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Effects of inoculation with *Phanerochaete chrysosporium* at various time points on enzyme activities during agricultural waste composting

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ABSTRACT

The effect of inoculation times on the enzyme activities during agricultural waste composting was determined. Four runs were used: without inoculation (Run A), inoculation with *Phanerochaete chrysosporium* (*P. chrysosporium*) during the first fermentation phase (Run B), inoculation during the second fermentation phase (Run C) and inoculation during both the first and the second fermentation phase (Run D). The results revealed that the effect of inoculation on carboxy methyl cellulase (CMCase) activities was negative during the first fermentation phase. The inoculation increased the activities of xylanase (almost 3000 U/g) during the first fermentation phase but no obvious difference among Runs A–D was observed during the second fermentation phase. The peak values of manganese peroxidase (MnP) in Runs C and D were three times higher than those of Runs A and B on day 21. The inoculation positively affected the lignin peroxidase (LiP) activities during the first fermentation phase and had a significant negative effect on the laccase (Lac) activities during the second fermentation phase. Therefore, the inoculation during the second fermentation phase was more effective than that during the first fermentation phase.

1. Introduction

When agricultural waste are composted, lignocellulose usually constitutes an important fraction of the total organic matter, as it is slowly decomposed (Shi et al., 2006; Tang et al., 2008; Tuomela et al., 2000; Yu et al., 2007). The inoculation with lignocellulolytic microorganisms is a strategy that could potentially enhance the lignocellulose degradation. Some species of basidiomycetes designated as white-rot fungi are able to break down all components of lignocellulose, including lignin, the polymer more refractory to microbial attack (Elorrieta et al., 2002; Huang et al., 2008; Vargas-García et al., 2006, 2007). Therefore, a lot of work has been carried out to study the effect of inoculation during composting (Higuchi, 1990; Kapich et al., 2004; López et al., 2007; Phil and Dan, 2007; Schoemaker and Leisola, 1990; Zeng et al., 2007). White-rot fungus, *P. chrysosporium*, is the most active ligninolytic organism described to date. Its ability to degrade lignin and a wide variety of aromatic compounds is due to a non-specific extracellular enzyme system, which involves manganese peroxidase (MnP), lignin peroxidase (LiP) and laccases (Lac) (López et al., 2007; Tang et al., 2006). Since inoculation with *P. chrysosporium* was usually done during the first fermentation phase of composting in the previous researches, the effect of different inoculation times is not known.

The phases of the composting process and the usefulness of compost as an organic amendment are determined by microbial activity (Goyal et al., 2005; Raut et al., 2008; Vargas-García et al., 2007; Zeng et al., 2006). The enzymes released by the microorganisms during composting also play a key role in the biological and biochemical transformations of the matrix. Microbial enzymes are responsible for the breakdown of several organic compounds characterized by a complex structure, finally produces simple water-soluble compounds (Benitez et al., 1999; Castaldi et al., 2008; Paola et al., 2008). Monitoring the presence and activities of specific intracellular and/or extracellular enzymes during composting may provide further insights into the development of the waste biodegradation processes (Benitez et al., 1999; Tang et al., 2006).

In this study, we analyzed the production of cellulase, xylanase and ligninolytic enzymes to evaluate the capacity of *P. chrysosporium* to improve the degree of lignocellulose decomposition in composting processes. The aim of this study was to understand the effect of different inoculation times on the enzyme activities.

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2. Methods

2.1. Agricultural waste collection and processing

Typical agricultural organic wastes (rice straw, vegetables, bran and soil) were collected from suburban areas. Rice straw, which was dried and cut to 10–20 mm lengths, was used as the recalcitrant organic composting material. Several kinds of vegetables chopped into 10–20 mm pieces were used as easily metabolizible materials. Bran was used to adjust the initial C/N ratio of composting. Furthermore, air-dried soil sieved through a 40-mesh screen to remove coarse plant debris, was added to increase microbial population and offer necessary nutrients.

2.2. Experimental set up

An experimental composting system with a weight of about 10 kg was set up in this study. Rice straw, vegetables, bran and soil were mixed at a ratio of 11:3:2:8 on a fresh weight basis and packed loosely in an open box. The organic matter content of this mixture was 60% and the initial C/N ratio was about 30:1 (Zeng et al., 2007). Moisture was monitored and adjusted to about 55% during the first fermentation phase and about 45% during the second fermentation phase by the addition of sterile water. To provide some aeration, the mixture was turned twice a week in the first 2 weeks and then once a week afterwards.

