Combined Water Extraction and Sodium Chlorite Pretreated Spent Mushroom Compost for Protease Production by Separate Hydrolysis and Fermentation and Simultaneous Saccharification and Co-fermentation

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A process of simultaneous saccharification and protease production was successfully established from spent mushroom compost (SMC) created through edible fungi cultivation. The combined water extraction and sodium chlorite pretreatment significantly ($p < 0.05$) improved enzymatic digestibility of SMC, which led to a reducing sugar yield of 0.759 g/g that was 12 times higher than raw SMC. The water extract from SMC was recycled for simultaneous saccharification and protease production from pretreated SMC by *Bacillus subtilis* DES-59, which promoted the protein concentration and neutral protease activity by 21.9% and 11.6%, respectively. The simultaneous saccharification and co-fermentation (SScF) of pretreated SMC by *Bacillus subtilis* DES-59 produced 5518 U/mL protease, which was superior to the separate hydrolysis and fermentation (SHF) process. Fermentation residues containing *Bacillus subtilis* cells could be further converted into fertilizer. The closed-loop utilization of SMC was achieved using established processes, which indicates potential for application in future biorefineries.

Keywords: Extract; Protease production; Spent mushroom compost; Sodium chlorite; Fertilizer

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INTRODUCTION

Protease, one of the three major industrial enzymes, accounts for approximately 60% of global enzyme sales (Hussain *et al*. 2017). It has been widely applied in food, detergent, antineoplastic drugs, and feed additives (Hmidet *et al*. 2009). Protease has been produced from inexpensive and readily available agricultural residues, such as wheat bran, rice bran, and sugarcane bagasse. As the world's largest solid-state fermentation industry, the annual edible mushroom yield is huge. Many industrial and agricultural wastes, such as sawdust, cottonseed husk, and corn cob, have been used as media for edible fungi cultivation. 5 kg SMC will be generated from 1 kg mushroom production. In 2013, the output of spent mushroom compost reached hundreds of millions of tons (Carrasco *et al*. 2018). Waste SMC has caused environmental concerns. However, compared to other residues, some nutrients remain in SMC. Meanwhile, SMC, as a kind of lignocellulose, is mainly composed of cellulose and hemicellulose, which can be

hydrolyzed into monosaccharide mixtures (glucose, xylose, and arabinose). *Bacillus subtilis* DES-59 (Zhu *et al*. 2013b), a protease producing strain, which can utilize both xylose and glucose, may be used to produce protease from SMC and improve the conversion efficiency of SMC compared with a single sugar utilization strain (Asada *et al*. 2011). Therefore, there is potential to produce protease from SMC by *Bacillus subtilis* DES-59.

The recalcitrant structure of SMC suggests that pretreatment is required prior to enzymatic hydrolysis. Organosolv, alkaline peroxide, dilute-acid, and steam-exploded pretreatment have been conducted on SMC (Asada *et al*. 2011; Kapu *et al*. 2012; Zhu *et al*. 2016; Lin *et al*. 2017). Dilute-acid pretreatment and steam-exploded pretreatment were both carried out at a high temperature. Organosolv pretreatment is expensive, and alkaline peroxide is difficult to recycle. Considering the efficacy and economy, it is preferable to develop an efficient pretreatment process. Acid-chlorite pretreatment has proven to be a valid process for removing lignin (Hubbell and Ragauskas 2010). During the pretreatment, the ClO_2 is released in an acidic environment and oxidizes lignin as a highly active bleach. Cellulose has been obtained from water hyacinth and softwood with high conversion rates by acid-chlorite pretreatment (Yu *et al*. 2011; Abdel-Fattah and Abdel-Naby 2012). Sodium chlorite pretreatment has also been employed to improve the digestibility of lignocellulose (Nan *et al*. 2018).

The water extract of lignocellulose is a non-negligible part composed of proteins, sugar, phenolics, and so on (Chen *et al*. 2007). The presence of water extract components is reported to impact not only the composition analysis, but also the effectiveness of pretreatment. Furthermore, the reactions between lignin and extract during the pretreatment impact the enzymatic hydrolysis efficiency of the pretreated samples (Martín *et al*. 2015). Therefore, the water extraction process before pretreatment is necessary. Because the water extract of SMC is composed of nitrogen, sugar, and metal ions, the water extract may be recycled for proteases production to avoid the loss of water-soluble nutrients. In this way, the production cost of protease can be reduced. However, the utilization of water extract for protease production has not been reported.

In addition, the fermentation process will affect the production of the desired product as well. Solid state fermentation is a traditional strategy for enzyme production utilizing agriculture by-products. However, in this study, the submerged fermentation was introduced due to its convenient product separation, sufficient oxygen transfer, lower fermentation time, and easy monitoring of fermentation parameters (Gomes *et al*. 1994; Cerda *et al*. 2019). Separate hydrolysis and fermentation (SHF) and simultaneous saccharification and co-fermentation (SScF) are traditional processes for lignocellulose utilization. SHF decouples enzymatic hydrolysis and fermentation steps as separate processes to be performed under two optimum conditions. The two separate steps require more reactors and present lower efficiency (Cannella and Jørgensen 2014). SScF is considered to be an ideal strategy due to the integration of enzymatic hydrolysis and fermentation, which could solve the aforementioned problems. In addition, the SScF could convert glucose and xylose together into desired products during fermentation (Xu *et al*. 2018). The lignocellulosic fermentation residues have been recommended for conversion into fertilizer (Zhu *et al*. 2013a; Quintanar-Orozco *et al*. 2018; Smitha *et al*. 2019). Such a product not only improves the soil fertility for sustainable agriculture, but also provides a viable means of waste disposal.

