An Effective Modification with Mild Alkali Pretreatment for Enhancing the Biodegradation of Wheat Straw by *Pycnoporus sanguineus* NFZH-1

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A delignification pretreatment is important for enhancing lignocellulose biodegradation. Alkali pretreatment is a promising approach. Fiber morphology, alkaline nitrobenzene oxidation, and ozonation were used to characterize the wheat straw modified by mild alkali pretreatment (2% sodium hydroxide (NaOH) at 121 °C for 30 min), and for studying the advantageous performance by *Pycnoporus sanguineus* NFZH-1 in the aspects of lignin and carbohydrate biodegradation. The results indicated a powerful and selective delignification in the mild alkali pretreatment process. The relative contents of the G unit and the T form both decreased with mild alkali pretreatment. Meanwhile, epicuticular wax removal and increased porosity was observed in the fibrous tissue of alkali-treated wheat straw. Thus, the biodegradation of the Klason lignin in alkali-treated wheat straw was clearly enhanced and reached 41.4% during the following 10 days of fermentation with *P. sanguineus* NFZH-1. In addition, the modification of fiber tissue with a mild alkali pretreatment enhanced the biodegradation of xylan. The biodegradation of the chemical constituents of the wheat straw was enhanced by the effective modification with a mild alkali pretreatment. The enhanced biodegradation will be helpful for improving the efficiency of straw return.

Keywords: Wheat straw; Biodegradation; Mild alkali pretreatment; Pycnoporus sanguineus; Straw return

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INTRODUCTION

Wheat straw, which is mainly composed of cellulose, hemicelluloses, and lignin, is one of the world's most important lignocellulosic biomasses for pulping, animal feeds, straw return, and biofuels (Tozluoglu *et al.* 2015). Determining strategies to use this valuable biomass and to prevent related environmental and ecological issues are urgent topics (Zong *et al.* 2016). There is a huge development potential for straw return through biodegradation by lignocellulolytic microorganisms (Tan *et al.* 2017). To partly replace chemical fertilizer, the straw is left to rot in the soil to replenish the nutrients for growth of the next generation, which can reduce haze pollution caused by the straw burning as well as alleviate water eutrophication caused by the use of chemical fertilizer (Fusi *et al.* 2014). The straw return is a two-way, cleaner production method that deeply attracts worldwide attention.

Pycnoporus sanguineus, a common species distributed in tropical and subtropical regions (Lesage-Meessen et al. 2011), is also named as *Trametes coccinea*. *P. sanguineus* is claimed to produce laccase as the predominant ligninolytic enzyme, and the fungus is described as a model organism for studying laccase and its implication in lignin degradation (Herpoël *et al.* 2000). Prior study indicated that 56.7% of Klason lignin and 36.6% of holocellulose in the wheat straw were biodegraded by *P. sanguineus* NFZH-1 within 30 days of incubation (Feng *et al.* 2014). However, the wheat straw that has not been biodegraded in a short time fails to improve soil fertility, hinders the germination of seeds, and delays the breeding of the next generation (Walsh and McDonnell 2012). Delignification is considered as a limiting step of the wheat straw biodegradation (Feng *et al.* 2016). Wheat straw lignin is composed of guaiacyl (G), syringyl (S), and hydroxyphenyl (H) structural units that are connected with ether bonds and C-C bonds (Freudenberg 1959). The cellulose and hemicellulose fractions in the wheat straw cell wall are surrounded by lignin. The hydrophobic lignin forms an interlaced network, which acts as a reaction barrier (Alvira *et al.* 2010) and results in a decrease in the accessible surface area and a consequently lower biodegradation. Therefore, the delignification pretreatment is expected to enhance the biodegradation of wheat straw.

Thermochemical pretreatment using alkali is noted as one of the most promising methods for the hydrolysis of lignocellulose, mainly due to its strong and rapid delignification ability (Kleppe 1970). During alkaline pretreatment, the nucleophilic reagent attracts the C_α site of lignin phenylpropane units and breaks the β-O-4 structures through neighboring group participation (Marton 1971). Molecular fragmentation causes lignin degradation. Meanwhile, alkaline hydrolysis improves the reactivity of the remaining polysaccharides, reduces acetyl groups and various uronic acid substitutions of hemicelluloses, removes epicuticular wax, swells the fiber cell wall, increases the porosity of fibrous tissue, and consequently increases the surface accessibility for exoenzymes (Karp *et al.* 2013). In addition, a number of studies have stated that the lignin of herbaceous plants contains hydroxycinnamic acids, ρ-coumaric, and ferulic acids (Ma *et al.* 2014). The hydroxycinnamic acids have alkali-labile ester bonds, forming lignin-carbohydrate complexes (LCC) by combining with lignin polymers and/or hemicelluloses (Zikeli *et al.* 2016). For this reason, wheat straw is easily delignified by using alkali under relatively mild pretreatment conditions (*e.g.*, low temperature, low alkali dose, and short treatment time), as compared with woody species.