A pure culture of *P. chrysosporium* was used as the inoculums. The heap was inoculated with 5 mL liquid inoculant per kg of waste during the first fermentation phase (day 2), and the concentration of the liquid inoculant was 1×10^9 CFU mL^{-1}. To obtain better final compost, a solid inoculant composed of a mixture of rice straw and bran in which *P. chrysosporium* was grown at 37 °C for 6 days to a concentration of 1×10^9 CFU g^{-1}, was added at a rate of 10 g per kg of waste during the second fermentation phase (day 15). The composting materials were turned fully after inoculating to spread the microbial consortium.

One control and three treatments, each in triplicate, were set up as shown in Table 1, in which Run A was the control and Runs B–D were the treatments.

2.3. Analysis

The temperature in the center of the composting materials was monitored every day. Samples were taken at various stages of the composting process (0, 2, 5, 8, 11, 15, 21, 28, 35 and 42 days) to measure the C/N ratio. The moisture content of samples was determined after drying at 105 °C for 24 h. The dried samples were ground and analyzed for total organic carbon (TOC) by dry combustion. The samples were weighed and combusted in a muffle furnace at 550 °C for 6 h. The ash content (%Ash) was calculated from the difference between the weight of samples before and after combustion. The carbon content (%C) was calculated from the ash content, according to the following formula (Jiménez and García, 1992):

$$\%C = (100 - \%\mathrm{Ash})/1.8 \quad (1)$$

The total nitrogen (TN) was measured by Kjeldahl method (Bremner and Mulvaney, 1982).

During the process of composting, 2 ± 0.0002 g fresh samples were extracted with 20 mL cold deionized water under rotary shaking (200 rpm) for 1 h. The homogenate was centrifuged (1315*g*) at 4 °C for 20 min, and the supernate was filtered through filter papers (Whatman NO. 1). The filtrate was analyzed for activities of LiP (Tien and Kirk, 1984), MnP (Rogalski et al., 2006), Lac (Giorgi et al., 2007), CMCase (Ghose, 1987) and xylanase activity (Saha et al., 2005). LiP unit activity was defined as the amount of the enzyme which led to the production of 1 μmol veratryl aldehyde from the oxidation of veratryl alcohol per minute. One unit of MnP activity was expressed as the amount of enzyme which led to the production of 1 μmol Mn^{3+} from the oxidation of Mn^{2+} per minute. One unit of laccase activity was defined as the amount of enzyme, which led to the oxidation of 1 μmol of 2,2-azino-bis-[3-ethyltiazoline-6-sulfonate] (ABTS) per minute. One unit of CMCase or xylanase activity was defined as the amount of enzyme required to produce 1 μmol of glucose or xylose per minute. Specific activities of these enzymes were expressed as units per gram of dry medium.

The hemicellulose, cellulose and lignin contents were determined according to Van Soest's method (Soest et al., 1991) by Foss Fibertec 2010 (Sweden). The hemicellulose content was estimated as the difference between neutral-detergent fibre (NDF) and acid-detergent fibre (ADF), and the cellulose as the difference between ADF and acid-detergent lignin (ADL). Lignin was estimated as the difference between ADL and ash content. Lignocellulose degrading ratio was calculated by the following formula:

$$R_n = \frac{m_0 - m_n}{m_0} \times 100\% \quad (2)$$

where, R_n is the degrading ratio for the nth day sampling, %; m_0 is the initial content of lignocellulose, *g*; m_n is the content of lignocellulose on the *n*th day, *g*.

All presented results are average values of triplicate and the maximum difference among triplicates results was 5%. In order to determine the effect of inoculation during different phases on the enzyme activities, paired-samples *T* tests were used for the content of the enzyme activities during each phase. Paired-samples *T* tests were completed by using SPSS 13.0 software.

Table 1
Experiment conditions for different runs.

