Reuse of Enzymatic Hydrolyzed Residues from Sugarcane Bagasse to Cultivate *Lentinula edodes*

Wen Wang,[a] Jing Liu,[a,b] Xinshu Zhuang,[a,*] Wei Qi,[a] Qiang Yu,[a] Qiong Wang,[a,b] and Zhenhong Yuan [a,*]

In the conversion process of lignocellulose into fuel ethanol, the enzymatic hydrolyzed or fermented lignocellulosic residues are produced as byproducts. In order to further recycle these byproducts, this study used the enzymatic hydrolyzed residues of sugarcane bagasse (EHR-SCB) as the media for culturing *Lentinula edodes*. The sugarcane bagasse (SCB), pretreated by liquid hot water (LHW), was hydrolyzed for 120 h with cellulase to obtain the EHR-SCB. The EHR-SCB was mixed with wheat bran and gypsum powder in a certain proportion to make six kinds of media. The media containing 50% and 60% EHR-SCB could cultivate *Lentinula edodes*, due to their rational ratio of carbon to nitrogen and mineral contents. Compared with *Lentinula edodes* sold in the market, the one cultured in the medium containing 50% EHR-SCB had a little higher content of amino acids except cysteine and proline, and mineral elements except zinc, while that cultured in the medium containing 60% EHR-SCB had a lower content of amino acids except serine, glutamic acid, glycine, methionine, tyrosine, and arginine, and mineral elements except selenium, copper, chromium, and manganese. After harvesting *Lentinula edodes*, the mushroom residues might be further used as media for obtaining value-added products or composting for manure.

Keywords: Sugarcane bagasse; Enzymatic hydrolysis; Lentinula edodes; Cultivation; Liquid hot water

Contact information: a: Guangzhou Institute of Energy Conversion, Key Laboratory of Renewable Energy and Gas Hydrate, Chinese Academy of Sciences, Guangzhou 510640, China; b: Graduate School of Chinese Academy of Sciences, Beijing 100039, China;
** Corresponding author: zhuangxs@ms.giec.ac.cn; yuanzh@ms.giec.ac.cn*

INTRODUCTION

There has been an increasing number of studies on the conversion of ligno-cellulose to fuel ethanol for many years due to a variety of reasons including renewable green energy sources, shortage of fossil fuels in a near or medium term future, reduction of greenhouse gas emission caused by utilization of fossil fuels, and so on (Galbe and Zacchi 2012; Huang *et al*. 2009). Lignocellulosic biomass mainly consists of cellulose, hemicellulose, and lignin, which are connected with non-covalent and covalent bonds (Zhang *et al.* 2009).

Two major pathways have been identified as the most promising routes for cellulosic ethanol production: thermochemical and biochemical conversion (Dwivedi *et al.* 2009; Gonzalez *et al.* 2012). In the thermochemical conversion process, all of the components of the lignocellulosic materials are gasified to produce syngas, which consists of CO, H_2, CO_2, and N_2. The syngas is cleaned up and reformed to be either fermented or catalyzed into ethanol. In the biochemical conversion process, cellulose and

hemicellulose are hydrolyzed to fermentable sugars through acid or enzymatic hydrolysis, while lignin still remains in the solid residues.

The biochemical conversion process has received more attention due to its similarity to the current ethanol production and expected lower capital cost (Čuček *et al.* 2011). The biochemical conversion of lignocellulosic materials into ethanol comprises the following four steps: (1) pretreatment to break down the compact structure of feedstocks and make the cellulose and hemicellulose more accessible to the subsequent steps; (2) acid and enzymatic hydrolysis of cellulose and hemicellulose into fermentable sugars; (3) fermentation of sugars to ethanol with microorganisms; (4) separation and concentration of ethanol from fermentation broth (Mussatto *et al.* 2010; Sánchez and Cardona 2008).

