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Enzymatic degradation of tetracycline and oxytetracycline by crude manganese peroxidase prepared from *Phanerochaete chrysosporium*

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ABSTRACT

Pharmaceuticals have been attracting increasing attention in recent years as emerging contaminants, of which the most frequently detected kind in various environments are antibiotics. In this study, crude manganese peroxidase (MnP) prepared from the *Phanerochaete chrysosporium*, a white rot fungi, was taken as a highly efficient biocatalyst to degrade tetracycline (TC) and oxytetracycline (OTC) which are widely used antibiotics. The results show that 72.5% of 50 mg/L of TC was degraded when added 40 U/L of MnP, while 84.3% of 50 mg/L of OTC was degraded with the same amount of the catalyst added, both within 4 h. The degradation rate was dependant on the pH and the temperature of the reaction system, and was likely sensitive to the concentration of H_2O_2. With the pH at 2.96–4.80, the temperature at 37–40 °C, the Mn^{2+} concentration higher than 0.1 mM and up to 0.4 mM, the H_2O_2 concentration of 0.2 mM, and the enzyme-substrate ratio above 2.0 U/mg, the degradation rate reached the highest. In addition, a separate series of experiments also show that the compensation of H_2O_2 during the reaction process could improve the degradation of TC by MnP.

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1. Introduction

Daughton and Ternes [1] coined the term PPCPs (pharmaceutical and personal care products) in 1999. And PPCPs have become emergent environmental research concerns attracting increasing attention ever since. Previous researches indicated that the highest concentration of PPCPs in the environment could be found at the effluent of biological wastewater treatment plants [2–4], due to the fact that the conventional biological treatment technologies are not designed for and cannot play effective roles in the removal of PPCPs [4–7]. Among various kinds of PPCPs, antibiotics are more frequently detected in the environment than the others [8,9]. They are especially difficult to be removed through common biological treatment methods since antibiotics are mainly designed in the purpose of exterminating bacteria [10–12]. Physical chemical methods, such as membrane contactor processes [13], activated carbon and palygorskite adsorption [9,14], ultraviolet technology [15] and ozone technology [16], have been proved effective to some extent in PPCPs removal, while further researches are yet to be conducted to make these methods low cost, safe and easy in operation.

The extracellular ligninolytic enzymes produced by white rot fungi, typically composed by one or more of the three principal ligninolytic enzymes (lignin peroxidases (LiP, EC 1.11.1.14), manganese peroxidases (MnP, EC 1.11.1.13) and laccases (EC 1.10.3.2)), are nonspecific and nonstereoselective enzymes that are capable of degrading lignin as well as a range of recalcitrate pollutants, and this capability of the enzymes gives them great application potential in the environment field [17]. Contrast to the treatment method of using the fungi directly, in vitro treatment with the ligninolytic enzymes can exclude some limiting factors, such as the difficulties in growth on a large scale, the long incubation processes, and the adsorption effects of the pollutants on the mycelia [18], and therefore may represent a simpler and more effective method [19]. Unfortunately, there have only been rare reports on in vitro degradation of pollutants by ligninolytic enzymes, and most of them have focused on the degradation of dyes [18,20–23]. In recent years, ligninolytic enzymes, especially manganese peroxidase (MnP), have been tested to degrade endocrine disrupters and polycyclic aromatic hydrocarbons, since MnP is produced by more species of white rot fungi [24–27] and so it is easier to obtain. For practical applications, crude enzymes are preferred because of the high cost related to enzyme purification. Therefore, the test on the capabilities of crude MnP to degrade PPCPs is highly called for and will lay the foundation of the application of the enzymatic degradation.

In this paper, crude MnP produced by *Phanerochaete chrysosporium* was used to degrade tetracycline (TC) and oxytetracycline (OTC) that were selected as the representatives of PPCPs because of their wide application, high-solubility in water, high residual toxi-

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city, and non-biodegradation. The objectives of this study were (1) to determine the capability of MnP to degrade TC and OTC; and (2) to optimize the reaction parameters of the degradation system.

2. Materials and methods

2.1. Chemicals

Veratryl alcohol and nitrilotriacetate (used in medium) were purchased from Fluka (Buchs, Switzerland). TC and OTC were obtained from Sino-American Biotec., Beijing. All other chemicals used were of analytical grade.