Heaps	Inoculant	
	First fermentation stage	Second fermentation stage
Run A	0.5% sterilized distilled water	1% sterilized solid inoculant
Run B	0.5% *P. chrysosporium* spore suspension	1% sterilized solid inoculant
Run C	0.5% sterilized distilled water	1% solid inoculant
Run D	0.5% *P. chrysosporium* spore suspension	1% solid inoculant

3. Results and discussion

3.1. Physico-chemical parameters

The changes of temperature during the composting are shown in Fig. 1. The composting process could be divided into two phases: (i) the first fermentation phase (day 0 to day 14) which included the mesophilic stage (day 0 to day 2) and the thermophilic stage (day 3 to day 14); (ii) the second fermentation phase (day 15 to day 42) which included the cooling stage (day 15 to day 22) and the maturation stage (day 23 to day 42). The temperatures were greater than 55 °C for more than 3 days in all the runs, which is the minimum requirement for a proper disinfection of waste materials from animal and plant pathogens (Yu et al., 2007). High temperature (exceeding 50 °C) was maintained for at least 10 days in the compost heaps during the first fermentation phase in the four runs. This showed that the inoculation during the first fermentation phase did not particularly affect the temperature in the compost heap. However, the inoculation during the second fermentation phase increased the temperature in the heaps (in

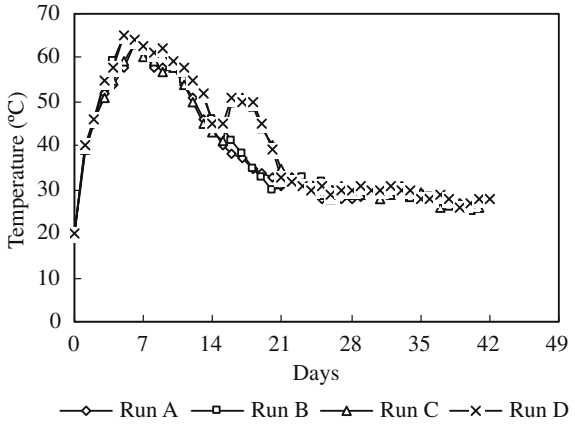

Fig. 1. Temperature evolution during the composting process. Runs A, B, C, and D are set up as shown in Table 1.

Runs C and D in comparison with Runs A and B). Abdelmajid et al. (2005) reported that more heat output probably was the result of biological activity in compost. So the result in this study indicated that the biological activity in compost was improved when the inoculant was present.

The C/N ratio decreased from 30 to 16 in all four runs during the composting process (Fig. 2). Nevertheless, some differences could be observed in the four runs. The C/N ratio in the heaps that were inoculated with *P. chrysosporium* during the second fermentation phase (Runs C and D) decreased to 16 after 35 days, while similar values were reached in Runs A and B but after 42 days. This observation suggests that inoculation with *P. chrysosporium* could promote the decrease of the C/N ratio during the second fermentation phase, but that the inoculation did not have an effect on the C/N ratio during the first fermentation phase.

3.2. Decomposition rate of lignocellulose

Changes of the lignocellulose degrading ratio during composting are shown in Fig. 3. Cellulose and hemicellulose were subjected to greater losses than lignin during composting, and their degrading ratios increased faster than that of lignin during the first fermentation phase. The ratios in lignocellulose degradation all showed an increase for four runs, with final values of about 40% as has been previously reported (Komilis and Ham, 2003).

Several studies have related the difficulty of lignocellulose degradation during composting (Baca et al., 1992; Tseng et al., 1996), with lignin being the more recalcitrant fraction (Lynch, 1993). Despite this reported difficulty, the main effect of inoculation was precisely in relation to this polymer as it has been previous reported (Vargas-García et al., 2007). Although, cellulose and hemicellulose decomposition could apparently not be enhanced by the inoculation in four runs, the presence of the inoculants led to higher lignin degrading ratio than those determined in non-inoculated runs. The inoculation during the second fermentation phase was the most effective since the degrading ratios of lignin were always significantly lower in the control heap. The result suggests that it is better to inoculate *P. chrysosporium* in composting during second fermentation phase.

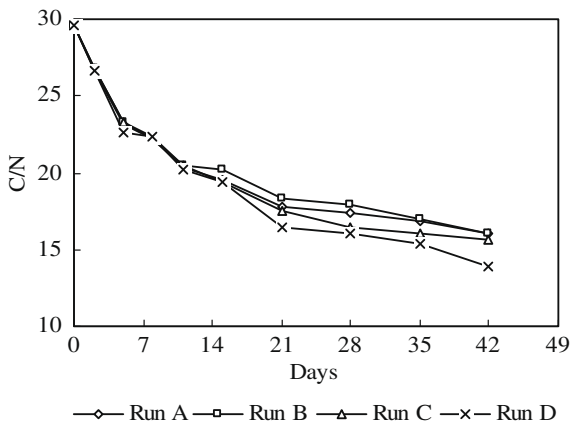

Fig. 2. Changes in C/N ratio during the composting process. Runs A, B, C, and D are set up as shown in Table 1.