The present study focused on the potential of SMC for protease production. Combined water extraction and sodium chlorite pretreatment was conducted to improve

the digestibility of SMC. The water extractives of SMC and pretreated SMC were used for protease production by SHF and SScF. The residues after SScF could be reused as fertilizer back to agricultural production, thereby achieving a closed-loop utilization of SMC.

EXPERIMENTAL

Materials

Flammulina velutipes SMC used in this study was kindly provided by Starway Biotechnology Co., Ltd. (Guangdong province, China). The medium for the cultivation of *Flammulina velutipes* mainly consisted of cottonseed hull, wheat bran, corn powder, corncob, and wood chips. SMC was ground and screened through 100-mesh sieves for further use. Cellulase (CTec 2, 120 FPU/mL) was purchased from Sigma Aldrich (St. Louis, MO, USA). The standard sugars for composition analysis were obtained from Aladdin (Shanghai Aladdin Bio-Chem Technology Co., Ltd., Shanghai, China). Sodium chlorite (80%), trichloroacetic acid, casein, and tyrosine were purchased from Macklin (Shanghai Macklin Biochemical Co., Ltd., Shanghai, China).

Methods

Water extraction of the raw SMC

A sample of 40 g SMC (dry weight) was extracted with 400 mL water at 55 °C for 2 h in a water bath with magnetic stirring. The water extracts were obtained by filtration and stored at -20 °C for further study. Subsequently, the filter cake was dried overnight at 55 °C to remove water. Water extract was collected for composition analysis and protease production, and the dried cake was used for subsequent pretreatment.

Orthogonal experiments design for pretreatment of SMC

The extractive-free SMC was pretreated with sodium chlorite. The effect of conditions on the pretreatment was investigated by $L_{16}(4^4)$ orthogonal design (Table 1). The main factors of pretreatment included solid-to-liquid ratio (factor A), treatment time (factor B), temperature (factor C), and sodium chlorite concentration (factor D). Four levels of each factor were selected for the experiment. The pretreated solid was separated from the liquid by leaching at the end of pretreatment. The separated solid was washed to neutral with water and dried overnight at 55 °C. A portion of the solid was used for composition analysis, and the rest was stored in sealed plastic bags for subsequent enzymatic hydrolysis. The lignin removal was calculated according to Eq. 1,

$$\text{Lignin removal (\%)} = (29.9\% - x)/29.9\% \times 100\% \quad (1)$$

where x is lignin content in pretreated SMC (%).

Enzymatic hydrolysis

A sample of 0.1 g pretreated SMC was soaked in 5 mL 50 mM sodium citrate buffer (pH 4.8). The enzymatic hydrolysis was performed at 55 °C and 150 rpm for 72 h with an enzyme loading of 20 FPU/g unless otherwise specified. The released reducing sugar concentration was determined using 3,5-dinitrosalicylic acid (DNS) for the enzymolysis efficiency calculation (Hong *et al*. 2019).

To investigate the impact of water extract of SMC on reducing sugar yield, 30%, 60%, 90% (v/v) sodium citrate buffer was substituted by an equal volume of water extract. The initial pH of enzymatic hydrolysis was 4.8. The solid loading and working volume were 2% (w/v) and 5 mL, respectively. To investigate the effect of water extract on the growth of *Bacillus subtilis* DES-59, the enzymatic hydrolysis of 1g pretreated SMC was conducted in 50 mL water extract and deionized water, respectively. The hydrolysates were used for media preparation.

The enzymatic hydrolysis of 5 g, 10 g, and 15 g pretreated samples was conducted at the same condition in 50 mL sodium citrate buffer. The enzymatic hydrolysis was performed for 96 h. The obtained hydrolysates were stored at -20 °C for further experiments.

Table 1. $L_{16}(4^4)$ Orthogonal Experiments Designed for the Study

Entry	A	B (h)	C (°C)	D (%)
1	2:20	1	80	9
2	2:25	1	70	10
3	2:15	1	65	7
4	2:30	1	75	8
5	2:30	2	70	9
6	2:15	2	80	10
7	2:20	2	65	8
8	2:25	2	75	7
9	2:15	3	70	8
10	2:30	3	80	7
11	2:20	3	75	10
12	2:25	3	65	9
13	2:15	4	75	9
14	2:25	4	80	8
15	2:20	4	70	7
16	2:30	4	65	10

A: ratio of solid to liquid; B: time (h); C: temperature (°C); D: sodium chlorite concentration (%)

Separate hydrolysis and fermentation of pretreated SMC

Bacillus subtilis DES-59 was cultured for protease production. Pure culture of *Bacillus subtilis* DES-59 from LB agar-slant was inoculated into LB medium at 30 °C for 12 h. The hydrolysates obtained at high solid loadings were diluted to 10 mg/mL, 20 mg/mL, and 30 mg/mL (total reducing sugar) as carbon sources for protease production. Proportions of 10.5 g/L K_2HPO_4, 4.5 g/L KH_2PO_4, 1 g/L $(NH_4)_2SO_4$, 0.5 g/L $Na_3C_6H_5O_7 \cdot 2H_2O$, and 0.2 g/L $MgSO_4 \cdot 7H_2O$ were dissolved in 47.5 mL of the hydrolysates as fermentation medium. The media were autoclaved at 115 °C for 30 min. Inoculation ratio was 5% (v/v). The 250 mL flask was cultivated at 180 rpm and 30 °C. During the fermentation, the enzyme activity, OD_{600}, glucose concentration, and xylose concentration were monitored. The number of bacteria was estimated by counting colony-forming units on agar plates.

Simultaneous saccharification and co-fermentation of pretreated SMC

The 47.5 mL hydrolysates in the above-mentioned media were replaced by equal volume of deionized water and water extracts for SScF with 20 mg/mL, 40 mg/mL, or 60 mg/mL of pretreated SMC, 2.5 mL inoculum, and 20 FPU/g cellulase. The 250 mL flask was cultivated at 180 rpm and 30 °C. The fermentation broth was sampled every 12 h to determine fermentation parameters. At the end of fermentation, the residues were collected for composition analysis.