The solid residues produced from the conversion process of cellulose to ethanol are often combusted to generate steam or electricity. After ethanol production, the solid residues still contain cellulose, which can be used to prepare the medium for culturing edible mushroom or single-cell protein to further recycle lignocellulosic residues. Mushroom mycelia can utilize the lignocellulosic wastes, such as paddy straw, cotton wastes, coffee waste, tree saw dust, and sugarcane bagasse, to reduce pollution through production of a group of complex extracellular degrading enzymes, and the spent substrate left after harvesting the mushrooms can be used as a livestock feed supplement due to its simpler and more readily digestible form after its modification by the mushroom enzymes (Chang 2008). The cultivation of edible mushrooms is not only environmentally-friendly, but also profitable. World production of cultivated edible mushrooms is estimated to be almost 5 million tonnes, valued at about $9.8 billion per year (Belay 1998). Edible mushrooms have high nutritive and medicinal values. They are rich in proteins, low in cholesterol, and contain various vitamins and minerals, which are advantageous for human health (Chang 2008; Wang 2004). Their extracts have been demonstrated to be beneficial for the cardiovascular system and to contribute anticancer, antiviral, antibacterial, antiparasitic, anti-inflammatory, and antidiabetic effects (Shah *et al*. 2011; Wang 2004). Therefore, it is a promising way to benefit from the cellulose ethanol process in culturing edible mushrooms through the utilization of hydrolyzed or fermented residues.

In our laboratory, liquid hot water is the main technology for pretreating lignocellulose. After LHW pretreatment, liquid and solid fractions can be obtained. Xylo-oligosaccharide will be refined from the liquid fraction to be used as a food additive or hydrolyzed to xylose for ethanol production. Sugar solution and solid residues can be obtained from the solid fraction through enzymatic hydrolysis. The sugar solution is fermented into ethanol, and the solid residue is used as a medium to culture edible mushrooms. After harvesting edible mushrooms, the leftover will be combusted for providing steam or electricity, or converted into fertilizer or forage.

In this study, the solid residues from enzymatic hydrolysis of LHW-pretreated SCB were prepared as media for culturing edible mushrooms. Due to the high lignin content in the enzymatic-hydrolyzed lignocellulose, wood-rotting edible mushrooms, which can degrade lignin into low molecule weight nutrients, were cultured. *Lentinula edodes* is one of the wood-rotting edible mushrooms; it was selected in order to determine whether the hydrolyzed residues of SCB are suitable to be media for culturing edible mushrooms.

EXPERIMENTAL

Raw Materials and Cellulase

SCB, provided by the National Engineering Research Center for Non-food Biorefinery, Guangxi Academy of Sciences, China, were milled and screened through 40-60 mesh sieves, then washed and dried at 60 ^{o}C to a constant weight. Wheat bran was bought on the Internet: http://item.taobao.com/item.htm?id=12988155081. *Lentinula edodes* was bought from the Changban vegetable market in Guangzhou, China.

Cellulase, produced from a strain of *Trichoderma reesei*, was purchased from KDN Biotech Group Co. Ltd. China. The filter paper activity of cellulase is 150 FPU/mL assayed according to Ghose (1987). Polyoxyethylene (20) sorbitan monooleate (Tween 80), chemical pure, was produced by Tianjin Kermel Chemical Reagent Co., Ltd. China.

Pretreatment and Enzymatic Hydrolysis

LHW pretreatment and fed-batch enzymatic hydrolysis were carried out as previously described (Wang *et al.* 2012a). The differences from the previous were that the substrate was SCB and the addition of polyoxyethylene (20) sorbitan monooleate was 0.125 mL/g dry material. The enzymatic hydrolyzed residues of SCB were stored at -20 ^{o}C for the following use.

