Nanoparticles Enhanced Ligninolytic Enzymes Activity of Rotten Wood Fungus *Phanerochaete chrysosporium*

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White-rot fungi are vital microorganisms that can decay lignocellulosic biomass. This study investigated the ability of white-rot fungus found on rotten wood to produce ligninolytic enzymes and their enhancement using nanoparticles (NPs). *Phanerochaete chrysosporium* was isolated from decayed wood. Its ability to produce ligninolytic enzymes, namely CMCase, FPase, Laccase, and MnPase, was recorded. The enzymes' productivity was enhanced *via* utilization of the surfactant polysorbate 80 with optimum concentration 0.20 mL/L for maximum CMCase (201.33 ± 2.31 U/mL) and FPase (66.33 ± 0.58 U/mL), whereas the corresponding value was 0.15 mL/L for laccase and MnPase production. Mn_2O_3NPs and CuONPs enhanced the enzymes' productivity but with different levels according to their concentrations, where the maximum productivity of CMCase (220.67 ± 2.31 U/mL) and (FPase 74.63 ± 3.51 U/mL) were at 50 ppm, but MnPase (0.52 ± 0.01 U/mL) and laccase (2.88 ± 0.01U/mL) were at 25 ppm and 75 ppm of CuONPs, respectively. Mn_2O_3NPs at 75 ppm enhanced the productivity of CMCase (219.33 ± 1.15 U/mL) and FPase (74.63 ± 3.51U/mL); but at 25 ppm and 75 ppm enhanced the productivity of laccase (2.40 ± 0.04U/mL) and MnPase (0.73 ± 0.03 U/mL), respectively. Thus, NPs were found to play a vital role in improving the productivity of ligninolytic enzymes.

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INTRODUCTION

The causative agent of white wood rot, namely fungi, is distinguished by having an enzymes class called ligninases, also referred to as ligninolytic enzymes, which are able to break down wood lignin. The distinctive brown color of wood is caused by lignin, which is preferentially broken down by these fungi, leaving behind the white cellulose, which explains how this type of rot received its name. These fungi utilize lignocellulosic material as an energy and carbon source. The three principal constituents of lignocellulosic materials are lignin, cellulose, and hemicellulose (Iram *et al*. 2021). Among these, lignin is of particular interest because of its complex structure, consisting of multiple aromatic rings. To break down lignin, fungi have produced a variety of extracellular oxidative enzymes. Because of the creation of extracellular oxidase and hydrolase enzymes (such as manganese peroxidase, cellulase, laccase, xylanase, and lignin peroxidase), lignocellulosic

residues, lignin, cellulose, as well as hemicellulose (the principal wood constituents) are broken down by lignocellulolytic fungi (Dao *et al.* 2021). Enzymes involved in reactions of non-specific oxidation and hydrolysis, known as ligninolytic and hydrolytic enzymes, are the hallmarks of wood-decay fungi.

Major components of commercial enzymatic cocktails used to convert pretreated lignocellulosic materials into plant biomass include hydrolytic enzymes (Adsul *et al.* 2020; Al-Rajhi *et al.* 2023). Modern biorefineries are built on the hydrolysis of cellulose as well as hemicellulose by means of optimized mixtures, *i.e.* "cocktails," of these enzymes. According to Ellila *et al*. (2017) and Adsul *et al*. (2020), to advance the economical production of plant biomass derivatives, however, these enzymatic cocktails still need to perform better. Endoglucanases, exoglucanases, and β-glucosidases are examples of enzymes associated with cellulolytic reactions, whereas lignin-degrading enzymes comprise manganese peroxidases (MnPs), phenol oxidases (laccases), lignin peroxidases (LiPs), besides versatile peroxidases (VPs), as reported in study of Andlar *et al*. (2018). Saldarriaga-Hernández *et al.* (2020) reported that these fungal enzymes have the potential to be used in a number of biofuel production processes, including the lignocellulosic biomass pretreatment, cellulose saccharification, and fermentation inhibitors removal.