Analytical methods

The composition of SMC was determined according to the procedure of the National Renewable Energy Laboratory (Sluiter *et al*. 2012). The content of monomeric sugars was quantified using a Waters 2414 HPLC (Waters Corporation, Milford, MA, USA) at a flow of 0.6 mL/min and 60 °C with 5 mM H_2SO_4 as the mobile phase, which was equipped with a refractive index detector and an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) (An *et al.* 2018). The surface morphological differences between SMC and pretreated SMC materials were observed by scanning electron microscope (SEM) (Merlin Compact, Carl Zeiss, Oberkochen, Germany). The magnification of the photographed images was 2000. The X-ray diffraction analysis (XRD) (Empyrean, PANalytical BV, Almelo, Holland) was performed according to a prior study (Zhang and Zhu 2016). The previous formula was applied to determine the crystallinity index (Lin *et al*. 2017). Fourier transformed infrared spectroscopy (FTIR) was conducted using a spectrometer (CCR-1; Thermo Nicolet, Shanghai, China) within the wavelength range of 400 cm^{-1} to 4000 cm^{-1}, as previously described (Hong *et al*. 2019). The protein content in water extract was determined as 6.25 multiplied by total nitrogen, which was measured by the Kjeldahl method (Elsamadony and Tawfik 2018). The enzyme activity was assayed by a traditional method (Borhani *et al*. 2018). One mL of diluted broth was mixed with 1 mL 10 g/L casein solution at 40 °C for 10 min. The reaction was stopped by 2 mL 0.4 mol/L trichloroacetic acid. The mixture was filtered after 10 min. Then, 1 mL filtrate was mixed with 5 m L of 0.4 mol/L sodium carbonate solution and 1 mL of Folin & Ciocalteu's phenol reagent. The solution was maintained at 40 °C for 20 min, and then the optical density at 680 nm was recorded. Meantime, in the control group, casein solution and trichloroacetic acid were added in reverse order. The protein concentration in the fermentation broth was determined using a BCA proteins assay kit from Thermo Fisher Scientific (Waltham, MA, USA). The previous method was conducted to explore the buffering capacity of water extractives (Linde *et al*. 2006). The pH was measured by a pH meter PB-10 (Sartorius, Göttingen, Germany).

Statistical Package for the Social Sciences for Windows (SPSS Inc., Chicago, IL, USA, version 17.0) was used for orthogonal design and all statistical analyses, and a value of $p < 0.05$ was considered statistically significant. Values shown are means of triplicate measurements ± standard deviation. The range method was introduced to analyze the results of the orthogonal experiment (Zhong *et al.* 2018). The number of bacteria was estimated by counting colony-forming units on LB agar plates in SHF and SScF. Filter paper activity was measured for the cellulase activity of CTec 2 according to a previous study (Chan *et al*. 1989).

RESULTS AND DISCUSSION

Chemical Composition Analysis and Water Extraction

To evaluate the potential of SMC as a feedstock for protease production, a composition analysis was performed. The SMC was composed of 24.8% cellulose, 17.6% hemicellulose, and 26.6% lignin (Table 2). Although the holocellulose content (42.4%) of SMC was lower than commonly used feedstocks, such as corn stover (37.8% cellulose and 28.1% hemicellulose) (Yang and Wyman 2004), the holocellulose of SMC could be used for protease production. Compared to other biomass, a higher lignin content (26.6%) of SMC results in a more recalcitrant structure of SMC. Therefore, it is necessary to adopt an efficient pretreatment method. The previously studied sodium chlorite pretreatment (6% w/v, 2 h, 80 °C, 1:10) was conducted on silvergrass (Nan *et al*. 2018). However, the enzymatic hydrolysis efficiency of pretreated SMC was 75.9% when the same pretreatment was performed on SMC. The higher enzymatic hydrolysis efficiency is supposed to be achieved under more severe conditions. Meanwhile, the water extract was reported to have significant impact on the pretreatment. Therefore, the water extraction of SMC was performed before pretreatment.

Previous studies have shown that the presence of water extract would affect the composition analysis of the material (Tamaki and Mazza 2010). Table 2 shows the composition of the water extractive-free SMC and raw SMC. The water-soluble extractive of SMC was 20.7%. There was 1.38 g/L protein in the water extract. The protein might be fungal extracellular secretion during the cultivation of mushrooms. 0.31 g/L xylose and 0.25 g/L arabinose were also detected in the water extract. In addition, 0.66 mg/mL of lactic acid was also detected in the water extract. The sugar and organic acid in water extract were also reported in a previous study (Asada *et al*. 2011). The content of cellulose, hemicellulose, and acid-insoluble lignin in the water extractive-free SMC significantly increased compared with that of the raw SMC, which was mainly attributed to weight loss during the water extraction process. In contrast, the content of acid-soluble lignin decreased, which might have been caused by the dissolution of water-soluble lignin and some chemicals with maximum absorption at around 240 nm. The total content of acid soluble and insoluble lignin (30.0%) in water extractive-free SMC was higher than that in raw SMC (26.6%), which was consistent with the previous report (Martín *et al*. 2015).

Sodium Chlorite Pretreatment

The extractive-free solid was pretreated by sodium chlorite. Orthogonal design was applied to optimize sodium chlorite pretreatment conditions. The four key factors of pretreatment were studied. Each level of the four factors in the investigation was independent: A (ratio of solid to liquid: 2:15, 2:20, 2:25, and 2:30), B (time: 1 h, 2 h, 3 h, and 4 h), C (temperature: 65 °C, 70 °C, 75 °C, and 80 °C), and D (the concentration of sodium chlorite: 7% w/v, 8% w/v, 9% w/v, and 10% w/v). All factors were evaluated by an orthogonal $L_{16}(4^4)$ design. The enzymatic efficiency was analyzed according to the statistical method.

