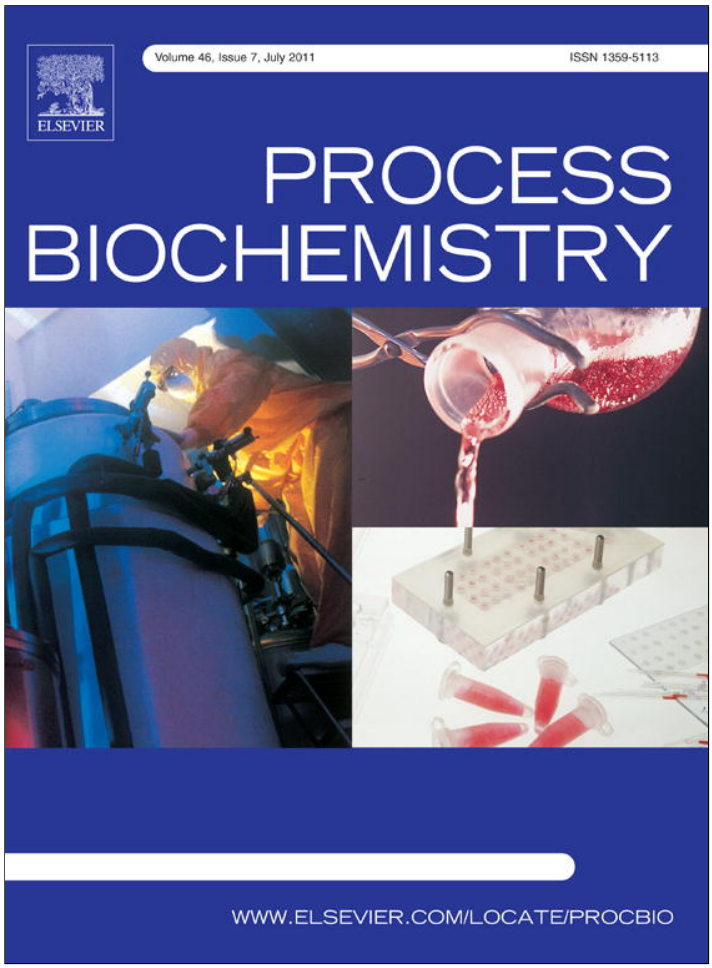

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Short communication

Effect of ligninolytic enzymes on lignin degradation and carbon utilization during lignocellulosic waste composting

ChongLing Feng[a,b], GuangMing Zeng[a,*], DanLian Huang[a,b], Shuang Hu[a,b], MeiHua Zhao[a,b], Cui Lai[a,b], Chao Huang[a,b], Zhen Wei[a,b], NingJie Li[a,b]

[a] College of Environmental Science and Engineering, Hunan University, Changsha, 410082, PR China
[b] Key Laboratory of Environmental Biology and Pollution Control (Hunan University), Ministry of Education, Changsha, 410082, PR China

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ABSTRACT

The objective of this study was to assess the response of carbon utilization profiles to addition of ligninolytic enzymes and the relationship between the carbon utilization and lignin degradation. Ligninolytic enzymes were added into the initial composting materials as treatment, and the inactive ligninolytic enzymes were added into the composting materials as control. The results showed that the treatment increased the degradation ratio of lignin and hemicellulose by 5.24% and 11.74%, respectively ($P < 0.05$), comparing to the result of control. Carbon utilization (measured by Biolog EocPlate™) revealed that, in the treatment, average well-color development (AWCD) of amino acids was significantly enhanced after day 6 ($P < 0.05$), while AWCD of carboxylic acids and polymers were increased after day 15. Principal component analysis confirmed the differentiation of the treatment and the control. The correlations between the lignin degradation and the Biolog indexes indicated that the presence of ligniolytic enzymes could enhance the activity of microorganisms.

1. Introduction

In the agricultural waste composting, lignocellulose accounts for the major part of biomass, and its degradation is important to the operation of composting [1,2]. Lignocellulose is a macromolecular complex consisted of lignin, cellulose and hemicellulose. Lignin is a highly irregular and insoluble polymer, chemically bonded by covalent linkages of hemicellulose. Therefore, the lignin–carbohydrate complexes enwrap cellulose in plant cell wall [3,4]. This complex structure inhibits lignocellulose transformation, and consequently slows down the lignocellulosic waste degradation process in nature. Therefore, it is generally accepted that lignin decomposition is the rate-limiting step during composting [5,6]. Recently, much attention has been drawn to the development of inoculation with fungi for the efficient treatment of lignocellulosic waste [7,8].