Culture of *Lentinula edodes*

The surface of *Lentinula edodes* was sterilized using 75% ethanol. The connection of cap and stipe was selected for inoculating in 150 mL flask containing potato dextrose agar (PDA) medium, which was made as follows: 20 g glucose and 25 g agar were added into potato juice pressed from 200 g peeled potato cooked in 800 mL boiling water for 30 min, and distilled water was supplemented until the volume was 1000 mL. After the medium was overgrown with the mycelia under the conditions of 25 ^{o}C and 65% relative humidity (RH), a piece of medium was dug out to inoculate in the 150 mL flask containing the primary culture medium (PCM) consisting of 78% (g/g dry medium) raw SCB, 21% wheat bran, and 1% gypsum powder. After the PCM was overgrown with the mycelia under the same conditions as before, several pieces of media were dug out to inoculate, respectively, in the aseptic bags containing the culture media (CM) as follows: (A) 50% EHR-SCB, 49% wheat bran, 1% gypsum powder; (B) 60% EHR-SCB, 39% wheat bran, 1% gypsum powder; (C) 70% EHR-SCB, 29% wheat bran, 1% gypsum powder; (D) 80% EHR-SCB, 19% wheat bran, 1% gypsum powder; (E) 90% EHR-SCB, 9% wheat bran, 1% gypsum powder; and (F) 99% EHR-SCB, 1% gypsum powder. When the CM were overgrown with mycelia under the same condition as before, they were separated from the bags and placed in the 250 mL sterilized beakers. One milliliter sterile water was added, and the CM were cultured at 25 ^{o}C and 65% RH during the daytime, while at 15 ^{o}C and 85% RH during the nighttime. This was repeated in the following days until the buttons grew out. Then *Lentinula edodes* were matured in the media for nearly one week under the conditions of 25 ^{o}C and 90% RH. The PDA medium and utensils were sterilized at 115 ^{o}C for 20 min, while the PCM and CM were sterilized at 121 ^{o}C for 60 min. The water contents of the PCM and CM were 55% to 65%.

Analytic Methods

Composition analysis was carried out according to a National Renewable Energy Laboratory (NREL) analytical procedure (Sluiter *et al.* 2008). The sugar concentrations

were measured as before (Wang *et al.* 2012a). The glycan conversion was calculated as before (Wang *et al.* 2012b). The carbon (C), nitrogen (N), and sulfur (S) contents of *Lentinula edodes* were measured with an elemental analyzer (Vario EL cube), while other mineral contents were analyzed by inductively coupled plasma emission spectrometer (ICP, OPTIMA 8000). These contents were the mass ratio of elements to dry materials. The amino acids contents and the mass ratio of amino acids to dry *Lentinula edodes* were determined by automatic amino acid analyzer (Hitachi L-8800).

RESULTS AND DISCUSSION

Composition of Feedstocks and Media

Compositional analyses of untreated and treated SCB, wheat bran, and media are shown in Table 1. After enzymatic hydrolysis, the EHR-SCB were washed three times, then used for compositions analyses. The washed EHR-SCB still contained cellobiose, glucose, and xylose with concentrations of 2.78 mg/g dry material (DM), 7.57 mg/g DM, and 0.83 mg/g DM, respectively. The contents of cellobiose, glucose, and xylose in the residues were too low to impact the composition analysis of EHR-SCB. From Table 1, it can be seen that the main components of EHR-SCB are cellulose and lignin, while those of wheat bran are cellulose and hemicellulose. With the proportion of EHR-SCB increasing, the cellulose and lignin contents of media rise up, while the proportion of hemicellulose decreases.

Table 1. Compositions of the Feedstocks and Media

Feedstocks and media	Cellulose (%)	Hemicellulose (%)	Klason lignin (%)
Raw SCB	43.95	25.82	17.93
Pretreated SCB	64.17	7.53	24.56
Hydrolyzed SCB	49.10	7.13	37.03
Wheat bran	28.08	27.87	9.29
Medium A	38.31	17.22	23.07
Medium B	40.41	15.15	25.84
Medium C	42.52	13.07	28.62
Medium D	44.62	11.00	31.39
Medium E	46.72	8.92	34.16