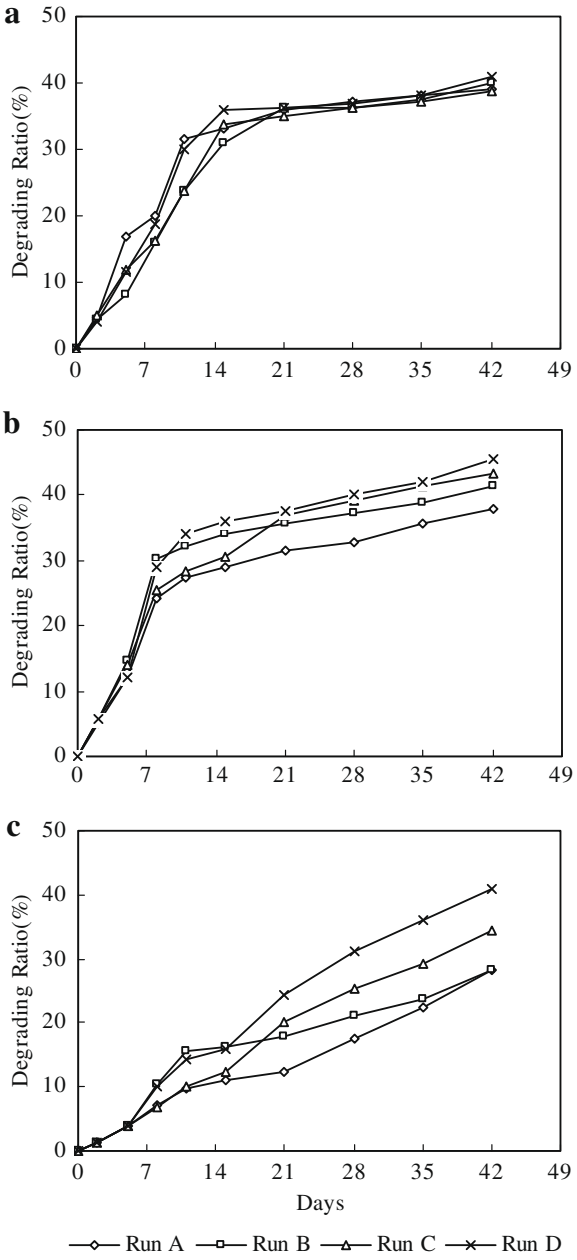

Fig. 3. Changes in degrading ratio of cellulose (a), hemicellulose (b), and lignin (c) during the composting process. Runs A, B, C, and D are set up as shown in Table 1.

3.3. Changes in enzyme activities during composting

3.3.1. CMCase activities

CMCase is one of the cellulases involved in the degradation of cellulose (Shi et al., 2006). Cellulose decomposition limits the rapid

production of compost more than any other substrates (Poincelot and Day, 1973). CMCase activity changes during the composting process are given in Fig. 4. In the four runs, CMCase activities peaked before day 14. The CMCase activities in Runs A and C reached the highest value on day 9, while the activities in Runs B and D inoculated with *P. chrysosporium* during the first fermentation phase reached the peak on day 12, and the values were lower than those in Runs A and C. A similar trend was also observed by Raut et al. (2008). Reports (Heilmann-Clausen and Boddy, 2005; Vasil-Chenko et al., 2004) suggest that white-rot fungi could produce an inhibitor during the degradation of cellulose. In the present investigation the lower values are probably due to the possible inhibitor secreted by *P. chrysosporium* inoculated during the first fermentation phase. The CMC activities were not much different among the four runs during the second fermentation phase. This result suggests that no obvious effect on the change of CMC activities was observed when the *P. chrysosporium* was inoculated during the second fermentation phase. Previous studies (Goyal et al., 2005; Schoemaker and Leisola, 1990) found that greater nitrogen availability could increase growth of microbial biomass and stimulate the secreting of CMCase, so the reason for the later increase in CMC activities might be the reduction in C/N ratio in the later stage of composting.