The objective of the present study is to investigate the effect of mild alkali pretreatment on wheat straw biodegradation for improving the efficiency of straw return. In this study, the wheat straw was hydrolyzed by 2% sodium hyroxide (NaOH) at 121 °C for 30 min, and subsequently biodegraded by *P. sanguineus* NFZH-1 within 10 days. Scanning electron microscopic and *in situ* analytical techniques, including nitrobenzene oxidation (NO) and ozonation (Z), were conducted for the purpose of testing the effect of alkali pretreatment on biodegradation.

EXPERIMENTAL

Materials

Mild alkali pretreatment of wheat straw

Wheat straw (*Triticum aestivum* cv. Yang *No. 4*), was collected from Jiangsu Academy of Agricultural Sciences, Nanjing, China. The straw was cleaned by the

removal of the leaves, spikes, sheaths, and fragments, subsequently ground in a Wiley mill, and a 0.25 mm to 0.42 mm fraction was obtained.

The wheat straw powder was treated with 2% NaOH solution (weight/volume ratio of 1/4) at 121 ℃ for 30 min. The alkali-treated wheat straw was cooled, washed with distilled water until a neutral pH (7) was reached, and then air-dried to a constant weight for fermentation. The alkali-treated wheat straw was named as AWS, and the yield of AWS was 71.5%.

Chemicals

N,O-Bis (trimethylsilyl) trifluoroacetamide with trimethylchlorosilane (BSTFA + TMCS) was purchased from Sigma-Aldrich, Co. (St. Louis, USA) and was of HPLC grade. Potatoes were obtained from a local market. All other chemicals were of analytical grade from Sinopharm Chemical Reagent Co., Ltd. (Nanjing, China).

Methods

Biodegradation by Pycnoporus sanguineus NFZH-1

P. sanguineus NFZH-1 was maintained on potato dextrose agar (PDA) slants at 4 °C, and deposited in the Culture Collection of Wood Rot Fungi, Nanjing Forestry University, China. The PDA medium contained 200 g/L potato, 20 g/L glucose, and 20 g/L agar.

50 mL of medium containing 200 g/L potato, 20 g/L glucose, 2 g/L $MgSO_4 \cdot 7H_2O$, 5 g/L $(NH_4)_2SO_4$ and 5 g/L KH_2PO_4 was added to a 250 mL Erlenmeyer flask, autoclaved at 121 °C for 30 min, and inoculated with the *P. sanguineus* mycelium taken from the PDA plates. After 3 days of growth at 28 °C on a rotary shaker (170 r/min, *Φ* 18 mm), the spores were harvested and aseptically filtered through a Buchner funnel which contained six layers of cheesecloth to remove mycelium. Spores were allowed to settle down by gravity overnight, followed by repeated washing with 0.9% (w/v) NaCl. The obtained spores were kept at 4 °C in the dark and used within 2 weeks of storage. The stored spore suspensions were adjusted to a concentration of about 1.0×10^7 spores mL^{-1} for inoculating.

Five grams of AWS (oven-dry weight) and 12 mL of Kirk nutrient solution were mixed in a 250-mL Erlenmeyer flask, followed by sterilization at 121 °C for 60 min, and inoculated with *P. sanguineus* NFZH-1. The Kirk medium contained 10 g/L glucose, 0.44 g/L ammonium tartrate, 6.0 g/L potassium dihydrogen phosphate (KH_2PO_4), 4.1 g/L magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), 0.3 g/L calium chloride ($CaCl_2$), 0.2 g/L sodium chloride (NaCl), 0.1 g/L manganese sulfate monohydrate ($MnSO_4 \cdot H_2O$), 6 mg/L vitamin B_1, and 0.5 mL/L Tween-80. After 10 days of growth at 28 °C without shaking, the biodegraded samples (residues) named as BAWS were washed with distilled water, and then air-dried for fiber morphology, lignin content, and chemistry structure analysis. The wheat straw was also biodegraded under the same conditions, and named as BWS. The BWS yield of 87.8% and BAWS yield of 82.0% were determinated in the biodegradation process, respectively.