2.2. Microorganism and culture conditions

P. chrysosporium strain BKM-F-1767 was maintained at 37 °C on potato dextrose agar (PDA) (200 g/L potato extract, 20 g/L glucose and 20 g/L agar) plates. *P. chrysosporium* was cultured in an immersed liquid culture system. The culture medium was prepared as described by Tien and Kirk [28] but with some modification. It contained (per liter) 10.0 g glucose, 0.2 g ammonium tartrate, 2.0 g KH_2PO_4, 0.175 g $MnSO_4{\cdot}2H_2O$, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.1 g $CaCl_2{\cdot}2H_2O$, 1.0 g Tween 80, 1.5 mL of veratryl alcohol (VA), 1 mg vitamin B1, 70 mL trace-element solution, 0.2 g yeast extract powder, 20 mM acetate buffer (pH 4.4). The trace-element solution contained (per liter) 1.5 g nitriloacetate, 3.0 g $MgSO_4{\cdot}7H_2O$, 1.0 g NaCl, 0.1 g $FeSO_4{\cdot}7H_2O$, 0.1 g $CoSO_4$, 0.1 g $ZnSO_4{\cdot}7H_2O$, 0.01 g $CuSO_4$, 0.01 g $AlK(SO_4)_2{\cdot}12H_2O$, 0.01 g H_2BO_3 and 0.01 g $Na_2MoO_4{\cdot}2H_2O$. The final spore concentration of 1×10^5 spores/mL was introduced into a 250 mL Erlenmeyer flask containing 100 mL medium. The flasks were then incubated at 37 °C in a rotary shaker with the agitation of 160 rpm/min at a 2.5-cm-diameter throw. The cultures were harvested when the maximum activity of MnP was detected, and centrifuged at 12,000 rpm/min for 30 min at 4 °C. Since the *P. chrysosporium* produces extracellular enzymes, the resultant supernate was stored in a refrigerator (0–4 °C) until it was used as crude ligninolytic enzymes in the following degradation experiments. Our former experiment had proved that the crude enzyme can keep its activity for at least 6 months under the storage condition described above.

2.3. Enzyme assay

The activity of MnP was measured according to the method described by Paszczynski et al. [29] using a Shimadzu UV-2401 spectrophotometer. The general procedure was as follow: at 25 °C, 0.4 mL tartaric buffer (1 M, pH 5.0) was first mixed in a cuvette with 0.4 mL 1 mM $MnSO_4{\cdot}2H_2O$ and 1 mL MnP solution. Then 0.4 mL 1 mM H_2O_2 was added to initiate the MnP catalyzed oxidation of Mn^{2+}. After a quick mixing, a plot of absorbance (A) at 238 nm versus the reaction time (t) was recorded against the corresponding reference (without MnP). The molar extinction coefficient of Mn(II) was 6500 M^{-1} cm^{-1}. From the slope of the linear portion of the A–t curve, the quantity of Mn(III) produced per minute was calculated, with 1 U defined as 1 μmol of Mn(II) oxidized to Mn(III) per minute.

2.4. Enzymatic degradation

If not specified, the enzymatic degradation reaction system consisted of the crude MnP (40 U/L), $MnSO_4{\cdot}2H_2O$ (1 mM), H_2O_2 (0.4 mM), and TC or OTC (50 mg/L) in 100 mM tartrate buffer. In the experiments testing the influence of pH, Mn^{2+} concentration, H_2O_2 concentration, enzyme-substrate ratio on the degradation rate, the tested parameter values were shown in the corresponding figures. The reaction was carried out in a total volume of 8 mL reaction system in a 20 mL tube at 37 °C and on a 120 rpm rotary shaker. The reaction was initiated by the addition of H_2O_2, and the final concentration of the antibiotics was measured 4 h after the commencement of the reaction, since the first experiment showed that the antibiotics concentration did not change after a 4 h reaction. 2 mL supernate from the degradation tube was taken and filtrated through a 0.45 μm membrane with a syringe filter. The filtrate was used for TC, OTC and their metabolites measurement. Control tests were conducted with the crude enzyme replaced by heat-inactivated crude enzyme with H_2O_2 addition. Control tests without adding H_2O_2 were also conducted. Experiments were performed in triplicate and the results are expressed as the mean values plus the standard errors.

