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Regular Article

Inherent antioxidant activity and high yield production of antioxidants in *Phanerochaete chrysosporium*

Liang Liu[a,b], Piao Xu[a,b], Guangming Zeng[a,b,*], Danlian Huang[a,b,*], Meihua Zhao[a,b], Cui Lai[a,b], Ming Chen[a,b], Ningjie Li[a,b], Chao Huang[a,b], Cong Wang[a,b], Min Cheng[a,b], Xiaoxiao He[a,b], Mingyong Lai[a,b], Yibin He[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

Incremental reactive oxygen species (ROS) under adversity environment could cause oxidative damages to microorganisms during environmental applications. In this study, we highlight a novel role of *P. chrysosporium* as antioxidants. The *P. chrysosporium* extracts possessed remarkable antioxidant activity, expressing a dose-dependent total antioxidant activity and accompanied with high reactive oxygen radical ($O_2^{\bullet-}$, $^{\bullet}OH$ and H_2O_2) scavenging capacity. In addition, it was established that, various compounds, such as antioxidant enzymes (catalase, superoxide dismutase and glutathione peroxidase) and low-molecular-weight antioxidant components (glutathione, phenolics and flavonoids) existed steadily in *P. chrysosporium*. Linear regression analysis and Pearson correlation coefficient analysis demonstrated that enzymatic and non-enzymatic antioxidants took part in processes and acted as "antioxidant network" in *P. chrysosporium* by confirming with the significant correlation coefficients among the tested antioxidants. Furthermore, those tested antioxidants were found sensitive response to Cd exposure, a 2.78-fold and 2.35-fold of stimulation has been found in SOD and phenolics, respectively, which provide a novel dimension to the involvement of antioxidants in the antioxidant defense system of *P. chrysosporium*. The paper proposes a new validation procedure to specifically validate the admirable tolerance and high efficiency of *P. chrysosporium* in environmental treatment application, taking advantage of the remarkable antioxidant activity.

1. Introduction

Reactive oxygen species (ROS) are common outcome of normal aerobic cellular metabolism implicated in all aerobic cells associated with oxidative processes. Reactive oxygen species are highly reactive molecules or molecular fragments as a consequence of aerobic respiration, which contain free radicals (such as superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$)) and some non-radicals (such as hydrogen peroxide (H_2O_2)) [1,2]. Increasing evidences suggest that contaminants exposure, such as heavy metal and xenobiotics, is considered as a key factor in intracellular ROS overproduction [3,4]. Excessive ROS lead to cellular aging, mutagenesis, DNA damage, oxidative damage, possibly through destabilization of membranes and oxidation of low-density lipoprotein, proteins, nucleic acids and lipids, due to the high reactivity of radicals with a wide range of molecules [5].

Since the spontaneous and/or induced production of ROS, antioxidants are critical to the control of ROS accumulation and thus the perpetuation or attenuation of ROS signals. Antioxidants are classified as exogenous (natural or synthetic) or endogenous compounds, in terms of the inhibition role in initiating or propagating of oxidizing chain reactions, scavenging ROS or their precursors, inhibiting formation of ROS, acting as the first-line defense against ROS intracellularly and also extracellularly [6,7]. Natural antioxidant system is classified into two major groups, enzymatic and non-enzymatic antioxidants.

Recent reports have described antioxidants and compounds with radical scavenging activity present in plant, vegetables and microorganisms [8–11]. The solvent extraction using different

* Corresponding authors at: College of Environmental Science and Engineering, Hunan University, Changsha 410082, PR China. Tel.: +86 731 88822754; fax: +86 731 88823701.
E-mail addresses: zgming@hnu.edu.cn (G. Zeng), huangdanlian@hnu.edu.cn (D. Huang).

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solvents has been the major method used to extract antioxidants or to obtain antioxidant extracts rich in antioxidants. For example, in the previous study conducted by Šeatović et al. [12], superoxide dismutases (SODs) were purified and partial characterized from the thermophilic bacteria *Thermothrix* sp., and the specific activity of the purified enzyme was 9191 U/mg. In addition, Xu and Zhu [13] investigated the dynamic extracellular and intracellular phenolic production and antioxidant activity in *Inonotus obliquus* extracts, and a dose-dependence of antioxidant activity of extracellular and intracellular phenolic compounds has been found. It was obviously that the natural existence these extracts were widely studied, however, the interactional biological systems of those antioxidants as an antioxidant network in the extracts was frequently neglected.