White-rot fungi are currently being used not only in the biodegradation of lignin but also in bioremediation of other lignin-related pollutants (such as industrial dyes, aromatic pollutants) [8–11], as they secrete the low specificity and strong oxidative ligninolytic enzymes which could oxidatively degrade lignin and mineralize them into CO_2 and water [12,13]. However, direct addition of fungi to degrade lignocellulosic waste may cause several problems. For example, diversity of microbial communities varies greatly in different phases of composting. It cannot be taken for granted that the inoculum can always remain in predominant status in a complex composting system, since it requires several proper growing conditions (such as pH, temperature and salinity range) as well as long biomass acclimatization and incubation processes [14,15]. In addition, the inoculum might be outcompeted by indigenous microorganisms or killed during composting. This might weaken the effect of inoculum in composting. By contrast, addition of ligninolytic enzymes may minimize these problems as a simpler and more effective method [15]. Recently, the use of ligninolytic enzymes as biocatalysts for environmental purposes presents a promising prospect. The degradation and removal of a wide range of toxic phenols and various synthetic dyes by ligninolytic enzymes have already been reported [9,11,12]. However, there is little information about addition of ligninolytic enzymes into composting for lignin degradation.

Previous studies reported that carbon utilization was remarkably influenced by addition of organic matter or microbial inoculation during composting [7,16]. Therefore, the studies on the carbon utilization could reveal the effects of ligninolytic enzymes during composting. Biolog assay has been considered an effective approach to detect the carbon utilization, which could differentiate the microbial communities based on the ability of microorganisms to oxidize different carbon substrates [17].

* Corresponding author. Tel.: +86 731 88822754; fax: +86 731 88823701.
E-mail address: zgming@hnu.cn (G. Zeng).

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The aim of this study is to assess the potential of ligninolytic enzymes to improve lignocellulose waste composting. Therefore, ligninolytic enzymes were added into the lignocellulosic waste composting in this study. During the composting process, lignin degradation and carbon utilization were investigated.

2. Materials and methods

2.1. Preparation of enzyme and composting material

Phanerocheate chrysosporium (*P. chrysosporium*) strain BKM-F-1767 was maintained on potato dextrose agar (PDA) slants at 4 °C, and then transferred to PDA plates at 37 °C for 4 days. The spores on the agar surface were gently scraped and blended in the sterile distilled water as spore suspension. After 5 days of incubation at 37 °C on a rotary shaker at 160 rpm, the culture mediums (reaching the maximum activities of the ligninolytic enzymes) were harvested and centrifuged at 9000 rpm for 10 min at 4 °C. The supernatant solution was used as crude ligninolytic enzymes [15], and the crude enzymes consisted of lignin peroxidase (LiP, 103 U L^{-1}) and manganese peroxidase (MnP, 72 U L^{-1}). LiP activity was measured as described by Hiromi et al. [18]. One unit (U) of LiP activity was defined as the amount of the enzyme required to produce 1 μmol veratryl aldehyde from the oxidation of veratryl alcohol per minute. MnP activity was measured as described by López et al. [19]. MnP unit activity was defined as the amount of enzyme required for producing 1 μmol Mn^{3+} from the oxidation of Mn^{2+} per minute.

Soil, wheat straw, root vegetable residues and bran were prepared as compost materials. Soil was air-dried and ground to pass through a 2 mm nylon screen, offering native microorganisms and some necessary nutrients. Wheat straw was air-dried and cut to 10–20 mm, using as the difficultly decomposed organic materials. Root vegetable residues chopped into 10–20 mm were used as easily metabolized materials. Bran was used to adjust the initial C/N ratio of the compost.

2.2. Composting condition

Soil, wheat straw, root vegetable residues and bran were mixed in the ratio of 75:80:28:30 (w/w). The experimental composting piles were built with open boxes (84 cm × 60 cm × 50 cm, with a 65% filling level) under indoor conditions. All the materials were fully mixed with the organic-matter content of 62.2% and lignocellulose content of 37.3%. The C/N ratio was about 31:1. The mixtures were evenly distributed into A and B. A was added with the inactive ligninolytic enzymes as the control. B was added with ligninolytic enzymes (in the weight ratio of 3.2%) as the treatment. The mixtures were watered to adjust the moisture contents at around 65% by adding distilled water during composting. The composting process lasted for 50 days. And the mixtures were turned every three days in the first 15 days and then turned every six days after 15 days to improve the O_2 level inside the mixtures.