Enzymatic Hydrolysis of LHW-pretreated SCB

The process of fed-batch enzymatic hydrolysis has been proved to be an efficient way to convert biomass to fermentable sugars. It was adopted in this study to convert LHW-pretreated SCB efficiently. From Table 1, it can be found that the main components of the pretreated SCB are cellulose and lignin. Lignin can adsorb cellulase, which reduces the enzymatic hydrolysis efficiency. Polyoxyethylene (20) sorbitan monooleate, which has the capacity to weaken the adsorption of cellulase to lignin, was added in this study. As shown in Fig. 1, after 120-h fed-batch hydrolysis of LHW-pretreated SCB, the concentrations of cellobiose, glucose, xylose, and arabinose were 3.4 g/L, 62.9 g/L, 5.2 g/L, and 1.6 g/L, respectively. The glycan conversion achieved 45.9%. The hydrolyzate can be further fermented to ethanol. From Table 1, it can be noted that, after enzymatic hydrolysis, the lignin content increased in the hydrolyzed SCB. Lignin can adsorb cellulase to reduce the amounts of reactive enzyme. The increasing content of lignin in hydrolyzed SCB indicates that more cellulase would be needed to enhance the

conversion of cellulose to glucose. This would not be economical. The solid residues obtained from the process of enzymatic hydrolysis was used to prepare media to cultivate *Lentinula edodes* in the subsequent research.

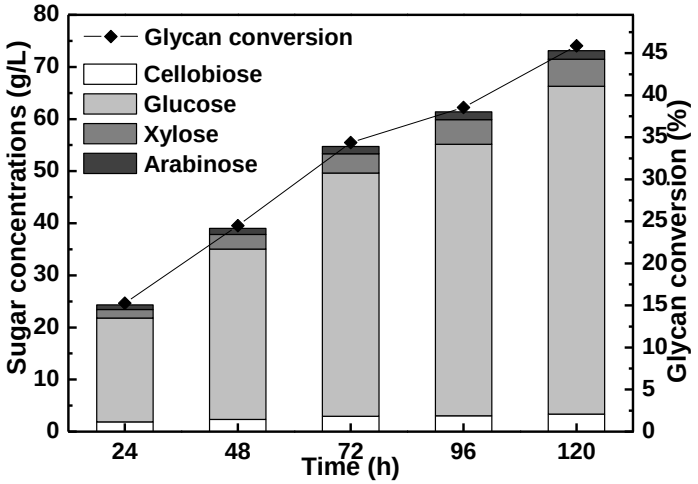

Fig. 1. Sugar concentrations and glycan conversion during 120-h fed-batch enzymatic hydrolysis of LHW-pretreated SCB

Growth of *Lentinula edodes*

Figure 2 shows that media A and B could cultivate *Lentinula edodes*, while others could not. However, media D and E germinated *Lentinula edodes* to form buttons marked with boxes in Fig. 2. There were not any buttons forming on the media C and F. Especially for medium F, mycelia spread more slowly than others. Through elemental analyses, the carbon content of EHR-SCB was 48.38% (g/g DM), which was 8.87% more than that of wheat bran, but the nitrogen content of EHR-SCB was 2.62%, which was 3.04% less than that of wheat bran.

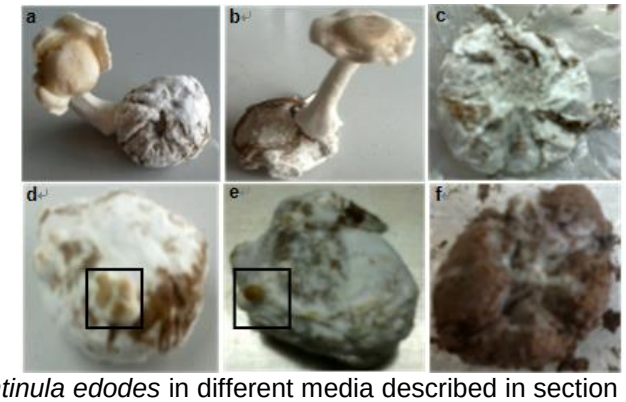

Fig. 2. Growth of *Lentinula edodes* in different media described in section of "Culture of *Lentinula edodes*". a: Medium A; b: Medium B; c: Medium C; d: Medium D; e: Medium E; f: Medium F