3.3.2. Xylanase activities

The changes in xylanase activities are shown in Fig. 5. The trends in xylanase activities are similar to that of CMCase activities. The activity of xylanase in the four runs increased until day 12 followed by a sharp decline. Mostly, fungi are involved in the decomposition of cellulose, hemicellulose and lignin present in the organic matter (Raut et al., 2008). The results of the present investigation showed a maximum degradation of hemicellulose within 14 days (Fig. 3b) and the activity of xylanase showed an increase during this period. Hemicellulose is the main substrate for the secretion of xylanase, therefore the increased activity of xylanase was consistent with the degradation of hemicellulose as reported by Paola et al. (2008). Early degradation of hemicellulose could be attributed to the increase of microbial biomass during the initial phase as the temperature increased (Fig. 1). High content of degradable organic compounds in the initial mixture might stimulate microbial growth and enzyme synthesis (Castaldi et al., 2008). Thus the available substrate as well as the enzyme activity decreased after day 10. Moreover, the activities in Runs B and D were higher (almost 3000 U/g) than those in Runs A and C from day 6–12, but no obvious difference was observed among the runs during the second fermentation phase. The result indicated that inoculating *P. chrysosporium* to compost had a more active effect on xylanase activity during the first fermentation phase than during the second fermentation phase.

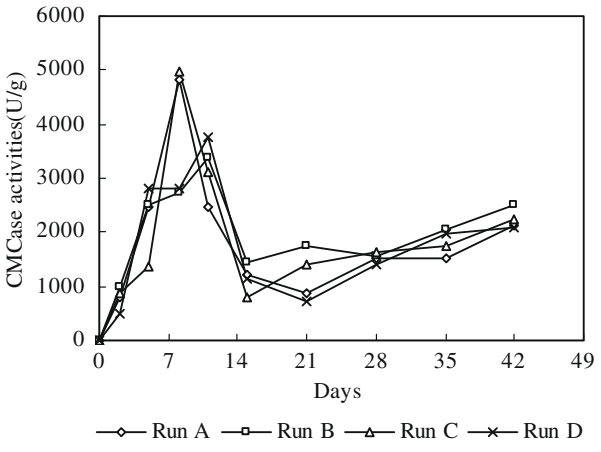

Fig. 4. Changes in the CMCase activities during the composting process Runs A, B, C, and D are set up as shown in Table 1.

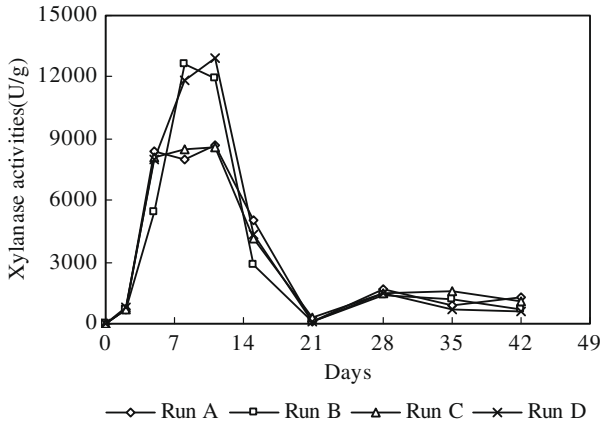

Fig. 5. Changes in the xylanase activities during the composting process Runs A, B, C, and D are set up as shown in Table 1.

3.3.3. Manganese peroxidase (MnP), lignin peroxidase (LiP) and Laccase (Lac) activities

Fig. 6 shows the changes in the MnP, LiP and Lac activities during composting of agricultural waste. LiP and MnP have been the most intensively studied extracellular enzymes of *P. chrysosporium*, and several reviews have summarized their biochemistry (Higuchi, 1990; Schoemaker and Leisola, 1990). In Fig. 6a, two peaks were observable from the changes of MnP activities during composting process in the four runs, the first time on day 10 and the second time on day 21. The results further indicated that the activities of MnP were high in samples inoculated with *P. chrysosporium*. Especially, the values of MnP in inoculated runs were three times higher than those of non-inoculated runs on day 21. MnP oxidizes Mn^{2+} to Mn^{3+}, using H_2O_2 as oxidant. Organic acids such as oxalic acid stimulate MnP activity by stabilizing the Mn^{3+}, and produce diffusible oxidizing chelates (Phil and Dan, 2007). In Miia et al. (2002) study it is reported that white-rot fungi could favor the accumulation of extracellular oxalic acid. In the present investigation MnP activity might have been stimulated the increasing levels of oxalate after *P. chrysosporium* was inoculated. The MnP increased sharply in the four runs during the later stage, while the lignin degrading ratio increased fast as shown in Fig. 3c. Kapich et al. (2004) have recently shown that the degradation products of lignin may have a role in the production of MnP.