2.3. Analytical methods

Homogeneous triplicates samples were obtained at different phases of the process (0, 3, 6, 15, 30, 40 and 50 days). Nine sub-samples were taken at three depths (0–10 cm, 10–30 cm and 30–40 cm) in the different sections of piles (left, middle and right). Then the samples were homogenized to prepare for analysis. The temperature of the mixtures was monitored throughout the whole process. They were monitored in the above nine sampling positions, and the mean value was considered as the temperature of mixtures. The standard errors of means of temperature were below 0.4 ($n = 9$). The moisture content was determined after drying at 105 °C for 24 h.

Carbon utilization was assessed using Biolog EcoPlate™ (Biolog Inc., California, USA). Each 96-well plate contains three replicates, each one comprising 31 sole carbon sources and one water blank (control well). 4 g sample was vigorously shaken in 36 mL sterilize water, and a series of 10-fold dilutions were made for this compost suspension. The 10^{-3} dilution (150 μL) was then used to inoculate into the micro-plates. The plates were incubated at 25 °C, and color development in each well was measured as optical density (OD) at 590 nm with a plate reader at regular 12 h intervals [17].

Carbon utilization in each microplate, expressed as average well-color development (AWCD) was determined as follows [18]:

$$\mathrm{AWCD} = \sum \frac{(\mathrm{OD}i - R)}{31}$$

where OD_i is the optical density value from each well, R is the optical density value from the control well. In addition to analyzing individual carbon utilization patterns, 31 carbon sources were subdivided into six substrate groups (Polymers, Miscellameous, Carboxylic acids, Carbohydrates, Amino acids, Amines) and the average absorbance of all carbon sources within each group was computed as follows:

$$\mathrm{Absorbance} = \sum \frac{(\mathrm{OD}i - R)}{n}$$

where $n_{\mathrm{polymers}} = 4$, $n_{\mathrm{miscellameous}} = 3$, $n_{\mathrm{carboxylic\ acids}} = 7$, $n_{\mathrm{carbohydrates}} = 9$, $n_{\mathrm{amino\ acids}} = 6$, $n_{\mathrm{amines}} = 2$.

The Shannon–Weaver index (H') was calculated using an OD of 0.25 as threshold for positive response. H' was calculated as follows:

$$H' = -\sum P_i(\ln p_i)$$

where p_i is the ratio of the activity on each substrate (OD_i) to the sum of activities on all substrates $\left(\sum \mathrm{OD}i\right)$.

The E was calculated as follow:

$$E = \frac{H'}{\ln R}$$

where R is the number of oxidized carbon substrates.

Plate readings at 48 h of incubation were used to calculate AWCD, H' and E, since it was the shortest incubation time that allowed the best resolution among treatments [20].

Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined [2]. Hemicellulose was estimated as the difference between NDF and ADF. Cellulose was estimated as the difference between ADF and ADL. Lignin was estimated as the difference between ADL and ash content.

2.4. Statistical analysis

All analyses were done in three replicates. The results to be shown were mean values of triplicates and the standard deviations were used to summarize experimental data. One way analysis of variance (ANOVA) and multiple comparisons were performed to compare the mean values for different sampling time, and to test whether there were any significant differences among the means at the 95% confidence level. Correlation analysis was carried out to determine the relationships among lignin degradation and AWCD, H' and E, respectively. All statistical analyses were performed with SPSS 13.0 software.

3. Results

3.1. Changes in temperature

The variation of temperature showed that there was a three-phase composting process in the control and treatment composting. In the initial phase, the temperature in the control and treatment reached 58 °C and 66 °C within 2 days, respectively. Afterwards, the two mixtures underwent the thermophilic phase. However, the thermophilic phase of the treatment was prolonged for 6 days compared with that of the control. Then, the temperature in both mixtures decreased steadily during the maturation phase.