Numerous white-rot fungi, *e.g.*, *Dichomitus* sp., *Ganoderma* sp., *Pycnoporus sanguineus*, *Trametes* sp., *T. villosa*, *T. versicolor*, *Lentinula boryana*, *Bjerkandera adjusta*, *Panus conchatus*, *Brunneoporus malicola*, *Antrodia neotropica*, *Stereum* sp., and *Laetiporus gilbertsonii*, perform an important function in biotechnological employments (Sánchez-Quitian *et al*. 2022). Sánchez-Corzo *et al*. (2021) studied the activities of lignin peroxidase (LiP), xylanase cellulase, and manganese peroxidase (MnP), from *Phlebiopsis flavidoalba*, *Trametes sanguinea*, *Phanerochaete sordida*, demonstrating their capability to oxidize lignin. In studies performed with white-rot fungus, such as *Lentinus sajor-caju*, little toxicity has also been observed in the Congo red degradation using manganese-dependent peroxidase (Yehia and Rodriguez-Couto 2017). As mentioned in numerous scientific papers, surfactants play an important role in an increasing the enzymatic activities of conversion of complex materials to simple molecules; furthermore, surfactants can possess a promising effect on the hydrolytic enzymes stability (Kamsani *et al.* 2018; Huang *et al.* 2022).

Many industries, including the chemical, medical, automotive, healthcare, cosmetics, and energy sectors, use and benefit from the application of nanoparticles (NPs) (Abdelghany *et al*. 2018a,b, Abdelghany *et al*. 2020; Al-Rajhi *et al.* 2022a,b, Alsalamah *et al*. 2023). The NPs generally have a high surface area-to-volume ratio and can find potential uses in several fields. Numerous investigations have documented the induction role of NPs for enhancing the cellulase activity, production, thermal stability, and pH of several enzymes (Srivastava *et al*. 2016; Sun *et al*. 2016; Nazir *et al*. 2021). Manganese dioxide (MnO_2) NPs are greatly attractive for biological applications due to their biocompatibility. CuO NPs and Mn_2O_3NPs have developed as a vital group of NPs for several applications – such as environmental, medicinal, and industrial uses – that have potential hazards to environment and organisms. However toxicity of CuO NPs was found to be dependent on several factors such as shape, surface modification, size, and applied dose (Naz *et al.* 2020). Aspects of toxicity of CuO NPs have been reported, such as immunotoxicity, oxidative stress, genotoxicity, cytotoxicity, inflammation and neuro-toxicity (Sajjad *et al.* 2023), so it is important to address their potential toxic effects. On the other hand, the synthesis and the utilize of Mn_2O_3NPs has developed in the last years, leading to a major hazard for environment and living organisms (Fernández-Pampín *et al.*

2022). According to scientific papers, to confirm that the CuO NPs and Mn_2O_3NPs are nontoxic, the poisonousness of these NPs must be decreased to the non-significant concentration. Criteria for sustainable applications of CuO NPs and Mn_2O_3NPs in different fields must be detected carefully. The purpose of this paper was to identify lignocellulolytic fungus isolated from decaying wood and investigate their prospective to produce lignocellulolytic enzymes, with induction of these enzymes activity using manganese (III) oxide NPs (Mn_2O_3NPs) and copper (II) oxide NPs (CuONPs).

EXPERIMENTAL

Wood Sample Collection and Isolation of Wood Decaying Fungus

A rotten wood specimen was collected from Egypt at a site rich in *Casuarina cunninghamiana* woods. A total of 3 g of wood was cleaned using 70% alcohol, and then crushed in a mortar. Then, 1 g of the powdered rotten wood sample was mixed with 2% of cellulose powder in sterile water to make suspension. Two mL of serial diluted suspension (10^{-1} to 10^{-5}) were sprinkled on the surface of plates containing medium of Potato Dextrose Agar (PDA), then incubated at for 6 days at 30 °C. The developed colony from the most diluted suspension was purified and identified.

Nanoparticle Source and Characterization by Transmission Electron Microscope

The used NPs in the current study, namely manganese(III) oxide (Mn_2O_3 NPs) and Copper(II) oxide (CuONPs) with particle size less than 100 nm and a purity level of over 99%, were obtained from Sigma-Aldrich Pty Ltd., Darmstadt, Germany. A transmission electron microscope (TEM) (JOEL, Tokyo, Japan) was employed to confirm the particle size of the obtained NPs.

Enzymes Preparation

Five grams of 3 mm-milled wood of health *Casuarina* sp. wood were put in 250-mL flasks. After addition of 20 mL of moisture agent to the substrate, it was autoclaved for 30 min at 121 °C. For each flask, three 9-mm fungus discs were used for inoculation. For 15 days, they were incubated at 30 °C. After the incubation time, each flask was fortified with distilled water (50 mL) and shaken (200 rpm) for 1 h on a shaker. Using muslin cloth on a glass funnel, the entire contents of the flask were filtered into a dry, clean flask. The filtrates were subjected to a 10-min cooling centrifugation at 80,000 rpm using a Sigma model 3 K30 centrifuge (Germany). Crude enzymes were made from the supernatants.