2.5. Analyses of TC and OTC

TC and OTC and their metabolites were monitored with high performance liquid chromatography (HPLC). The characteristic peak areas of the TC and OTC HPLC prints remained the same when tested within the range of pH values indicated in the degradation experiments. The degradation of TC and OTC was expressed as a concentration percentage which was calculated based upon the peak area of known standards.

The HPLC procedure described in the China Codex (2005) was adopted. A Shimadzu HPLC system (Japan) consisting of a LC-10AT pump, a CTO-10A column oven, and a SPD-10A UV–vis detector, was used. The system was equipped with a HYPERSIL ODS C18 (5 μm, 4.6 × 250 mm, No. 1217290). The oven was set to 35 °C with the column oven. Separations were run using 68% (v/v) 0.1 M oxalic acid ammonium, 27% (v/v) N,N-dimethylformamide, 5% (v/v) 0.2 M diammonium phosphate in high purity water with a constant flow rate of 1.0 mL/min for TC, and 75% (v/v) 0.05 M oxalic acid ammonium, 20% (v/v) N,N-dimethylformamide, 5% (v/v) 0.2 M diammonium phosphate in high purity water with a constant flow rate of 0.8 mL/min for OTC. Eluted substances were both detected at 353 nm.

3. Results and discussion

3.1. Production of MnP

The production profiles of the ligninolytic enzymes in the medium during the immersed liquid culture of *P. chrysosporium* are depicted in Fig. 1. Activities of MnP first appeared on day 3, peaked on day 10, and then decreased gradually until the end of the culture. The peak activity of MnP was 588 U/L while the lignin peroxidase (LiP) activity, which might be produced under some other culture conditions, was not present. The product was used as the crude MnP in the following experiments.

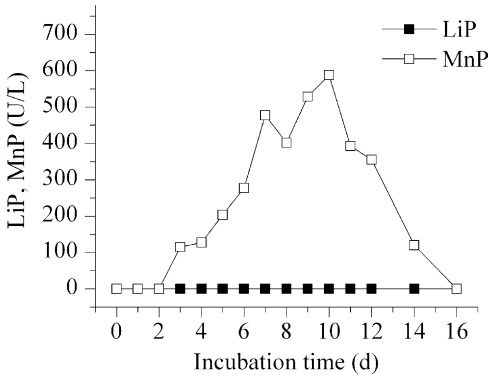

Fig. 1. Production profiles of the ligninolytic enzymes in liquid culture of *P. chrysosporium* in air at glucose/NH_4^+ mol ratios of 56/8.8.

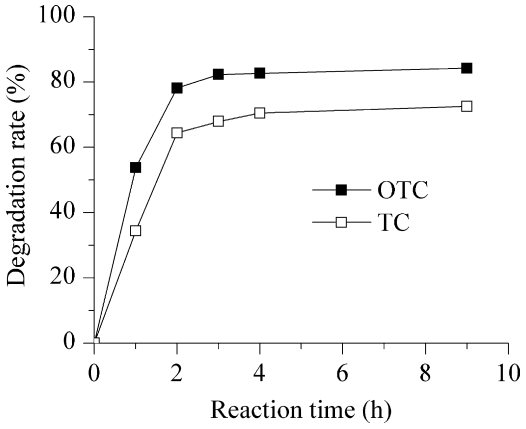

Fig. 2. Time course of the disappearance of TC or OTC in an enzymatic reaction mixture. Reaction mixture: pH 4.8, 0.1 mM Mn^{2+}, 0.1 mM H_2O_2, 50 mg/L TC or OTC, 40 U/L MnP. The reaction took place in a tube with a volume of 8 mL at 37 °C and 120 rpm.

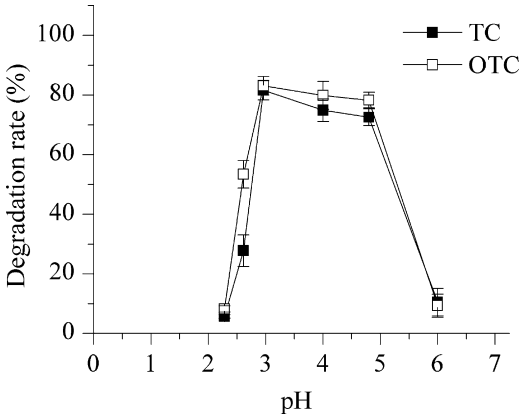

Fig. 4. Effect of pH on degradation of TC or OTC by crude MnP. Reaction mixture: 0.1 mM Mn^{2+}, 0.1 mM H_2O_2, 50 mg/L TC or OTC, 40 U/L MnP. The reaction was taken in a tube with a volume of 8 mL at 37 °C and 120 rpm.