The elemental analyses of the media are shown in Table 2. With the proportion of EHR-SCB rising, carbon contents of the media increased from 43.55% to 47.90%, while N contents decreased from 1.70% to 0.41%, and thus the ratios of carbon to nitrogen (C:N) increased from 25.67 to 115.74:1. It was reported that the C:N ratio in the substrate

should be in the range from 25 to 40:1 in the vegetative growth stage of *Lentinula edodes*, and from 40 to 73:1 in the reproductive stage (Chang 2008). This means that media A, B, and C are fit for vegetative growth of *Lentinula edodes*, and might grow *Lentinula edodes*. However, there were not any *Lentinula edodes* or buttons growing on the medium C; this might be ascribed to the improper cultivation management, because media D and E had buttons.

The contents of zinc (Zn) and selenium (Se) were almost identical among these six media. As the proportion of EHR-SCB increased, the S, magnesium (Mg), calcium (Ca), and manganese (Mn) contents decreased from 0.30% to 0.20%, 0.019% to 0.0007%, 0.089% to 0.082%, and 0.00085% to 0.00002%, respectively, while the copper (Cu), chromium (Cr), and iron (Fe) contents increased from 0.008% to 0.016%, 0.00019% to 0.00037%, and 0.015% to 0.029%, respectively. All of these minerals can enhance the growth of *Lentinula edodes*. Among these minerals, Mg is more important (Wang 2004). The buttons on the media D and E were unable to mature, which could be due to the small Mg contents and inappropriate ratios of carbon to nitrogen. On the medium F, sporocarp and button of *Lentinula edodes* could not be formed and the mycelia spread extremely slowly, the main reason for which might be the extremely inappropriate C:N ratio. These results indicate that the EHR-SCB has to be mixed with wheat bran, which can provide nitrogen nutrient to prepare the media for culturing *Lentinula edodes*. Meanwhile, the EHR-SCB and wheat bran must be mixed rationally to maintain the proper C:N ratio.

Table 2. Nutrient Elements Contained in the Media

Media	C (%)	N (%)	C/N	S (%)	Zn (%)	Mg (%)	Cu (%)	Fe (%)	Cr(%)	Ca(%)	Mn (%)	Se (%)
A	43.55	1.70	25.67	0.30	0.0074	0.019	0.008	0.015	0.00019	0.089	0.00085	0.0029
B	44.43	1.43	30.97	0.28	0.0074	0.015	0.010	0.018	0.00023	0.088	0.00068	0.0029
C	45.32	1.17	38.64	0.26	0.0073	0.012	0.012	0.020	0.00026	0.086	0.00051	0.0030
D	46.21	0.91	50.72	0.24	0.0072	0.008	0.013	0.023	0.00030	0.085	0.00034	0.0030
E	47.10	0.65	72.53	0.22	0.0072	0.004	0.015	0.026	0.00034	0.083	0.00017	0.0030
F	47.90	0.41	115.74	0.20	0.0071	0.0007	0.016	0.029	0.00037	0.082	0.00002	0.0031

Nutrient Comparison with the Market *Lentinula edodes*

Lentinula edodes harvested from media A and B were compared with the sample from the market in terms of nutritious components. Amino acids contents of *Lentinula edodes* are shown in Fig. 3. Seventeen standard amino acids, which include aspartic acid (Asp), threonine (Thr), serine (Ser), glutamic acid (Glu), glycine (Gly), alanine (Ala), cysteine (Cys), valine (Val), methionine (Met), isoleucine (Ile), leucine (Leu), tyrosine (Tyr), phenylalanine (Phe), lysine (Lys), histidine (His), arginine (Arg), and proline (Pro), could be detected. Eight of them, noted with asterisk in Fig. 3, are essential amino acids that cannot be synthesized from other compounds in the human body. Therefore, the eight essential amino acids must be taken in from foods. When the amino acids are assimilated by the human body, they can be either used to synthesize proteins or oxidized to urea and carbon dioxide as sources of energy (Sakami and Harrington 1963). Due to their significant biological function, amino acids are commonly used as nutritional additives. From Fig. 3, it can be seen that the amino acid contents of *Lentinula edodes* cultured in medium A were highest, except that Ala, Cys, and Pro were 0.007%, 0.074%, and 0.031% less than those of the market sample, respectively, while Ser and Met were