Protein and Reducing Sugar Assessment

The reagent of 3,5-dinitrosalicylic acid (DNS) (1 mL) was introduced into each of the tubes holding 1 mL of sugar solution and 1 mL of water serving as a reference. The tube containing the reaction mixture was kept in a boiling water bath for 10 min. Subsequently, the tubes were cooled at 25 °C. Each tube was completed with 5 mL of distilled water. At 540 nm, the color shifted from yellow to orange and was recorded. Glucose was employed as standard (Miller 1959). As per Lowry *et al*. (1951), the amount of soluble proteins in the fungal filtrate (supernatant) was measured.

Carboxymethyl Cellulase (CMCase) Assessment

Carboxymethyl cellulose (CMC) (1 g) was mixed with 100 mL of sodium acetate buffer (pH 5.0). One mL of the enzyme-containing supernatant and 1 mL of 1% CMC were combined in a tube. After 30 min of incubation at 63 °C, the tube's liberated reducing sugar was measured employing Miller's (1959) DNS approach, which gave an absorbance reading of 540 nm. Rather than employing 1 mL of enzyme (supernatant), the blank contained one mL of distilled water. Using the standard curve of glucose, the quantity of the resulting sugar was ascertained. The micromole of sugar liberated per mL of fungus medium filtrate / minute is one unit of enzyme (CMCase) (Wang *et al*. 2008).

Laccase Assessment

In a 1-cm quartz cuvette, laccase activity was measured in accordance with Garzillo *et al*. (2001). Two mg of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) in a buffer of Mcllvaine (pH 5.0) were present in a mL reaction mixture. A total of 100 µL of centrifuged extracellular supernatants were added to the assay mixture. Through measuring the shift in absorbance (420 nm) (ABTS), with α = 36 $mM^{-1}cm^{-1}$, employing a spectrophotometer (JENWAY) Model 6300, EU at 30 °C, the enzymatic activity was calculated in units of identification (IU) (Garzillo *et al*. 2001).

Manganase Peroxidase (MnPase) Assessment

A 1 cm quartz cuvette was used to test MnPase activity. Buffer of Mcllvaine (pH 5.0) comprising 1 mM Mn^{2+} besides 2 mM ABTS made up the 1 mL reaction mixture. Then, 100 µL of centrifuged extracellular fluid (supernatants) were added to the assay mixture. Afterwards, 0.4 mM H_2O_2 was added to start the peroxidase activity (Garzillo *et al*. 2001). Through measuring the change in absorbance at 420 nm (ABTS), with α = 36 mM^{-1cm-1}, using a spectrophotometer (JENWAY, Model 6300, Jasco, Japan) at 30 °C, the enzymatic activity was calculated in units of identification (IU) (Garzillo *et al*. 2001).

Filter-paperase (FPase) Assessment

One mL of the enzyme-containing supernatant (pH 4.8), 2 mL of 0.1 M buffer with pH 4.8, and 0.05 g of ground Whatman filter paper No. 1 were mixed in a tube (Gadgil *et al*. 1995). For 1 h, mixture was held at 50 °C. Next, Miller's (1959) DNS approach was employed to measure the released sugar. At 540 nm, the absorbance was calculated. The substitute for the enzyme was 1 mL of distilled water. The micromole of sugar liberated per mL of fungus medium filtrate/minute is one unit of enzyme (FPase).

Ligninolytic Enzymes Production at Different Concentrations of Surfactant agent and Nanoparticles

Polysorbate 80 (Tween 80) as surfactant was added at different concentrations ranging from 0.05 up to 0.25 µL. The productivity of ligninolytic enzymes were detected as mentioned previously. At optimum concentration of polysorbate 80, different concentrations of Mn_2O_3NPs and CuONPs up to 100 ppm were added to substrate to study their inducer role for enzymes production.

Statistical Study

Standard deviation was computed *via* SPSS version 25 (IBM Corp., Armonk, NY, USA) and Minitab version 19 (Minitab, PA, USA).