Determination of chemical constituents

The washed residues were measured for their chemical constituents according to the standard methods from the Technical Association of the Pulp and Paper Industry. These protocols were processed for Klason lignin (TAPPI T222 om-88 (1988)), acid-

soluble lignin (TAPPI T13wd-74 (1974)), and carbohydrate constituents (TAPPI T249 cm-00 (2000)).

Gas chromatography (GC; GC9800, Kechuang Instruments Technology Co., Ltd., Shanghai, China) was used for the carbohydrate constituents analysis. The analytical column was a fused-silica capillary column (SGE Analytical Science BP5, Melbourne, Australia, 0.25 mm i.d. × 30 m). Nitrogen was used as a carrier gas with a flow rate of 1 mL/min. The column temperature programme was 60 ℃ (1 min) and 60 °C to 220 °C (15 °C/min), held for 15 min at 220 °C. The injector and detector temperatures were maintained at 225 °C and 250 °C, respectively.

Fiber morphology observation

The fiber morphology observation employed in this work was based on previous reports (Feng *et al.* 2015). The washed residues were dehydrated and critical point dried for fiber surface and cross-section analysis *via* scanning electron microscopy (SEM; FEI Quanta 200, Portland, USA) after gold-plating.

Alkaline nitrobenzene oxidation

Alkaline nitrobenzene oxidation was performed according to previous literature (Feng *et al.* 2016). The samples were degraded, extracted, and dried for GC analysis (GC9800, Kechuang Instruments Technology Co., Ltd., Shanghai, China). The column temperature programme was 160 °C (0 min) and 160 °C to 260 °C (4 °C/min), held for 3 min at 260 °C. The injector and detector temperatures were maintained at 260 °C and 270 °C, respectively. Other conditions were the same as described in the carbohydrate constituents analysis.

Ozonation

Ozonation was performed according to previous literature (Feng *et al.* 2016). The samples were degraded, filtered, saponified, washed, and dried for GC analysis (GC9800, Kechuang Instruments Technology Co., Ltd., Shanghai, China). The column temperature programme was 120 °C (0 min) and 120 °C to 260 °C (4 °C /min), and held for 3 min at 260 °C. The injector and detector temperatures were maintained at 260 °C and 270 °C, respectively. Other conditions were the same as described in the carbohydrate constituents analysis.

Degradation calculation and statistics

The degradation of chemical constituents in AWS, BWS, and BAWS was calculated with Eq. 1,

$$\text{Degradation (\%)} = \frac{C_a - (Y \times C_b)}{C_a} \times 100 \quad (1)$$

where Y (%) is the yield of the wheat straw degradation, C_a (%) is the chemical constituents of wheat straw before degradation, and C_b (%) is the chemical constituents of wheat straw after degradation.

The tests were performed in triplicate. The data values are presented as means ± standard error (SE). A statistical analysis was performed by an analysis of variance (ANOVA) using Origin Pro (Version 8.0, OriginLab, Northampton, USA), and the probability values of $p \leq 0.05$ were considered a statistically significant difference.

RESULTS AND DISCUSSION

Effective Modification with Mild Alkali Pretreatment

Chemical constituents

Table 1 shows the impact of mild alkali pretreatment on the chemical constituents of wheat straw. The total lignin, the sum of Klason lignin and acid-soluble lignin, fell from 23.1% in the raw material to 16.2%, an overall 49.9% degradation. Mild alkali pretreatment led to a 49.0% degradation in Klason lignin, resulting in a decrease from 20.9% in the raw material to 14.9%. The degradation of acid-soluble lignin was more likely to happen. The content of acid-soluble lignin decreased from 2.2% in the raw material to 1.3%, a 57.8% degradation. Lower lignin content in the fermentation process for enhancing the biodegradation should be expected (Bikovens *et al.* 2010).

Glucan and xylan suffered weak attacks in the mild alkali pretreatment process. For glucan, the content increase occurred from 39.6% in the raw material to 48.3%; for xylan, the content also increased from 23.8% in the raw material to 25.7% (Table 1). This was a typical effect, caused by lignin degradation. The degradation of the hemicellulose side chain was high, including the degradation of 49.0% arabinan, 100.0% mannan, and 100.0% galactan. However, the degradation effect was virtually negligible. The degradation of carbohydrates is known to generate inhibitors, such as furfural from pentoses degradation, and 5-hydroxymethylfurfural from hexoses (Rasmussen *et al.* 2014). Lower carbohydrate degradation should thus lead to lower inhibitor generation. Lower inhibitor content might be good for the following biodegradation.

