for 30 min, followed by enzymatic hydrolysis, led to the highest total sugar yield of 32.35 g/L after 96 h, representing an increase of about 114% over the untreated control. Hydrolysis of the pre-treated MS with ammonia at 121 °C for 30 min gave a higher total sugar yield (13.50 g/L) at 96 h of about 108% compared with that of the pre-treated MS at ambient temperature (6.50 g/L). As expected, SMS material had higher total sugar yield and required milder pre-treatment conditions than the MS material.

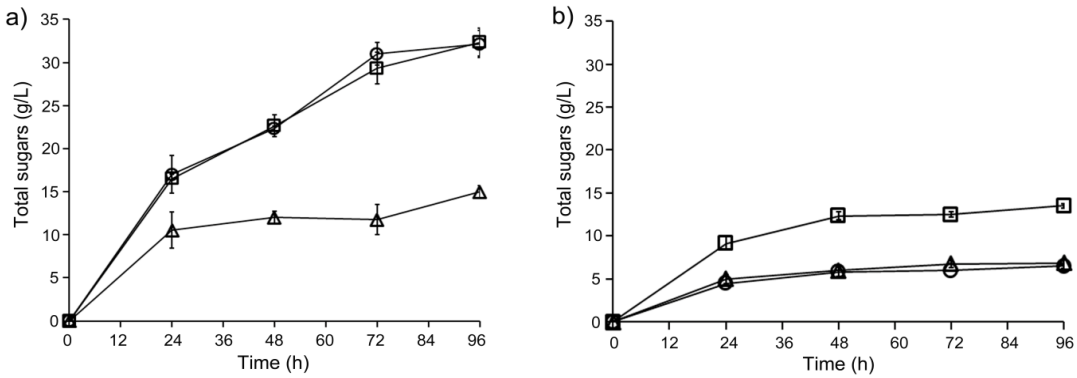

Fig. 2. Total sugars profile after enzymatic hydrolysis of the ammonia pre-treated SMS (a) and the ammonia pre-treated MS (b) at 121°C (square), and at ambient temperature (circle), compared with the untreated control (triangle). Values represent mean ± standard deviation from three independent experiments.

Hiyama et al. (2011) reported that waste mushroom medium and stored waste mushroom medium after *Lentinus edodes* cultivation were more susceptible to cellulase activity than mushroom medium. *L. edodes* is a type of white-rot fungi, which can disrupt the lignocellulosic structure by partially breaking down the lignin and carbohydrate complex. The maximum glucose yield of the partially-degraded stored waste mushroom medium was 200.50 mg/g of biomass, which was 4.5 times higher than the value of MS. Lee et al. (2008) also reported that the sugar yield of waste mushroom logs after *L. edodes* cultivation was higher than that of normal wood. Balan et al. (2008) found that subjecting *P. ostreatus* SMS to an ammonia fiber expansion (AFEX) showed significantly higher glucan and xylan conversion than when treating MS with AFEX directly. The higher yield of sugars released from SMS could be due to the removal of lignin content following cultivation of *P. ostreatus*. During the cultivation process, mushrooms released hydrolyzing and oxidizing enzymes to degrade material for growth, thereby weakening the compact lignocellulosic networks and promoting the formation of holes in the cell wall of SMS. Partial delignification and the exposed celluloses and hemicelluloses structures of SMS provide easily degradable structures for pre-treatment and enzyme hydrolysis compared with the rigid structure of MS (Balan et al. 2008, Castoldi et al. 2014, Corrêa et al. 2016, Hiyama et al. 2011).

The results in this study suggest SMS required a mild pre-treatment before enzymatic hydrolysis, and was more easily hydrolysable into sugars than MS. Considering the energy conserved, SMS is an economically-feasible source for ethanol production. However, operating conditions for pre-treatment and hydrolysis, including the type of raw material, influence the generation of the saccharized sugars (Asada et al. 2011, Teoh et al. 2014). Therefore, optimization of the pre-treatment and hydrolysis conditions is required to increase sugar yield.