3.2. Changes in AWCD, H′ and E

The values of AWCD, H' and E in the treatment were significantly ($P < 0.05$) different from those of the control during composting (Fig. 1 and Fig. 2). In the control, the AWCD increased rapidly and reached the maximum (1.12) on day 3, and then gradually decreased, while, the value of AWCD in the treatment kept increasing and reached the maximum (1.19) on day 6. The absorbance within each substrate group (e.g., polymers, carboxylic acids, amines) exhibited different dynamic patterns in the two mixtures. Comparing with the utilization of carbon in the control, amino acids and polymers utilization in the treatment significantly increased after day 6 ($P < 0.05$), while carboxylic acids utilization increased after day 15. The utilization of the other groups showed insignificant difference during composting. No significant differences between the control and treatment were found in the index of H' (Fig. 2). However, multiple comparison test of the E showed that there was a significant difference ($P < 0.05$) on day 30.

The principal component analysis was conducted to evaluate the microbial metabolic characteristics in the composting samples. The percentage values of the total variance accounted for the first two principal components (PC1 and PC2) are 34.5% and 21.5%, respectively. The result indicated some interesting trends of carbon utilization in the two mixtures (Fig. 3). From days 0 to 15, samples from the treatment, which showed high PC1 loading scores, have similar carbon utilization characteristics with those from the con-