In Fig. 6b, the LiP in Runs B and D increased more markedly than that in Runs A and C before day 10. After the second inoculation, the LiP in Runs C and D did not increase as fast as it did during the first fermentation phase. On day 35, the LiP in Run D peaked again, and among the four runs the value in Run D was the highest. The results indicated that inoculating twice was better for the secretion of LiP. The reason may be that, based on the inoculants in the first fermentation phase, inoculating during the second fermentation phase allowed the inoculants to avoid a thermophilic phase as it has been previously reported (Vargas-García et al., 2007). Another reason may be that the C/N ratio fell in the later stage during composting, which could induce the secretion LiP (Raut et al., 2008; Vargas-García et al., 2006).

Fig. 6c presents the change in Lac activities during composting of agricultural waste. In the first 7 days, a sharp increase in Lac activity was observed, and it was found that the inoculation during the first fermentation phase did not have much influence since the

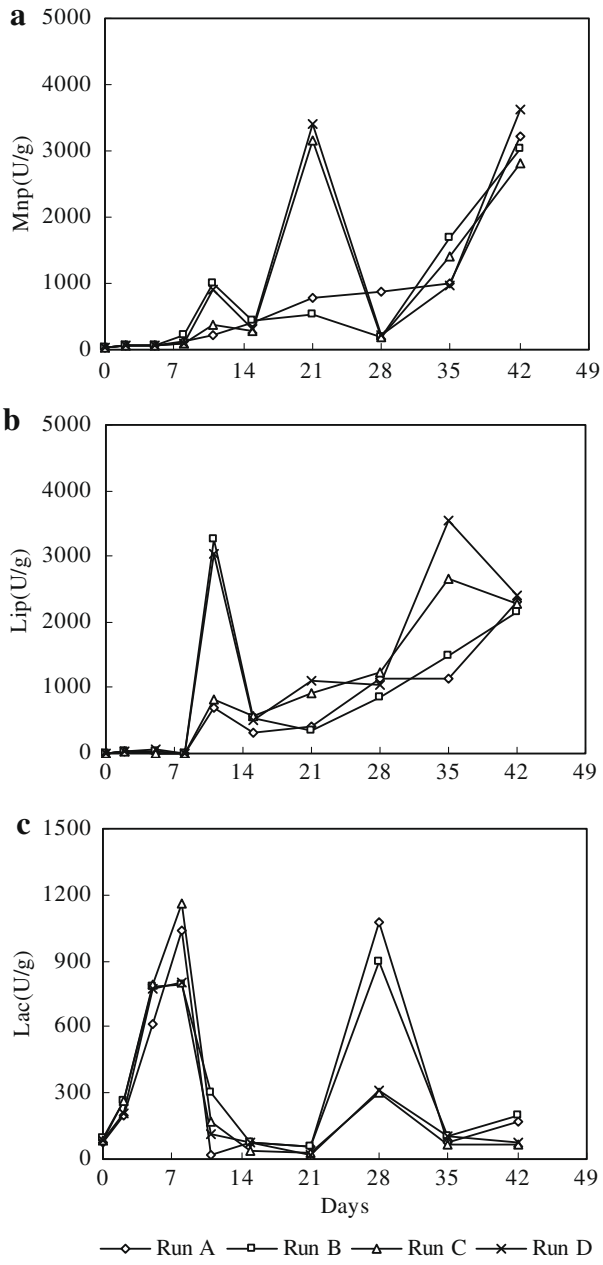

Fig. 6. Changes in the MnP (a), LiP (b) and Lac (c) activities during the composting process Runs A, B, C, and D are set up as shown in Table 1.