The average values of K for each factor at each level in the range analysis were named K_i (Table 3). According to the value of K_i, the optimal pretreatment condition was 2:25, 4 h, 80 °C, and 8% (w/v). Based on the R value, the four factors influencing pretreatment were concluded to increase in this order: D < A < B < C. The increase of cellulose content might have been attributed to the lignin removal. The enzymolysis

efficiency of the pretreated SMC was 98.7% under the optimal condition, which was 3.5 and 6.9 times higher than that of extractive-free SMC and raw SMC, respectively. The lignin removal (81.6%) was higher than that (69.5%) reported in previous research (Zhu *et al.* 2013a). The reducing sugar yield reached 759 mg/g, which was much higher than that (323 mg/g) of dilute-acid pretreated SMC (Kapu *et al.* 2012).

Table 2. Composition of Raw and Extracted SMC

Composition (% Dry Weight)	Raw SMC	Water Extracted SMC
Cellulose (%)	24.78 ± 0.62	30.29 ± 0.07
Hemicellulose (%)	17.59 ± 0.52	21.59 ± 0.11
Acid Insoluble Lignin (%)	17.65 ± 0.11	24.25 ± 0.05
Acid Soluble Lignin (%)	8.98 ± 0.11	5.70 ± 0.35
Ash (%)	9.23 ± 0.21	8.62 ± 0.16
Petroleum Ether Extractives (%)	4.41 ± 0.75	/
Water Soluble Extractives (%)	20.74 ± 0.76	/
Enzymolysis Efficiency (%)	12.56 ± 0.21	21.84 ± 1.15

Table 3. Results of Orthogonal Design

Entry	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Lignin Removal (%)	Enzymolysis Efficiency (%)
1	45.94 ± 0.61	19.17 ± 0.34	12.57 ± 0.16	57.95	56.58 ± 10.59
2	44.02 ± 8.64	18.82 ± 3.97	12.85 ± 0.33	57.02	55.25 ± 5.56
3	39.27 ± 4.18	18.56 ± 1.94	17.93 ± 0.63	40.03	36.42 ± 0.53
4	45.66 ± 0.91	19.46 ± 0.18	12.89 ± 0.54	56.89	53.00 ± 7.87
5	51.01 ± 4.87	21.01 ± 1.98	11.34 ± 0.64	62.07	63.18 ± 5.69
6	45.44 ± 0.15	19.04 ± 0.18	8.57 ± 0.10	71.34	64.96 ± 2.01
7	42.14 ± 5.19	18.64 ± 2.14	12.10 ± 0.74	59.53	51.50 ± 0.40
8	48.17 ± 9.34	19.76 ± 4.05	10.39 ± 5.89	65.25	72.21 ± 1.60
9	44.75 ± 1.36	20.27 ± 0.68	12.59 ± 0.98	57.89	52.58 ± 7.99
10	45.30 ± 1.42	18.88 ± 0.45	5.87 ± 0.20	80.37	80.37 ± 3.93
11	46.88 ± 1.46	17.98 ± 0.57	9.42 ± 0.42	68.49	76.24 ± 1.68
12	58.39 ± 0.07	24.41 ± 0.26	17.27 ± 1.09	42.24	37.42 ± 2.45
13	44.96 ± 1.38	19.52 ± 0.61	11.52 ± 0.15	61.47	65.82 ± 0.32
14	49.56± 0.93	22.92 ±4.40	5.50 ± 0.10	81.61	98.71 ± 6.54
15	48.20 ± 0.93	20.57 ± 0.27	11.41 ± 1.27	61.84	58.73 ± 5.85
16	50.11 ± 0.47	20.38 ± 0.78	10.66 ± 0.34	64.34	59.08 ± 8.96
K_1	47.85	60.76	65.9	63.91	/
K_2	50.31	62.96	61.65	70.59	/
K_3	46.11	57.44	66.82	75.16	/
K_4	61.93	63.95	55.75	63.88	/
R	18.05	20.27	29.05	8.2	/

Pretreatment was also conducted on raw SMC under the above optimal conditions. An enzymolysis efficiency of 89.5% was achieved, while the recovery of cellulose (80.7%) and hemicellulose (48.4%) decreased (Table 4). The content of cellulose and hemicellulose after pretreatment on extractive-free SMC was higher. Moreover, cellulose and hemicellulose recovery for pretreatment on extractive-free SMC was also slightly enhanced, which was consistent with the previous report (Ballesteros *et al*. 2011). The reducing sugar yield of pretreated extractive-free SMC increased 19.3% compared to pretreated SMC without water extraction. The enzymatic hydrolysis

efficiency was also improved from 89.5% to 98.7%. The water extract was found to be able to buffer H^+ (Fig. S1), and the ash had the same function as well (Vera *et al*. 2015; Li *et al*. 2016), both of which might reduce the pretreatment intensity. Additionally, the removal of xylose and arabinose by water extraction could reduce the formation of furfural, which was related to the decrease of enzyme activity (Chen *et al*. 2007). Therefore, the combination of water extraction and sodium chlorite pretreatment was an effective pretreatment process to produce a highly digestible SMC solid.