0.003% and 0.020% less than those of *Lentinula edodes* cultured in medium B. This might be ascribed to the high nitrogen content in the medium A (Table 2). The amino acid contents of *Lentinula edodes* cultured in medium B were higher in Asp, Ser, Glu, Gly, Met, Tyr, His and Arg, while less in Thr, Ala, Cys, Val, Ile, Leu, Phe, Lys, and Pro than those of the market sample. Eight essential amino acid contents of *Lentinula edodes* cultured in medium A were highest. This indicates that medium A, which contains more wheat bran, can cultivate more nutritious *Lentinula edodes*.

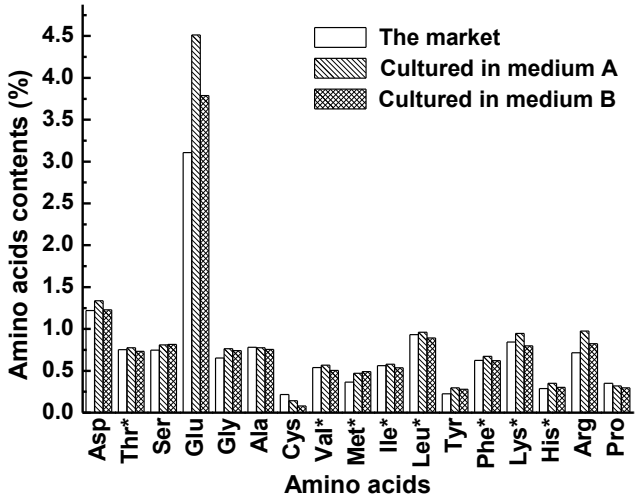

Fig. 3. Nutrient comparison of *Lentinula edodes* between this study and the market in the contents of amino acids. *Essential amino acids.

Table 3. Nutrient Elements Contained in *Lentinula edodes* (mg/g dry weight)

Lentinula edodes	C	N	Zn	Mg	Cu	Fe	Cr	Ca	Mn	Se
The market	388.9	36.9	0.127	0.874	0.00456	0.200	0.0153	1.104	0.0121	0.0296
Cultured in medium A	378.4	46.3	0.108	0.927	0.0107	0.325	0.0469	1.457	0.0119	0.0302
Cultured in medium B	377.6	43.3	0.111	0.794	0.0126	0.139	0.0287	0.905	0.00998	0.0303
Daily recommended or adequate intakes of minerals (mg)[a]			1.5-19[b]	30-400[c]	0.4-2.0[c]	0.3-35[c]	0.01-0.05[c]	200-1300[c]	3.5[c]	0.015-0.065[b]
Daily tolerable upper intakes of minerals (mg)[a]			13-45	200-700	1.5-8.0	10-60	0.2-0.5	2000	10	0.055-0.4

[a] Chinese Dietary Reference Intakes (Chinese DRIs 2001); [b] Daily recommended intakes; [c] Daily adequate intakes

Table 3 shows the nutrient elements contents of the three *Lentinula edodes*. C, N, Zn, Mg, Cu, Fe, Cr, Ca, Mn, and Se were detected. It can be seen that C content of the market *Lentinula edodes* was 10.5 and 11.3 mg/g dry weight more than those of *Lentinula edodes* cultured in media A and B, respectively, while N content of the market *Lentinula edodes* was 9.4 and 6.4 mg/g dry weight less than those of *Lentinula edodes* cultured in media A and B, respectively. These indicate that the carbohydrate content of the market *Lentinula edodes* was higher than those of *Lentinula edodes* cultured in media A and B, while the protein content of the market *Lentinula edodes* was lower than those of *Lentinula edodes* cultured in media A and B, which is corresponding to the contents analyses of amino acids (Fig. 3). The daily recommended intakes of protein were from