RESULTS AND DISCUSSION

Rotten wood provides an abundant environment for fungi that can produce lignocellulolytic enzymes, which aid in the breakdown of wood. In the present work, the decayed woods appeared with deeper shades of brown to yellow, in agreement with Sablík *et al.* (2016), who reported that the color changes occurred due to rot fungi and depends on the kind of wood. In the current study, ligninolytic fungus was isolated from rotten wood (Fig. 1 A and B) because this is a valuable niche for fungal biodegradation. From the observation of fungus growth, it was found that the color of its colony was white, resembling cotton (Fig. 1C). The isolated fungus was identified as *Phanerochaete chrysosporium.* According to other studies, *P. chrysosporium* has been isolated from decayed woods (Hervé *et al*. 2016; Schmerling *et al*. 2022).

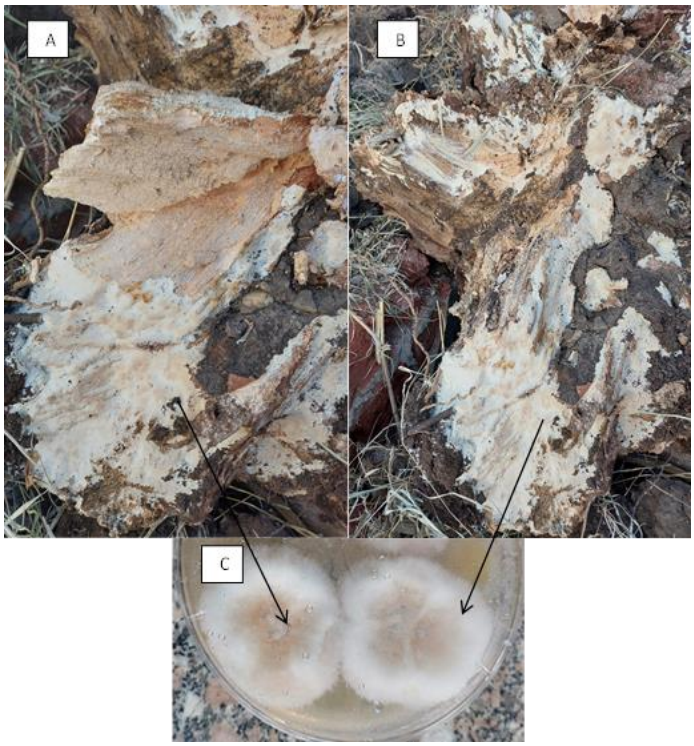

Fig. 1. Decayed woods samples (A and B) and the isolated fungus colonies (C)

Based on previous investigations, the potential of white-rot fungus *P. chrysosporium* to create ligninolytic enzymes such as manganese peroxidases (MnPs) and lignin peroxidases (LiPs) was documented (Singh and Chen 2008; van der Made *et al*. 2023). Therefore, *P. chrysosporium* was then evaluated for its lignolytic capability to degrade powder of undecayed wood. Different ligninolytic enzymes, namely CMCase, FPase, laccase, and MnPase were produced by *P. chrysosporium* as shown in Table 1.

Table 1. Enzymes Production by *P. chrysosporium* using Health Wood as Substrate

Enzymes Productivity (U/mL)			
CMCase	FPase	Laccase	MnPase
113.33 ± 2.89	26.0 ± 1.73	2.57 ± 0.73	0.25 ± 0.02

There are some scientific reports stating that ligninolytic enzymes creation depends strongly on the substrate composition, fungal strain, and cultivation condition (Dogaris *et al*. 2009; Adsul *et al*. 2020). In the present experiment, the additive of polysorbate 80 was studied for enhancing the creation of CMCase, laccase, MnPase, and FPase. The highest CMCase (201.33 ± 2.31 U/mL) and FPase (66.33 ± 0.58 U/mL) were recorded at 0.20 mL/L of polysorbate 80, while laccase (2.22 ± 0.17 U/mL) and MnPase (0.48 ± 0.02 U/mL) activities were recorded at 0.15 mL/L of polysorbate 80. Maximum protein productivity was at 0.2 mL/L of the polysorbate 80 by *P. chrysosporium*. Based on literature of Wang *et al*. (2008), the definite mechanism by which the surfactants improve the production of extracellular enzymes in multicellular fungi has not been explained. Zheng and Obbard (2001) noted that surfactants, particularly polysorbate 80, have the ability to increase the fungal spores' growth and bioavailability of less soluble substrates. Furthermore, Rodrigues *et al.* (2013) reported that polysorbate 80 can influence the structure of the cell membrane and facilitate the excretion of ligninolytic enzymes from fungal cells into the medium. Several reasons were reported in other studies which explained the increasing of enzymes production by surfactants. For instance, the nature of the substrate may change by the surfactant *via* increment of the available surface of cellulose or *via* eliminating the inhibitory lignin. Moreover, the interaction between the enzyme and substrate may be affected by the surfactant which accelerates the separation of enzymes from its substrate (Huang et al. 2022). Surfactants, such as Tween 20, Triton X 100, and Polysorbate 80, were tested to improve productivity of ligninolytic enzymes; polysorbate 80 was the highest stimulator of enzyme creation from white-rot fungus *Stereum ostrea* (Usha *et al*. 2014).