Table 4. Comparison of Pretreated SMCs

Samples	Pretreated SMC [a]	Pretreated SMC Without Extraction [b]	Pretreated SMC with Extraction [c]
Solid Recovery (%)	45.86 ± 0.74	45.00 ± 0.45	54.34 ± 0.46
Cellulose Recovery (%)	95.75 ± 0.21	80.74 ± 0.17	88.92 ± 1.09
Hemicellulose Recovery (%)	61.42 ± 0.59	48.40 ± 0.10	57.98 ± 0.28
Cellulose (%)	50.92 ± 1.44	44.46 ± 0.9	49.56 ± 0.93
Hemicellulose (%)	23.19 ± 0.75	18.92 ± 4.40	22.92 ± 4.40
Lignin (%)	10.57 ± 1.70	6.8 ± 1.2	5.50 ± 0.10
Enzymolysis Efficiency (%)	75.94 ± 1.64	89.54 ± 1.79	98.71 ± 2.10

[a] The pretreatment conditions were: sodium chlorite concentration 6% (w/v), ratio of solid to liquid 1:10, temperature 80 °C, time 2 h (Nan *et al.* 2018).
[b] The pretreatment condition was consistent with this study.
[c] Combined water extraction and sodium chlorite pretreatment were conducted on SMC under the condition optimized in this study.

Structure of the Pretreated SMC

The microstructures and crystallinity of the materials were characterized by SEM and XRD. The SEM images (Fig. S2) showed the difference between raw SMC and pretreated SMC. The surface of raw SMC was intact, flat, and compact, and the appearance of pretreated SMC was porous. According to microstructures of pretreated samples, it could be deduced that the lignin was partly removed and some of the cellulose was exposed, which improved the accessibility of enzyme. Thus, the enzymatic hydrolysis efficiency was improved. The crystallinity index (CrI) of the lignocellulose is a primary obstacle against its accessibility to cellulase. To further investigate the recalcitrance of cellulose against the enzyme, X-ray diffraction was performed to determine the CrI of the materials (Fig. S3). The CrI of pretreated SMC (54.01%) increased compared to raw SMC (39.8%). This was attributed to the reduction of lignin content (Table 3). Additionally, the increased CrI might also be attributed to the extractive removal, as well as the removal of amorphous substances (Nan *et al*. 2018).

The changes of functional groups in raw and pretreated SMC were probed through FTIR (Fig. S4). The FTIR analysis showed that the carbonyl vibration peak (1712 cm^{-1}) of pretreated SMC was enhanced, indicating that hemicellulose content increased after pretreatment (Diaz *et al*. 2015), which was consistent with the component analysis of pretreated samples. The signal peak (1437 cm^{-1}) of the CH_3 asymmetric groups in lignin decreased, which indicated the removal of lignin. Meanwhile, the absorption peak of C-O stretching (1305 cm^{-1}) in syringyls and C-C bending (1506 cm^{-1}) in aromatic groups were also significantly reduced (Diaz *et al*. 2015). These results suggested that sodium chlorite could remove lignin efficiently to facilitate enzymolysis of pretreated SMC.

SHF of Protease Production

To optimize reducing sugar concentration, high sugar concentration hydrolysates were obtained at high-solid substrate loading. The eventual reducing sugar concentration reached 63.8 mg/mL, 126.5 mg/mL, and 169.0 mg/mL at substrate loadings of 10%, 20%, and 30%, respectively; the glucose concentration (46.19 mg/mL, 92.76 mg/mL, 122.79 mg/mL, respectively) was superior to other pretreatment methods (Lu *et al*. 2010). The sugar concentration increased with increasing solid loading. The obtained hydrolysate at 30% solid loading was diluted to the desired concentration for protease production. As shown in Fig. 1b, the enzyme activity increased significantly ($p < 0.05$) with the increase of sugar concentration. In contrast, the obtained protease yield (U protease/mg reducing sugar) decreased with increasing sugar concentration (Table 5), which was consistent with previous studies (Singh *et al*. 2004).