1.5 g/kg for babies to 85 g/kg for 18-year-old adults (Chinese DRIs 2001). This indicates that the more protein the *Lentinula edodes* contains, the better for human health it is. The over-intakes or insufficient intakes of nutrient minerals would be disadvantage for human health. The daily recommended or adequate intakes of minerals are listed in Table 3, and so are the daily tolerable upper intakes of minerals. The data at low levels were collected from babies, and those at high levels were collected from different ages of adults. From Table 3, it can be seen that the Cr and Se contents of the three *Lentinula edodes* were higher than their lowest adequate or recommended intakes, but lower than their daily tolerable upper intakes. *Lentinula edodes* cultured in medium A had the highest Cr content which nearly equals to its highest adequate intake. Because the data of Cr and Se contents were analyzed from one gram *Lentinula edodes*, and the one-time intake of *Lentinula edodes* would be more than one gram, the lower contents of Cr and Se would be better for human health. Except for the Fe content of *Lentinula edodes* cultured in medium A which is as much as its lowest adequate intakes, other mineral contents of the three *Lentinula edodes* are less than their lowest intakes, especially for the contents of Mg and Ca. Although the contents of Mg, Cu, Fe, and Ca contained in *Lentinula edodes* cultured in medium A are more than the two others, the high Cr content makes its nutritious value lower than the two others. Based on the comparison of the mineral contents between *Lentinula edodes* cultured in medium B and the market one, *Lentinula edodes* from the market is superior to the one cultured in medium B.

Composition Analysis of Mushroom Residues

After harvesting *Lentinula edodes*, the leftover media are the mushroom residues that consist of media residues and mycelium. As shown in Table 4, there were still a little cellulose and hemicellulose existing in mushroom residues. The cellulose and lignin contents in mushroom residue A were less than those in mushroom residue B, while the hemicellulose content in mushroom residue A was more than that in mushroom residue B. These corresponded to the compositions of media A and B shown in Table 1. Compared with the compositions of media A and B, the content of cellulose and hemicellulose in mushroom residues A and B decreased, while that of lignin increased. This indicates that *Lentinula edodes* mainly assimilated cellulose and hemicellulose, while it hardly assimilated the lignin. The C:N ratios of mushroom residues A and B were in the range of 25 to 40:1, which suggested that it was probable to reuse the mushroom residues A and B as media for culturing *Lentinula edodes*. According to the analyses of compositions and C:N ratios, mushroom residues might be further used as media for producing edible mushrooms or single cell protein, or composted for manure.

Table 4. Compositional and Elemental Analyses of the Mushroom Residues

Mushroom residues	Cellulose (%)	Hemicellulose (%)	Klason lignin (%)	C (%)	N (%)	C/N
A	21.79	14.13	36.63	45.81	1.59	28.81
B	28.90	12.82	37.35	45.83	1.22	37.57

CONCLUSIONS

1. This study provides a feasible way to use enzymatic-hydrolyzed residues of LHW-pretreated SCB to prepare a medium for culturing *Lentinula edodes*. The results indicate that the C:N ratio ranging from 25 to 40 is important, and the enzymatic-hydrolyzed residues of SCB should be mixed with the wheat bran in the range from 50% to 60% to maintain the C:N ratio in the appropriate range to cultivate *Lentinula edodes*. Taking the contents of amino acids and minerals into account simultaneously, the medium containing 60% enzymatic-hydrolyzed residues of LHW-pretreated SCB is more suitable for cultivating *Lentinula edodes*. The mushroom residues may be further used for producing value-added products.
2. As for the process of converting LHW-pretreated SCB into ethanol, it is possible to improve the recycling and to achieve economic benefit from making EHR-SCB into medium for culturing edible fungi.

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