Table 1. Degradation of Chemical constituents by Mild Alkali Pretreatment

	Wheat Straw (%)	AWS (%)	Degradation (%)
Total lignin	23.1 ± 0.4	16.2 ± 0.3	49.9 ± 1.9
Klason lignin	20.9 ± 0.5	14.9 ± 0.4	49.0 ± 2.6
Acid-soluble lignin	2.2 ± 0.1	1.3 ± 0.1	57.8 ± 5.5
Arabinan	3.9 ± 0.1	2.8 ± 0.1	49.0 ± 3.6
Xylan	23.8 ± 0.2	25.7 ± 0.1	22.8 ± 1.0
Mannan	0.5 ± 0.1	ND[a]	100.0
Glucan	39.6 ± 0.3	48.3 ± 0.3	12.8 ± 1.2
Galactan	1.3 ± 0.1	ND	100.0
ND: not detected			

Fiber morphology

Figure 1 shows the SEM images of the fiber surface and cross-section of wheat straw modified by mild alkali pretreatment. Figures 1a and 1c are the SEM images of the fiber surface and cross-section of wheat straw, respectively. Figures 1b and 1d present the SEM images of fiber surface and cross-section of AWS, respectively. The AWS had a broken fiber surface, and the removal of the epicuticular wax in the mild alkali pretreatment process was obvious (Fig. 1b). Some pores formed in the compartment layers of the cell wall during mild alkali pretreatment of wheat straw (Fig. 1d).