3.2. The degradation of TC and OTC by MnP

About 72.5% and 84.3% of the initial TC and OTC, respectively, were removed during the course of the reaction (Fig. 2). In fact, the concentration of TC and OTC did not change much after the first 4 h of the incubation. Controls using heat-inactivated MnP and controls without H_2O_2 addition did not show any decrease in the TC and OTC concentration throughout the entire experiment, proving that the removal of TC and OTC was enzymatic. As shown in Fig. 3, no new peaks appeared in the HPLC graphs for samples from the TC degradation system, indicating that intermediate compounds might not be produced in the degradation process. However, the OTC degradation system has one conspicuous peak between 3 and 4 min elution time (Fig. 3), and this indicates that smaller molecular weight intermediate compounds were produced in the degradation process. It would be interesting to further illustrate the intermediate compounds.

3.3. Optimization of degradation system parameters

3.3.1. Effects of pH

Fig. 4 shows that the TC and OTC removal percentage reached a maximum when the pH was at about 2.96 (final degradation rate about 82%) and remained at a high and stable level when the pH was between 2.96 and 4.80. When the pH was set below 2.61, the percentage of degraded TC and OTC decreased rapidly to 28% and 53%. Since the enzymatic activity of MnP has its peak at pH 4.8 and can be higher at pH 3.5–5.0, the pH effect on the degradation can be explained by the pH dependence of the enzyme activity. The properties of TC and OTC may also have some effects on the result. There has been no research on in vitro degradation of TC and OTC by MnP. The results from the experiments conducted confirm that in vitro degradation of TC and OTC by crude MnP is highly dependent on the pH.

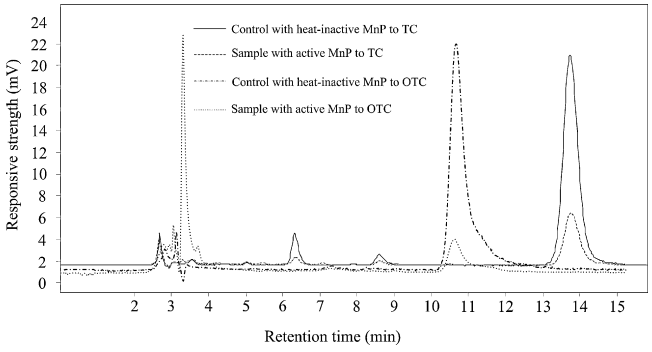

Fig. 3. HPLC elution profiles of the TC-containing or OTC-containing reaction solution after 2 h of incubation at 37 °C and 120 rpm.

3.3.2. Effects of temperature

At 20 °C, the degradation percentage of TC reached about 43% and the value of OTC was 57% (Fig. 5). Both percentages increased with the increase of the temperature and reached a maximum at 37–40 °C which is the temperature that the enzyme has its highest activity.

3.3.3. Effects of Mn^{2+} concentration

The addition of Mn^{2+} promoted the degradation of TC and OTC (Fig. 6). When the concentration of Mn^{2+} reached 0.1 mM, the degradation percentage became stable. The maximum degradation percentages were 78% for TC and 81% for OTC. Mn^{2+}, an ideal reducing substrate, served as a redox mediator in MnP catalysis [30]. The mediation of Mn^{2+} required the participation of some organic acid to form a stable Mn^{3+}–acid complex. High levels of degradation can only take place when the concentration of Mn^{2+} and the concentration of the organic acid satisfied a certain relationship [21]. It can be deduced that an Mn^{2+} concentration of 0.1 mM is the best for the degradation.