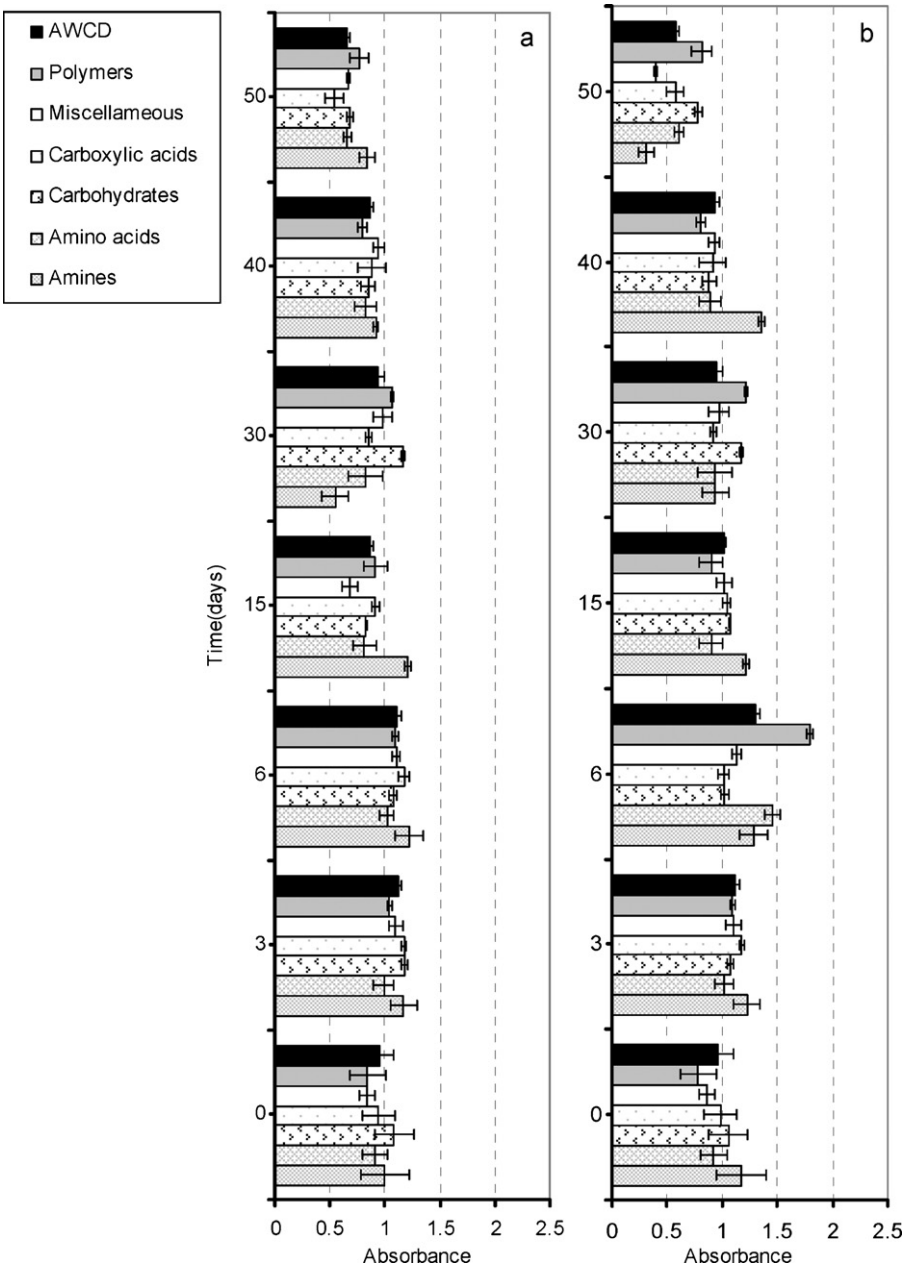

Fig. 1. Average well-color development in Biolog EcoPlateTM during lignocellulosic waste composting. (a) Control with inactive ligninolytic enzymes, (b) treatment with ligninolytic enzymes. The bars represent the standard deviations of the means ($n = 3$).

trol, as indicated by the same cluster. After day 15, samples from the control and treatment appeared to show the markedly different carbon metabolic characteristics, as indicated by the wide spread of the two data clusters. Samples showing high PC2 loading scores were mainly from the treatment (B15d, B30d, B40d and B50d). The metabolism of substrates such as Pyruvic acid methyl ester (carboxylic acid), D-Galacturonic acid (carboxylic acid) and Glycogen (polymers) were enhanced by the treatment mixture microbial communities after day 15. This result was consistent with AWCD.

3.3. Changes in lignocellulose degradation

As shown in Fig. 4, lignocellulose was gradually degraded during composting. The decomposing trends of cellulose were similar in two mixtures. Cellulose were degraded slightly during the initial phase of composting (0–6 days), while rapid decomposition were identified during the thermophilic phase. The significant differences between the control and treatment were detected in hemiclellulose and lignin degradation during composting. The degradation rate of hemiclellulose and lignin in the treatment increased by 5.24% and 11.74% ($P < 0.05$), respectively, comparing to the result of control.

3.4. Relationship between lignin degradation and the Biolog indexes

The possible roles of the microbial communities as degrader during the lignin degradation process were studied by correlation analysis of the lignin degradation with AWCD, H' and E (Table 1). No correlation was found between lignin degradation and AWCD in the control, whereas lignin degradation was significantly positively correlated with AWCD ($P < 0.05$) in the treatment. There was a negative correlation between the H' and the lignin degradation in the control. The highly significant positive correlations between E

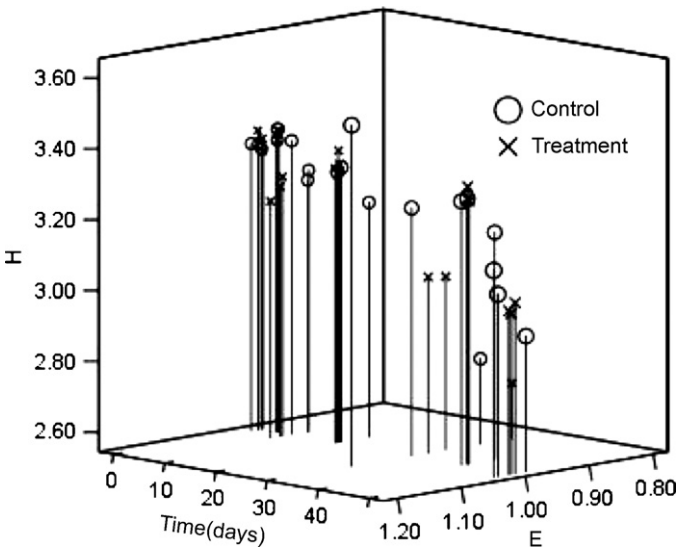

Fig. 2. Shannon–Wiener index (H') and Evenness (E) of carbon utilization in Biolog EcoPlateTM during lignocellulosic waste composting. (○) Control with inactive ligninolytic enzymes, (×) treatment with ligninolytic enzymes.

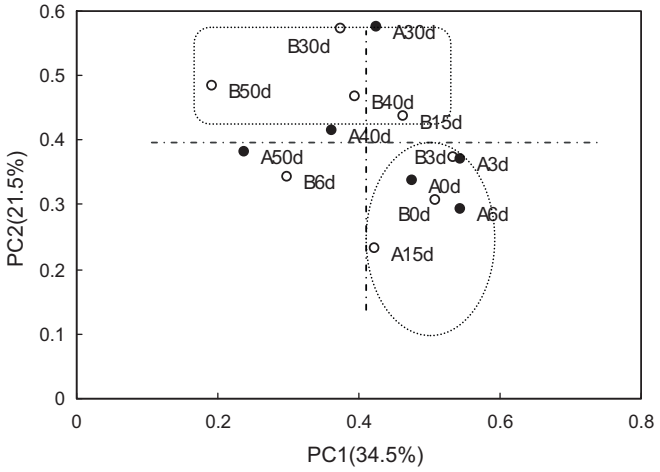

Fig. 3. Princinpal component analysis of composting samples based on carbon utilization profiles. (○) Control with inactive ligninolytic enzymes, (●) treatment with ligninolytic enzymes.