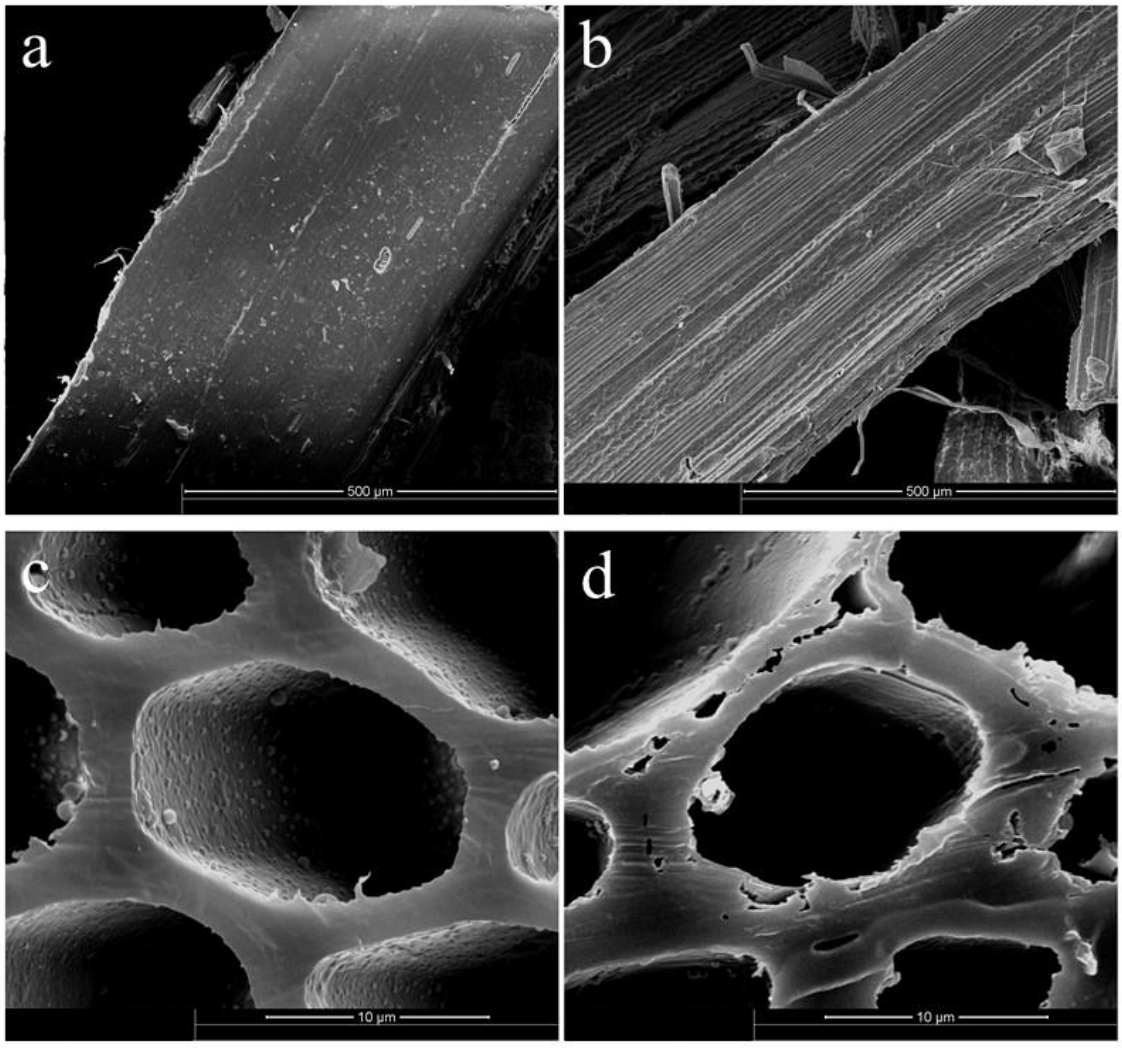

Fig. 1. SEM images of fiber surface and cross-section of wheat straw modified by mild alkali pretreatment, wheat straw (a and c), and AWS (b and d)

Lignin chemical structure

Alkaline nitrobenzene oxidation is important in terms of the characterization of lignin by providing information on the relative amounts of the uncondensed lignin units (Chen 1992). When applied to wheat straw, NO converts the lignin to identifiable nitrobenzene oxidation productions (NOP), which are mostly a mixture of aromatic aldehydes comprising vanillin (G), syringaldehyde (S), and ρ-hydroxybenzaldehyde (H) (Chang and Allan 1971). Table 2 shows the effect of mild alkali pretreatment on the uncondensed lignin units of wheat straw. After mild alkali pretreatment, NOP degradation of wheat straw was 32.4%, which was from 35.0% uncondensed G unit degradation, 43.9% uncondensed H unit degradation, and only 27.2% uncondensed S unit degradation. Lower S unit was degraded by mild alkali pretreatment, that differed from kraft pulping (Ventorim *et al.* 2014). Lower S unit degradation may have been attributed to the mild pretreatment conditions. The degradation merely occurred at the middle lamella, while the S-rich second wall (Sjöström 1993) was relatively undamaged (Fig. 1d).

The β-O-4 structures are predominant linkage types in lignin (Henriksson 2009). The linkage type can either be in the *erythro* (E) or *threo* (T) forms, and their stereochemistry can be characterized by ozonation (Akiyama *et al.* 2003). The degradation of β-O-4 structures and their stereochemistry are listed in Table 2. The 51.4% β-O-4 structures were degraded by mild alkali pretreatment. The E form was cleaved faster than T form under alkaline conditions. Faster E cleavage was explained by steric hindrance and intermolecular hydrogen bonding (Ueda *et al.* 2015), and was consistent with Shimizu's report (Shimizu *et al.* 2012).

Table 2. Degradation of Uncondensed Units and β-O-4 Structures in Lignin by Mild Alkali Pretreatment

		Wheat Straw (%)	AWS (%)	Degradation (%)
Uncondensed units	NOP	39.5 ± 0.1	37.5 ± 0.3	32.4 ± 1.0
	G	20.5 ± 0.5	18.7 ± 0.3	35.0 ± 2.9
	S	16.3 ± 0.4	16.5 ± 0.5	27.2 ± 3.6
	H	2.7 ± 0.2	2.1 ± 0.1	43.9 ± 6.8
β-O-4 structures	E+T	22.2 ± 0.3	15.1 ± 0.2	51.4 ± 1.4
	E	14.1 ± 0.3	8.7 ± 0.1	55.9 ± 3.0
	T	8.1 ± 0.0	6.4 ± 0.1	43.5 ± 0.9