Simultaneous saccharification and fermentation (SSF)

Based on the obtained saccharized sugar yield and the economics of pre-treated conditioning, alkaline pre-treated biomass under ambient temperature conditions was selected for the SSF experiment to evaluate the feasibility of SMS for ethanol production. The time profiles of the total sugars and ethanol yields with SSF are shown in Fig. 3. After 16 h of pre-saccharification, a total sugars amount of 25.33 g/L and 16.00 g/L were released from pre-treated SMS and MS, respectively, from an initial 10% (w/v) solids (on a dry matter basis). The maximum ethanol concentration of SMS by *S. cerevisiae* ATKU132 was 10.00 g/L, while the corresponding values for MS was 4.12 g/L. It is clearly demonstrates that SMS, which has less lignin, does improve the hydrolysis process and ethanol production.

The concentration of ethanol decreased after 24 h and 36 h of SMS and MS fermentation, respectively. These results suggested that ethanol can be utilized as a source of carbon by *S. cerevisiae* ATKU132, and also partly due to ethanol evaporation at 40 °C. Hideno et al. (2007) reported that the maximum ethanol concentration produced in SSF from hot water treated spent sawdust after cultivation of *Grifola frondosa* was 16.00 g/L. Oguri et al. (2011) reported that maximum ethanol concentrations were 5.50 g/L and 17.70 g/L when 5% (w/v) and 20% (w/v) corncob-based spent mushroom waste, respectively, were used as substrate for saccharification and fermentation.

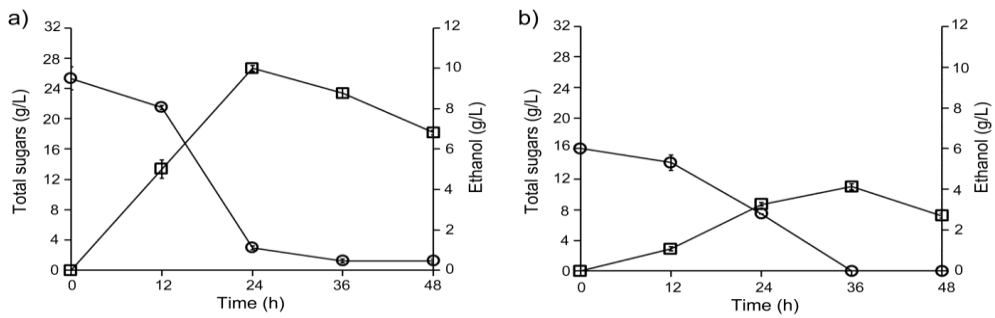

Fig. 3. Ethanol production in SSF by *S. cerevisiae* ATKU132 from SMS (a) and MS (b). Total sugars (circle), ethanol (square). Values represent mean ± standard deviation from three independent experiments.

The type of biomass material, pre-treatment, and hydrolysis conditions, type of ethanologenic yeast strains, and fermentation conditions affected the saccharized sugars and ethanol yields (Asada et al. 2011, Thammasittirong et al. 2012, Teoh et al. 2014). Based on the results from the current study, it is useful to report the advantages of waste mushroom sawdust after *P. ostreatus* cultivation as a potential biomass resource for ethanol production.

CONCLUSION

SMS produced from cultivation of *P. ostreatus* contained less lignin content than MS, but it still had a high amount of cellulose and hemicellulose. According to the results of the reduced lignin content and the appearance of pores in SMS, this partially-degraded agricultural waste was then easily converted to fermentable sugars using ammonia pre-treatment under mild conditions. In SSF, SMS produced more ethanol than MS, implying that SMS, a cost-effective form of biomass, could be used as a potential material for the production of fermentable sugars related to ethanol production. Further study is required on the optimization of the pre-treatment and hydrolysis conditions to improve the yield of released sugars.

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