Table 2. Production of Ligninolytic Enzymes on Wood Inoculated with *P. chrysosporium* and Incubated at 30 °C

Polysorbate 80 (mL/g)	Ligninolytic Enzymes (U/mL)				Protein (µg/mL)
	CMCase	FPase	Laccase	MnPase	
0.05	119.67 ± 4.04	42.67 ± 1.53	2.03 ± 0.01	0.23 ± 0.01	281.33 ± 1.15
0.10	160.67 ± 1.15	45.33 ± 1.15	2.04 ± 0.02	0.27 ± 0.02	285.33 ± 3.06
0.15	176.33 ± 3.79	50.33 ± 1.53	2.22 ± 0.17	0.48 ± 0.02	301.67 ± 1.53
0.20	201.33 ± 2.31	66.33 ± 0.58	2.07 ± 0.06	0.44 ± 0.03	298.67 ± 2.31
0.25	166.67 ± 2.89	58.67 ± 1.15	1.53 ± 0.06	0.39 ± 0.03	232.33 ± 1.15

The results in Table 3 reflected the inductive role of CuONPs (Fig. 2A) or enhancement in the productivity of ligninolytic enzymes. It was found that 50 ppm was the optimum concentration for CMCase (220.67 ± 2.31 U/mL) and FPase (74.63 ± 3.51 U/mL), while 25 ppm and 75 ppm was the optimum concentration for MnPase (0.52 ± 0.01 U/mL) and laccase (2.88 ± 0.01U/mL), respectively. The increased productivity of enzymes at optimum concentrations of CuONPs was 8.33, 9.45, 12.51, and 29.73% for MnPase, CMCase, FPase, and laccase, respectively, indicating the vital role of CuONPs in laccase activity. According to Usha *et al*. (2014), 300 µM of copper sulphate has a positive impact on the production of laccase, increasing 2-fold compared to the control. In contrast, it is clear that 75 ppm of Mn_2O_3NPs (Fig. 2B) was the optimum concentration for CMCase (219.33 ± 1.15 U/mL) and FPase (68.33 ± 2.31U/mL) according to data in Table 4 at 75 ppm (Table 4). The increased productivity of enzymes at optimum concentrations of CuONPs was 8.11, 8.95, 9.56, and 29.73% for laccase, CMCase, FPase, and MnPase, respectively, indicating the vital role of Mn_2O_3NPs in MnPase activity. The results matched other investigations and reflected the effect of NPs on enzymes activity. For example,

Salunke *et al*. (2015) registered the inductive role of silver NPs to degrade the cellulose by cellulase. Srivastava *et al*. (2016) found enhancement of the pH, activity, production as well as thermal stability of cellulase in the existence of zinc oxide NPs. Zyrina *et al*. (2017) reported the cofactor role of copper for superoxide dismutase activation. Moreover, the activity of CMCase was improved using $CaCl_2$ NPs (Yousef *et al*. 2019). In a recent investigation, silver NPs (50 ppm) exerted an improvement in the activity of cellulose (two-fold compared to the activity by untreated culture) by *Bacillus subtilis*, while FeONPs showed a suppressive effect on cellulase activity at all concentrations (Hussain *et al*. 2023). In the present work, high concentration of applied NPs (100 ppm) reduced the enzymes productivity; it may be affecting the fungus growth.