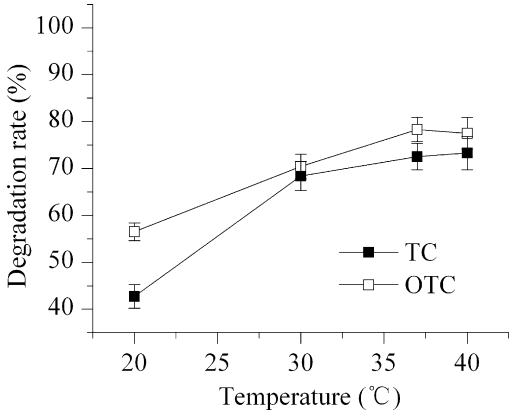

Fig. 5. Effect of temperature on the degradation of TC or OTC by crude MnP. Reaction mixture: pH 4.8, 0.1 mM Mn^{2+}, 0.1 mM H_2O_2, 50 mg/L TC or OTC, 40 U/L MnP. The reaction took place in a tube with a volume of 8 mL at 120 rpm.

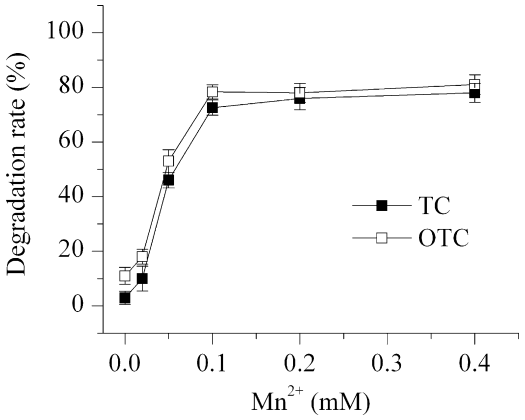

Fig. 6. Effect of Mn^{2+} on the degradation of TC or OTC by crude MnP. Reaction mixture: pH 4.8, 0.1 mM H_2O_2, 50 mg/L TC or OTC, 40 U/L MnP. The reaction took place in a tube with a volume of 8 mL at 37 °C and 120 rpm.

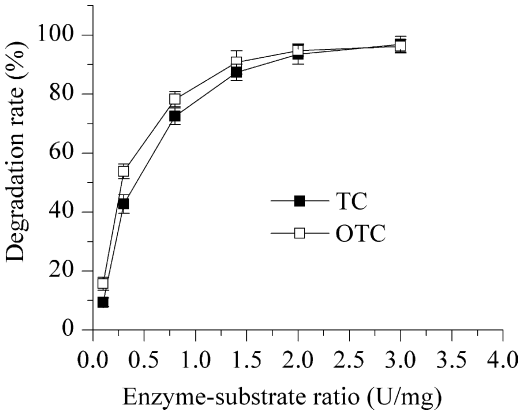

Fig. 8. Effect of enzyme-substrate ratio on the degradation of TC or OTC by crude MnP. Reaction mixture: pH 4.8, 0.1 mM Mn^{2+}, 0.1 mM H_2O_2, 50 mg/L TC or OTC. The reaction took place in a tube with a volume of 8 mL at 37 °C and 120 rpm.

3.3.4. Effects of H_2O_2 concentration

Fig. 7 shows that the degradation did not occur without the addition of H_2O_2, and was enhanced greatly with the addition of 0.1 mM H_2O_2. The degradation percentages of both TC and OTC reached the maximum (around 80%) with the H_2O_2 concentration at 0.2 mM.

3.3.5. Effects of the enzyme-substrate ratio

The TC and OTC degradation percentage by MnP quickly increased with the increase of the enzyme-substrate ratio, and remained at a maximum value after a threshold ratio was achieved (Fig. 8). This ratio was 2.0 U/mg for crude MnP used in this research. Higher enzyme-substrate ratios are beneficial for the degradation, but there is a threshold ratio beyond which the benefits decrease. When the enzyme-substrate ratio passed the threshold, the degradation rate could not be elevated further. It is thus recommended that the enzyme-substrate ratio of about the threshold value be applied to obtain efficient and cost effective degradation.

3.3.6. Effects of the H_2O_2 compensation

Considering that the H_2O_2 in the system was consumed through the degradation process and would be used up at the time of the first sampling (1 h after reaction), the same concentration of H_2O_2 as applied in the beginning of the reaction (0.1 mM) was added into the TC degradation system as compensation at the time. Due to the compensation of H_2O_2, the degradation percentage of TC at the second sampling was enhanced from 72% to 87% (Fig. 9).