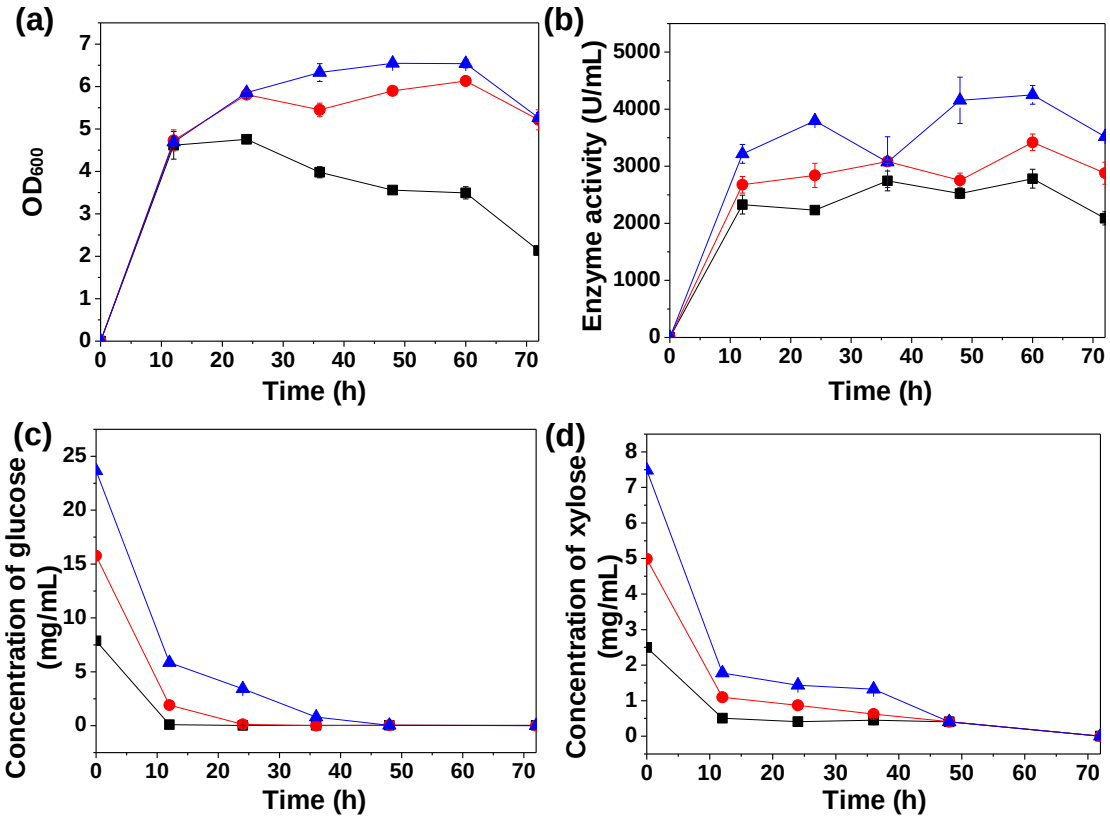

Fig. 1. Fermentation parameters at different initial total reducing sugar concentrations: OD_{600} (a); enzyme activity (b); concentration of glucose (c); and concentration of xylose (d)
Legend: ■ 10 mg/mL; ● 20 mg/mL; ▲ 30 mg/mL

Table 5. SHF Process for Protease Production

Sugar Concentration (mg/mL)	Protease Activity (U/mL)	Time (h)	Protease Productivity (U/mL/h)	Yp/s (U/mg)	Cell Density (CFU/mL)
10	2782 ± 164	60	46.36	278.2	1.8×10^{10}
20	3419 ± 144	60	56.98	170.95	3.1×10^{10}
30	4250 ± 164	60	70.83	141.67	3.3×10^{10}

It was reported that under high initial glucose concentration, some glucose would be converted into viscous material in the cultures, which led to the fermentation becoming unusable. Furthermore, the metabolites would affect the formation of protease. During the fermentation, glucose, as a preferred carbon, did not repress the utilization of xylose, but its consumption rate was much higher than xylose (Fig. 1c and 1d), which was consistent with a previous study (Zhang *et al*. 2016). There was no significant difference ($p > 0.05$) in the maximal OD_{600} (60 h) between 20 mg/mL and 30 mg/mL. It was speculated that higher glucose concentration would impact the growth of *Bacillus subtilis* DES-59 (Mehta *et al*. 2006). However, protease productivity of SHF was low due to the 96-h enzymatic hydrolysis. Furthermore, the initial high sugar concentration affected protease production. Hence, the SScF process was introduced for protease production, during which the sugar was slowly released.

Effect of Water Extract of SMC on the Enzymatic Hydrolysis and the Growth of *Bacillus subtilis* DES-59

The 30%, 60%, and 90% (V/V) water extract was added to investigate the effect of water extract on enzymatic hydrolysis of pretreated SMC. There was no significant difference ($p > 0.05$) in reducing sugar yield (Fig. 2a). Phenols in water extractive were reported to hinder degradation of the substrate. In this study, the water extract showed no influence on digestion of pretreated SMC according to the reducing sugar yield. This could be due to the presence of proteins in the water extract, which could promote the digestion of cellulose (Smit and Huijgen 2017). In addition, the water extract showed buffering capability. Hence, water extract was recycled as an alternative to sodium citrate buffer.

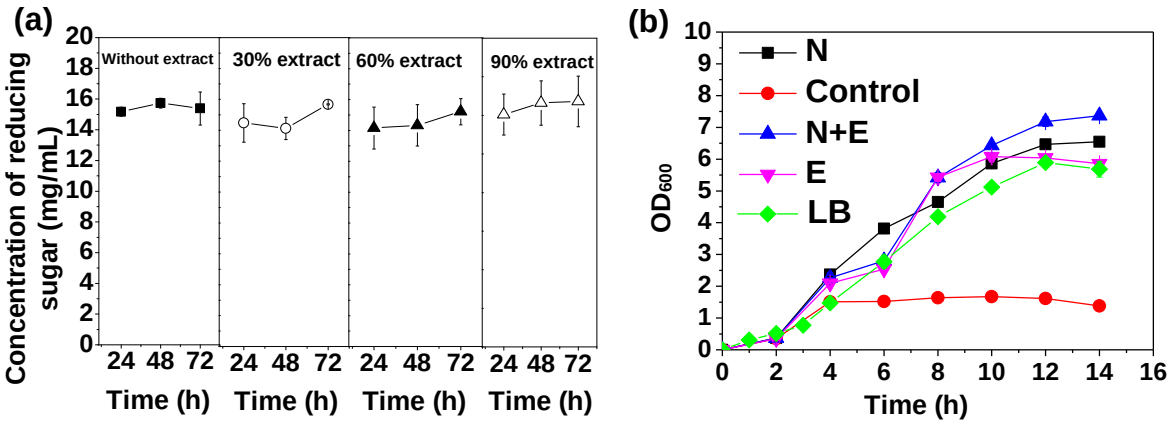

Fig. 2. Effect of water extractives on enzymatic hydrolysis of pretreated SMC and growth of *Bacillus subtilis* DES-59: (a) Concentration of reducing sugar during the enzymatic hydrolysis, (b) OD_{600} of *Bacillus subtilis* DES-59 during the fermentation. Control represents the medium without $(NH_4)_2SO_4$ and water-extract liquid; N represents the medium with $(NH_4)_2SO_4$ addition; N+E represents the medium with $(NH_4)_2SO_4$ and water-extract liquid addition; and E represents the medium with water-extract liquid addition; LB represents the LB medium.