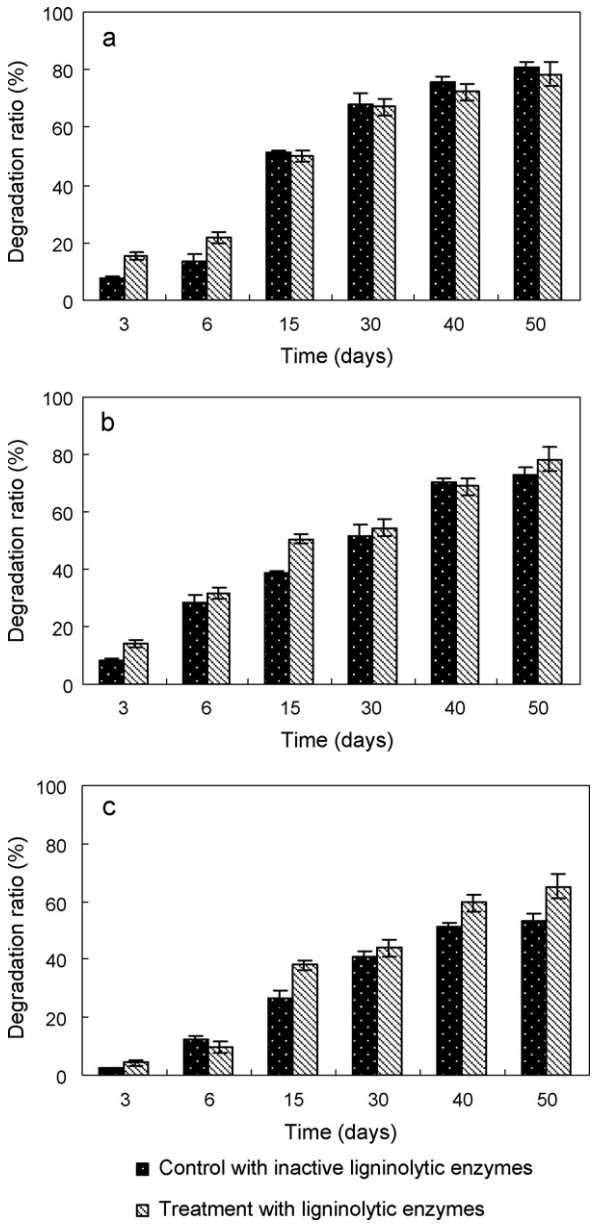

Fig. 4. Changes of degradation rates of cellulose (a), hemicelluloses (b) and lignin (c) during lignocellulosic waste composting. The bars represent the standard deviations of the means ($n = 3$).

Table 1
Correlation coefficients between carbon utilization and lignin degradation.

	AWCD	H'	E	%LD
Control				
AWCD	1.000	0.279	−0.327	0.048
H'		1.000	0.999*	−0.946*
E			1.000	0.928**
%LD				1.000
Treatment				
AWCD	1.000	−0.207	0.638*	0.772**
H'		1.000	0.885*	0.781
E			1.000	0.982*
%LD				1.000

**, * Indicate correlations significances at 0.01 and 0.05 probability levels, respectively.
AWCD: Average well-color development; H': Shannon–Wiener index; E: Evenness; %LD: Lignin degradation.

and lignin degradation were observed in the control and treatment (0.928 and 0.982, respectively; $P < 0.05$).

4. Discussion

Temperature is the most dominant factor of controlling composting reaction as its effect on microbial metabolic [21]. According to the study of López et al. [6], inoculating the composting of agricultural waste can result in a larger amount of heat output. Liang et al. [22] reported that the enhancement of microbial activities was induced by increasing temperature. In the present study, the temperature in both mixtures rose immediately after the onset of composting. However, the maximum temperature in the control was lower than that in the treatment during the mesophilic phase. Furthermore, higher temperature was maintained for a longer time in the treatment during the thermophilic phase. It indicated that adding enzymes in the composting could enhance

the microbial activities. These results could be explained by phenomenon that straw treated with ligninolytic enzymes can produce the polysaccharide-derived products which was easily utilized by more microorganisms [23].