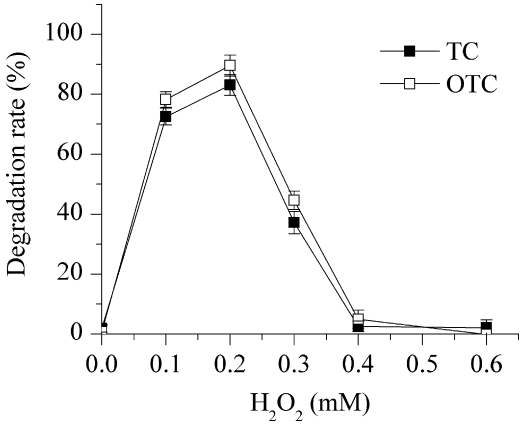

Fig. 7. Effect of H_2O_2 on the degradation of TC or OTC by crude MnP. Reaction mixture: pH 4.8, 0.1 mM Mn^{2+}, 50 mg/L TC or OTC, 40 U/L MnP. The reaction took place in a tube with a volume of 8 mL at 37 °C and 120 rpm.

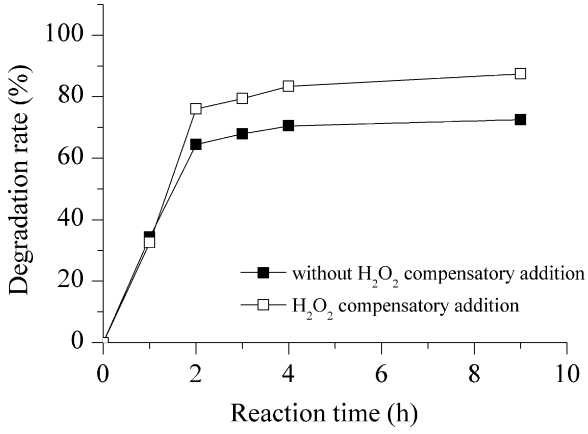

Fig. 9. Effect of H_2O_2 compensatory addition on the degradation of TC by crude MnP. Reaction mixture: pH 4.8, 0.1 mM Mn^{2+}, 0.1 mM H_2O_2, 50 mg/L TC, 40 U/L MnP. The reaction took place in a tube with a volume of 8 mL at 37 °C and 120 rpm.

The catalytic cycles of the ligninolytic peroxidases consist of a two-electron oxidation of the native ferric enzyme to compound I by hydrogen peroxide and two single-electron reductions via an intermediate compound II to its resting state by appropriate reducing substrates (redox mediators or target compounds) [30,31]. Compound II may be further oxidized to an inactivated form, compound III, in the presence of excess hydrogen peroxide [32]. Therefore, H_2O_2 plays a pivotal role in the catalytic cycle of MnP, so the concentration and addition method of H_2O_2 is very important when MnP is used in the degradation directly. Data collected from this study indicate that low H_2O_2 levels led to low degradation rates. When more H_2O_2 was added in one batch, a rapid, but limited, degradation was observed. However, if too much H_2O_2 was added at once, the structure of MnP could be disrupted. Therefore, it is crucial to control the level of H_2O_2 in the MnP catalyzed oxidative system. This study confirmed that the subsequent addition of H_2O_2 is one possible way to improve the effectiveness of the degradation by MnP. Furthermore, measures that can generate and control the level of H_2O_2 should be tested in the future.

4. Conclusions

This study tested the feasibility of MnP to degrade TC and OTC in vitro and optimized the degradation system parameters. The following conclusions can be drawn from the experiments.

1. MnP can effectively degrade TC and OTC. 72.5% of 50 mg/L of TC was degraded when added 40 U/L of MnP, while 84.3% of 50 mg/L of OTC was degraded with the same amount of the catalyst added, both within 4 h.
2. The degradation rate was dependant on the pH and the temperature of the reaction system, and was likely sensitive to the concentration of H_2O_2. With the pH at 2.96–4.80, the temperature at 37–40 °C, the Mn^{2+} concentration higher than 0.1 mM and up to 0.4 mM), the H_2O_2 concentration of 0.2 mM, and the enzyme-substrate ratio above 2.0 U/mg, the degradation rate reached the highest.
3. The compensation of H_2O_2 during the reaction process could improve the degradation of TC by MnP.

Acknowledgements

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