Bacillus subtilis DES-59 was cultured in hydrolysates with and without water extract. The OD_{600} of the control, N, E, and N+E were 1.62, 6.47, 6.04, and 7.18 at 12 h, respectively. OD_{600} in LB medium (5.895) was lower than that in N+E medium (7.18) at 12 h. This may due to the different carbon sources. Reducing sugar was more conducive to the growth of *Bacillus subtilis*. In Fig. 2b, cell density in the E and N+E media was

higher than the control and N mediums, respectively, which might be attributed to the proteins, inorganic salt, organic acid and sugars in the water extract. From 2 h to 12 h, the average specific growth rates of 0.297 h^{-1} and 0.285 h^{-1} were achieved in N+E and E media, respectively, indicating that water extract of SMC is beneficial to the growth of *Bacillus subtilis*. Previous research mainly focused on the antibacterial effect of water extract (Ahn *et al*. 2007). In this study, the water extract of SMC was demonstrated to increase the growth of *Bacillus subtilis*.

SScF of Pretreated SMC

For SScF, 2773 U/mL protease was obtained with a specific activity of 561 U/mg under 20 mg/mL solid loading (Table 6). 2675 U/mg protease was produced at 12 h with 20 mg/mL substrate in SHF. Considering the 72-h enzymatic hydrolysis, the productivity of SHF was 32 U/mL/h, which was significantly lower to 231 U/mL/h of SScF. In addition, with the addition of water extract, enzyme activity (3096 U/mL) increased 11.64% at 20 mg/mL solid loading. Metal ions in the water extract might have had a positive influence on enzyme activity *via* improving stability of protease as co-factors (Hansen *et al*. 2015). Meanwhile, protein concentration (6.02 mg/mL) was also increased by 21.9%, possibly due to increasing cell density (2.16×10^{10} CFU/mL) and then promotion of the secretion of proteins. Higher enzyme activity of the culture was achieved by increasing the solid loading of the medium. The 4692 U/mL and 5518 U/mL (60 h) proteases were obtained at 40 mg/mL and 60 mg/mL solid loadings, respectively. In contrast, enzyme productivity declined due to extended fermentation time. The absence of sugar during SScF fermentation indicated that sugar in the water extract was also consumed. Furthermore, the productivity was improved compared with SHF. Therefore, it is feasible to apply SScF for protease production. Considering the process cost, the lower enzyme loadings were investigated under 60 mg/mL solid loading. The 5227 U/mL, 5306 U/mL, and 5331 U/mL (60 h) proteases were obtained at 5 FPU/g, 10 FPU/g, and 15 FPU/g enzyme loadings, respectively. The comparative enzyme activity was achieved with a lower enzyme loading of 5 FPU/g. Compared with the addition of 8 FPU/g enzymes for lactic acid production, it was more economical (Zhang *et al*. 2016).

Table 6. SScF Process for Protease Production

	Without Water Extractives	With Water Extractives		
Solid Loading (mg/mL)	20	20	40	60
Protein Concentration (mg/mL)	4.94 ± 0.33	6.02 ± 1.22	12.92 ± 1.83	13.70 ± 0.99
Protease Activity (U/mL)	2773 ± 136	3096 ± 219	4692 ± 64	5518 ± 103
Specific Activity (U/mg protein)	561	514	363	403
Time (h)	12	12	60	60
Protease Productivity (U/mL/h)	231.08	258.92	78.2	91.96
Cell Density (CFU/mL)	1.79×10^{10}	2.16×10^{10}	1.70×10^{11}	2.01×10^{11}

At the end of fermentation, the obtained residues were mainly composed of ash, lignin, and *Bacillus subtilis*. Previous study showed that *Bacillus subtilis* was beneficial to plant growth (López-Valdez *et al*. 2011). Lignin exhibited excellent adsorption capacity for heavy metals, and could be used as a natural urease inhibitor to promote the retention of urea in soil. In addition, as a humic acid precursor, lignin could be degraded

into humus to improve soil fertility (Legras-Lecarpentier *et al*. 2019). Considering the above advantages, it is feasible to convert fermentation residue into fertilizer that provides nutrients and essential elements for plant growth.

In this study, the SScF was conducted to produce protease more efficiently than previous studies (Kshetri *et al*. 2016; Mishra 2016; Singh and Bajaj 2016). Furthermore, the water extract of SMC was recycled for protease production in SScF. The pretreated SMC was the only carbon source in present research, while previous studies required additional carbon sources (Singh and Bajaj 2016; Hussain *et al*. 2017). The established process provided an alternative method for closed-loop utilization of an inexpensive and not easily degradable substrate.

Mass Balance

It was determined that 0.79 kg extractive-free SMC was obtained from 1 kg raw SMC. After the pretreatment, 0.45 kg substrate was recovered, which could be used for protease production by SScF with *Bacillus subtilis* DES-59. Meanwhile, 0.11 kg fertilizer was obtained. The co-production of protease and fertilizer was achieved by SScF. Traditionally, the residues of enzymatic hydrolysis should be blended with bacteria for fertilizer production. Here, residue of SScF might be directly used for fertilizer. Therefore, the developed SScF process exhibited potential application in future biorefinery.

CONCLUSIONS

1. The enzymatic hydrolysis efficiency of SMC was improved significantly with the established pretreatment.
2. The water extract of SMC was recycled to promote protease production by *Bacillus subtilis* DES-59.
3. The residues after SScF were recovered as fertilizer.
4. A closed-loop process of simultaneous saccharification and protease production from SMC by *Bacillus subtilis* DES-59 was successfully established.