change trend and values of Lac activities were similar in the four runs. On day 28, the Lac activities reached a peak again, but this time the activities of Lac in Runs A and B which were not inoculated during the second fermentation phase, were much higher than those in Runs C and D. This result indicates that the activity of Lac was limited by the inoculants. Since exhaustive searches of the *P. chrysosporium* genome provide evidence that conventional laccases are absent in this efficient lignin degrading fungus (Tuomela et al., 2000), it is generally assumed that *P. chrysosporium* cannot secrete laccases. This observation should not be construed to exclude laccases involvement in lignin degradation by other species during composting. Indeed, considerable evidence supports that laccase is involved in ligninolysis (Phil and Dan, 2007). Maybe *P. chrysosporium* displaced other Lac producers and therefore the Lac activities were lower in inoculated heaps. However, these situations are not comparable to those that microorganisms have to deal with in real composting processes, where there is relatively high competition between species. Thus, it is not possible to know if *P. chrysosporium* would exhibit the same behaviour in relation to Lac activities.

3.4. Effect of inoculation during different phases on the enzyme activities

To reveal the effect of inoculation on the enzyme activities during different phases of agricultural waste composting process, paired-samples *T* tests were used to analyze the enzyme activity data for each phase, and it was found that different inoculation time had different influence on the enzyme activities (Table 2). The changes of CMCase activities in Runs B and D were significantly different compared with Run A during the first fermentation phase. The effect of inoculation on CMCase was negative as shown in Table 2. In fungi, CMCase is often low and synthesized with difficulty (Goyal et al., 2005; Schoemaker and Leisola, 1990). The difficulty was probably from the complex media composition since *P. chrysosporium* was able to secrete CMCase in pure culture as reported in previous studies (Liu et al., 2008; István et al., 1996). No significant difference was found in xylanase activities from Runs B–D compared with Run A. The changes of MnP and LiP activities during the first fermentation phase and the second fermentation phase in different runs were both significantly different. The inoculation positively affected the MnP and LiP activities. Since the MnP and LiP are the main extracellular ligninolytic peroxidases from *P. chrysosporium*, it is to be expected that the enzyme activities may be stimulated after inoculation (Tang et al., 2006; Tien and Kirk, 1984). The inoculation during the second fermentation phase resulted in a significant negative effect on the Lac activities.

The use of inoculants to speed up the composting process or to obtain better final compost has been a controversial subject for a long time and, in fact, contradictory results have been described by different authors (Elorrieta et al., 2002; Faure and Deschamps, 1991; Gaind et al., 2005). Nevertheless, controversy should not be surprising if we consider the complexity of the biological events that take place and the many factors the process depends on. The results in the study by Vargas-García et al. (2006) revealed that the usefulness of inoculation in composting depends on the conditions in which the process is carried out, in particular the characteristics of the raw material and inoculant. *P. chrysosporium* was able to increase the decomposition of lignocellulose whatever the characteristics of the waste, as it has been stated by other authors (Vargas-García et al., 2006). Thus, to make the ability of *P. chrysosporium* as inoculant more effective, the inoculating time may be an influential factor. In this study, the results suggested that the inoculation during the second fermentation phase was more effective.

Table 2
Effects of inoculation on enzyme activities during each phase.

Heaps	CMCase	Xylanase	MnP	LiP	Lac
The first fermentation phase (0–14 days)					
Run B-A I	−0.795*	0.290	1.179*	1.120*	0.679
Run C-A I	0.378	0.207	0.013	0.403	0.367
Run D-A I	−0.727*	0.292	1.182*	1.129*	0.623
The second fermentation phase (15–42 days)					
Run B-A II	−0.385	0.312	−0.365	0.167	−0.673
Run C-A II	−0.278	0.275	0.586	1.742*	−1.321*
Run D-A II	−0.513	0.231	0.792*	1.482*	−1.163*

Results are the mean of three replicates.
* Values are significantly different at $P < 0.05$ according to the paired-sample *T* test.

4. Conclusion

Inoculation can be a useful tool to accelerate the degradation of lignocellulose in agricultural waste composting. It is evident from the results that the presence of the inocula led to higher degrading ratio in lignin than those determined in non-inoculated heaps. This is in accordance with the conclusions drawn from enzyme activities. The inoculations had different effects when carried out during different phases of composting. The inoculation during the second fermentation phase was more effective than that during the first fermentation phase, since it improved the temperature and sped up the composting process.

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