Enhancing the Biodegradation of Wheat Straw

Lignin biodegradation

Table 3 shows the impact of mild alkali pretreatment on lignin biodegradation. Total lignin biodegradation of BWS was 14.6%; 13.7% Klason lignin biodegradation and 17.7% acid-soluble lignin biodegradation were found during 10 days of fermentation of the wheat straw. Total lignin biodegradation of BAWS reached 38.7%, which was approximately 2.7 times the amount of the biodegradation in BWS. The considerable biodegradation of total lignin mainly resulted from a 41.4% biodegradation of Klason lignin in BAWS. In consideration of the starting mass of wheat straw, 69.3% degradation of total lignin was found in the entire process, including mild alkali pretreatment and fermentation. The high degradation resulted from 70.1% degradation of Klason lignin and 61.1% degradation of acid-soluble lignin. As compared with wheat straw biodegradation, the lignin degradation was obviously enhanced by combining mild alkali pretreatment with fermentation. The G unit and T form were resistant to lignin biodegradation (Ander 1990). It was interesting that their relative contents both decreased after mild alkali pretreatment. The decreasing content of G unit and T form was the reason why the biodegradation of Klason lignin was enhanced. The removal of epicuticular wax promoted the strain growth on the fiber surface, and the increased porosity of fiber tissue allowed the enzyme proteins to penetrate into the cell wall. It laid another foundation for the lignin biodegradation. Lower biodegradation of acid-soluble lignin was found in the BAWS because acid-soluble lignin was more easily dissolved in the alkali pretreatment of wheat straw before the fermentation (Table 1).

Table 3. Lignin Biodegradation by *P. sanguineus* NFZH-1

	Degradation (%)		
	BWS	BAWS[a]	BAWS[b]
Total lignin	14.6 ± 0.9	38.7 ± 1.5	69.3 ± 0.7
Klason lignin	13.7 ± 0.2	41.4 ± 1.6	70.1 ± 0.8
Acid-soluble lignin	17.7 ± 1.2	7.9 ± 0.3	61.1 ± 0.1
a, based on the AWS; b, based on the start mass of wheat straw			

Carbohydrate biodegradation

Prior study reported that xylanase and carboxymethyl cellulase were able to be produced by *P. sanguineus* NFZH-1 (Feng *et al.* 2014). Table 4 shows the impact of mild alkali pretreatment on carbohydrate biodegradation. Xylan and glucan were the main carbohydrates in wheat straw (Table 1), but the impacts of mild alkali pretreatment were different. For xylan, the biodegradation increased from 16.3% to 22.6%, and 44.7% degradation of xylan was found in the entire process. This increase was because of epicuticular wax removal and increased porosity in the mild alkali pretreatment process, which improved the surface accessibility of xylanase. For glucan, 35.6% degradation was found in the entire process. However, a slight decrease of biodegradation might result from the increased crystallinity in AWS. It is widely believed that the degradation of crystalline cellulose was the key problem to further improve the biodegradation efficiency of alkali-treated lignocellulose (Zhao *et al.* 2010).

Table 4. Carbohydrate Biodegradation by *P. sanguineus* NFZH-1

	Degradation (%)		
	BWS	BAWS[a]	BASW[b]
Xylan	16.3 ± 0.3	22.6 ± 0.2	44.7 ± 0.1
Glucan	11.0 ± 0.2	9.9 ± 0.1	35.6 ± 0.1
a, based on the AWS; b, based on the start mass of wheat straw			

Usually, at least 30 days of cultivation was required for straw return. The long cultivation time limited the efficiency of straw return. As compared with wheat straw biodegradation, the cultivation time of straw return was reduced from 30 days to 10 days, and an increased proportion of the lignin was biodegraded after return of the alkali-treated straw to the soil. The improvement of biodegradation by mild alkali pretreatment was believed to promote the straw return. Besides, in consideration of the limited carbohydrate biodegradation, a co-cultivation of lignin- and carbohydrate-degrading strain may further improve the efficiency of lignocellulose biodegradation. The studies on the biodegradation of alkali pretreatment wheat straw using a co-cultivation are currently underway.

CONCLUSIONS

1. Modification with mild alkali pretreatment enhanced the biodegradation of wheat straw by *P. sanguineus* NFZH-1 within 10 days of fermentation. The enhanced biodegradation of the chemical constituents in wheat straw is expected to improve the efficiency of straw return to the soil.

2. The degradation of Klason lignin in the alkali-treated wheat straw reached 41.4% in fermentation process and 70.1% in entire process, respectively. The enhanced biodegradation of Klason lignin resulted from powerful and selective lignin degradation, especially for the G unit and T form in the mild alkali pretreatment process. Meanwhile, epicuticular wax removal and increased porosity of fibrous tissue with mild alkali pretreatment were also beneficial for the following biodegradation of Klason lignin.
3. The degradation of xylan in the alkali-treated wheat straw reached 22.6% in fermentation process and 44.7% in entire process, respectively. The epicuticular wax removal and increased porosity of fiber tissue with mild alkali pretreatment were significant to enhance xylan biodegradation.

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