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APPENDIX

Supplementary Information

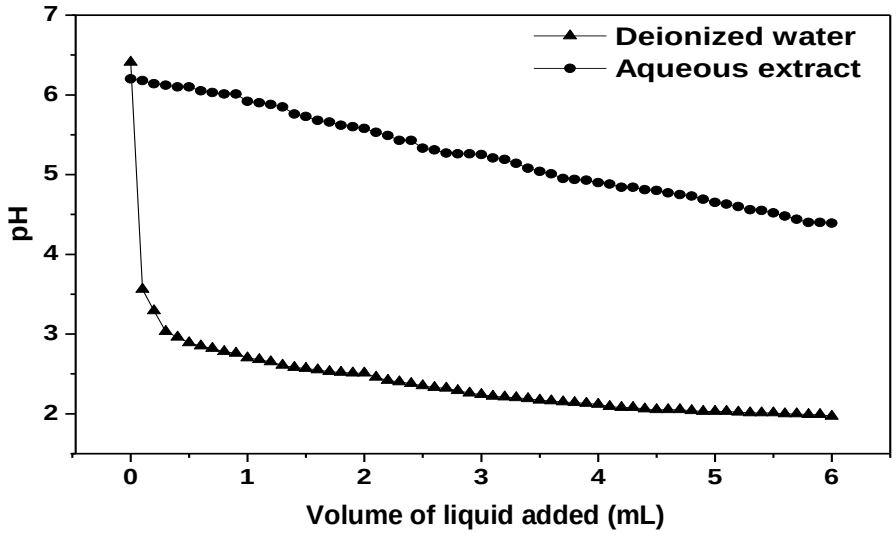

Fig. S1. Titration curve with 0.1 M H_2SO_4 for deionized water and aqueous extract

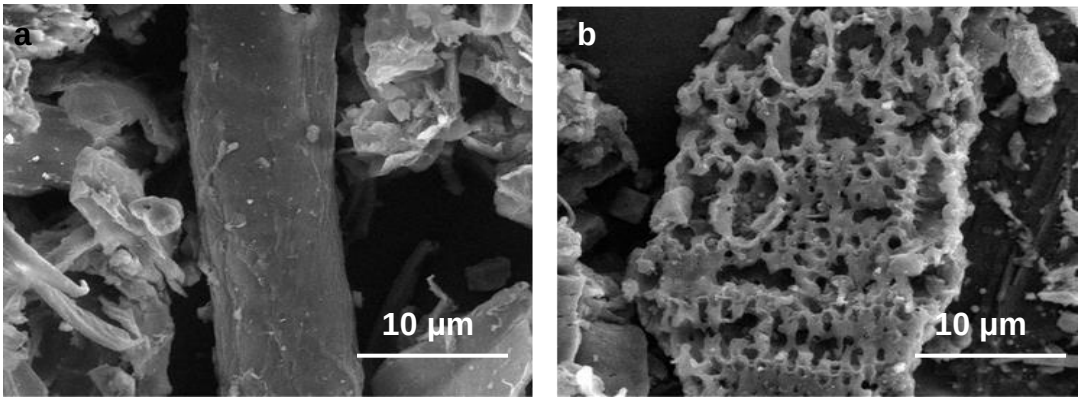

Fig. S2. SEM images of raw (a) and pretreated SMC (b). The images correspond to 2000× magnification

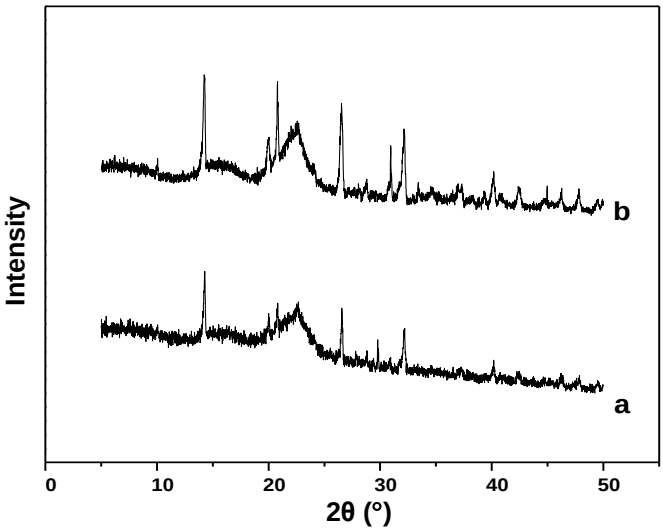

Fig. S3. X-ray diffraction pattern of raw SMC samples: (a) Raw SMC; (b) Pretreated SMC

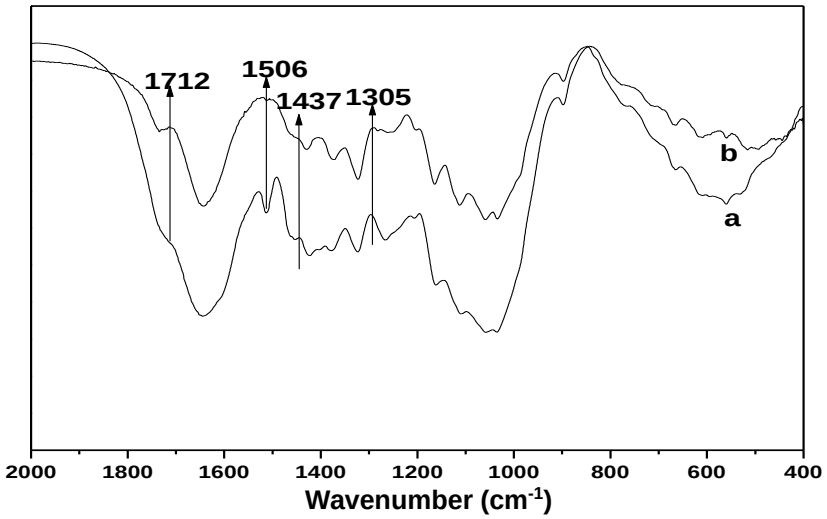

Fig. S4. The FITR spectra of SMC samples: (a) Raw SMC; (b) Pretreated SMC