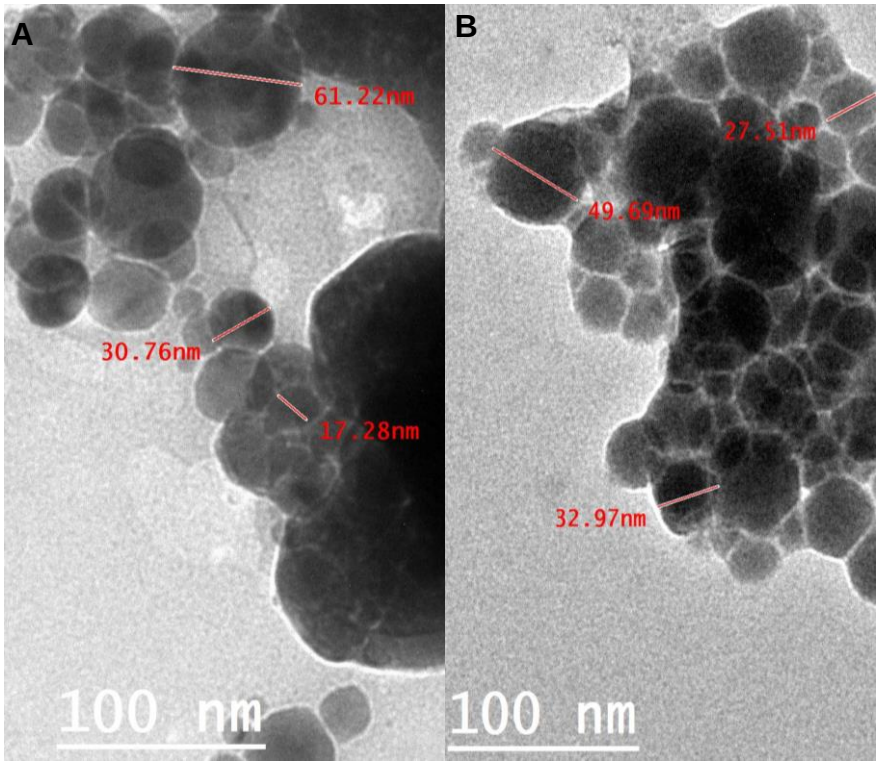

Fig. 2. TEM image of used Mn_2O_3NPs (A) and CuONPs (B)

Table 3. Effect of CuONPs on the Production of Ligninolytic Enzymes on Wood Inoculated by *P. chrysosporium* with 0.20 mL/g of Polysorbate 80 and Incubated at 30 °C

CuONPs (ppm)	Ligninolytic Enzymes (U/mL)				Protein (µg/mL)
	CMCase	FPase	Laccase	MnPase	
0.0	201.33 ± 2.31	66.33 ± 0.58	2.22 ± 0.17	0.48 ± 0.02	278
25	217.67 ± 2.31	71.33 ± 0.58	2.57 ± 0.02	0.52 ± 0.01	282
50	220.67 ± 2.31	74.63 ± 3.51	2.84 ± 0.03	0.47 ± 0.47	280
75	212.33 ± 2.08	67.33 ± 1.15	2.88 ± 0.01	0.47 ± 0.01	277
100	211.33 ± 1.53	63.67 ± 1.53	2.41 ± 0.06	0.45 ± 0.01	275

Table 4. Effect of Mn_2O_3NPs on the Production of Ligninolytic Enzymes on Wood Inoculated by *P. chrysosporium* with 0.20 mL/g of Polysorbate 80 and Incubated at 30 °C

Mn_2O_3NPs (ppm)	Ligninolytic Enzymes (U/mL)				Protein (µg/mL)
	CMCase	FPase	Laccase	MnPase	
0.0	201.33 ± 2.31	66.33 ± 0.58	2.22 ± 0.17	0.48 ± 0.02	278
25	202.67 ± 2.89	71.33 ± 1.53	2.40 ± 0.04	0.56 ± 0.02	281
50	215.67 ± 2.31	72.67 ± 2.31	2.39 ± 0.01	0.64 ± 0.03	285
75	219.33 ± 1.15	68.33 ± 2.31	2.36 ± 0.02	0.73 ± 0.03	282
100	213.33 ± 2.31	65.67 ± 2.52	2.34 ± 0.01	0.67 ± 0.02	276

CONCLUSIONS

1. Wood-decaying *P. chrysosporium* was able to produce various ligninolytic enzymes including CMCase, FPase, laccase, and MnPase.
2. Polysorbate 80, Mn_2O_3NPs, and CuONPs each played a vital role in enhancement of the productivity of ligninolytic enzymes but at specific concentrations (0.15 mL/g polysorbate 80 for laccase and MnPase; 0.20 mL/g of polysorbate 80 for CMCase and FPase; 25 ppm of CuONPs for MnPase; 50 ppm of CuONPs CMCase and FPase; 75 ppm of CuONPs for laccase; 25 ppm of Mn_2O_3NPs for laccase; 50 ppm of Mn_2O_3NPs for FPase; and 75 ppm of Mn_2O_3NPs for CMCase and MnPase.
3. High concentrations of Mn_2O_3NPs and CuONPs reduced the productivity of enzymes and therefore may be used as a controlling agent for growth of fungi wood or wood products.

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