The AWCD reflects the overall metabolic activity for a microbial community, and could be used as an indicator of microbial activity. A high AWCD was obtained when more carbon sources were used [24]. The addition of enzyme significantly increased AWCD in the treatment. This indicated that the addition of ligninolytic enzymes stimulated the microbial activity and the utilization of carbon substrates in the composting, which might be due to the improved lignocellulose degradation by ligninolytic enzymes [25]. This result was consistent with the temperature effect (Section 3.1) and principal component analysis. H' is a measure of actual richness and evenness of the microorganism population, while E reflects the comparability of substrate utilization between all utilized substrates, and the expected distribution of microorganism groups within the community. The multiple comparison tests showed that a significant change occurred on day 30 (Fig. 2), which indicated that the addition of ligninolytic enzymes could lead to different use of carbon substrates between the control and the treatment. Similar result was reported by Gomez et al. [20].

According to PCA, the samples at day 0 and 3 in the control and those in the treatment were classified as a closed group. This might suggest that the ligninolytic enzymes did not cause a notable change of carbon utilization during the first 3 days. After day 15, the metabolism of carboxylic acid and polymers were enhanced in the treatment, which indicated that ligninolytic enzymes affected carbon utilization after 15 days of composting. The reason might be that, after the easily degradable carbon sources have been consumed, more resistant compounds such as cellulose, hemicellulose and lignin are degraded and partly transformed into humus [1]. Ligninolytic enzymes can oxidize the lignin evolved in a lot of different reactions [26]. In the study on the ability of ligninolytic enzymes to degrade natural lignin by Thompson et al. [27], it was found that ligninolytic enzymes decreased the solid mass by 11%, decreased the lignin content by 5%, and released some typical low molecular weight lignin-derived products.

Lignin biodegradation is a key process during composting lignocellulosic waste. This polymer is difficult to be biodegraded and hampers the availability of lignocellulose. Ligninolytic enzymes have been widely used to degrade recalcitrant environmental pollutants, such as pesticides, organochlorines, polycyclic aromatic hydrocarbons and synthetic dyes. Alam et al. [28] used crude ligninolytic enzymes to remove methylene blue, and reported that the decolorization process gave a removal of 90% with incubation time 60 min in the presence of enzymes. Wen et al. [29] demonstrated that crude lignin peroxidase has a strong degrading ability towards tetracycline and oxytetracycline with the degradation rate of 95% in 5 min. In the present study, the results showed that adding enzymes significantly enhanced the degradation of lignin and hemicellulose (Fig. 4). This might be due to the ligninolytic enzymes which can act as one-electron oxidants, thereby generating cation radicals of the lignin. The cation radicals may undergo spontaneous chemical reactions such as C–C cleavage or hydroxylation, resulting in more hydrophilic products [30,31]. After then, those products could be transformed into CO_2, thermoenergy and humus-like end-product [1]. Furthermore, lignin is chemically bonded by covalent linkages with hemicelluloses, so the hemicelluloses degrading rate was high when lignin was degraded effectively [3]. The high degradation of lignocellulose could provide good conditions for the growth of indigenous microorganisms, which might be responsible for the prolonged thermophilic phase and high value of AWCD observed in the treatment.

The relationship between the carbon utilization and lignin degradation showed that the addition of ligninolytic enzymes in the composting caused a significant increment in correlation coefficients. This result suggested that the increase in lignin degradation might be explained by an increase in carbon utilization as a consequence of ligninolytic enzymes incorporation, which indicated that the presence of ligniolytic enzymes could enhance the activity of microorganisms.

5. Conclusion

With adding ligninolytic enzymes into the composting of lignocellulosic waste, the lignin degradation was enhanced, and the carbon utilization ability of microbial communities was also improved. It might be due to the ligninolytic enzymes which catalyze lignin degradation. All results suggested that it is feasible to apply ligninolytic enzymes to improve lignocellulosic waste composting. Further studies are needed to investigate on the mechanisms of ligninolytic enzymes accelerating the composting process, and to develop efficient composting technology with ligninolytic enzymes for the effective treatment of lignocellulosic waste.

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