In recent years, it is interesting to note the development of programs using microorganisms in wastewater treatment. Among these, *Phanerochaete chrysosporium* (*P. chrysosporium*) possesses the excellent biodegradation and biosorption ability which enable them to degrade a wide range of xenobiotics and passivate heavy metals [14–17]. Hence, besides its environmental significance, among the special problems incurred by *P. chrysosporium* is the need to effectively eliminate the excessive intermediate ROS generated during normal metabolic activity or as a consequence of various exogenous environmental insults. However, according to our knowledge, no previous study focused on the natural existence of antioxidants and inherent antioxidant activity of *P. chrysosporium*.

What is needed, therefore, is a thorough understanding of the antioxidant systems and underlying mechanisms in *P. chrysosporium*. It is self-evident that knowledge is required both on the inherent activity antioxidant of *P. chrysosporium* intracellularly or extracellularly setting and the identification of antioxidant components for antioxidant system. Meanwhile, the information on the relationship between antioxidant activity and antioxidant composition of *P. chrysosporium* is essential. Such information is interesting in and of itself, it is also essential in any future attempts to raise tolerance to environmental oxidative stress in organisms and to reduce cellular damage by active O_2 or extraneous oxidative stress.

To understand these mechanisms, it is essential to identify the antioxidant activity and to understand their biosynthesis, metabolism, and regulation. Herein, in order to develop dietary supplements and preventative treatments for offsetting the adverse biological effects of ROS, the present study was conducted with the aim of investigating the inherent antioxidant activity and the composition of antioxidants in *P. chrysosporium* and their roles in providing antioxidant defenses. Total antioxidant activity (TAA) and reactive oxygen radical scavenging activity (RSA) ($O_2^{\bullet-}$, $^{\bullet}OH$ and H_2O_2 scavenging activity) were studied to determine the inherent antioxidant activity. In addition, various antioxidants, involving antioxidant enzymes and low-molecular-weight antioxidant components, were quantified. Furthermore, the relationships between antioxidant activity and various antioxidants were evaluated to examine potential antioxidant mechanisms involving in *P. chrysosporium*.

2. Materials and methods

2.1. Strains

The white-rot basidiomycete, *P. chrysosporium* BKMF-1767 was purchased from the China Center for Type Culture Collection (Wuhan) and maintained by subculturing on potato dextrose agar (PDA) slants at 4 °C. All the chemicals and reagents were of analytical grade and used without further purification. Distilled water was used for the preparation of all the solutions throughout this study.

2.2. P. chrysosporium extract preparation

The *P. chrysosporium* biomass was collected after 5 days incubation at 30 °C, and then filtered and centrifuged at 12,000 rpm for 10 min, then collected and rinsed with sterile ultrapure water. A series mass of *P. chrysosporium* biomass (0–0.5 g wet biomass) was weighed, and homogenated in glass homogenizer after addition of 5 ml phosphate buffer solution (PB solution, 50 mM, pH 7.0) or ultrapure water. The soluble fraction was centrifuged at 12,000 rpm, 4 °C, for 10 min, and then filtered for the preparation of *P. chrysosporium* PB solution extract (PPE) and *P. chrysosporium* water extract (PWE).

2.3. Total antioxidant activity assay

To measure the total antioxidant activity (TAA) of *P. chrysosporium* extracts, scavenging of $ABTS^{\bullet+}$ was determined as previously described [18]. 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was dissolved in H_2O_2 solution and incubated for 1 h in dark at room temperature, and then diluted with acetate buffer to the absorbance of 0.70 (±0.02) at 734 nm for the preparation of $ABTS^{\bullet+}$ radicals. The total antioxidant activity (ΔA/min) was assessed by plotting the decrease in absorbance of $ABTS^{\bullet+}$ at 734 nm vs time for 3 min, after adding 100 μl *P. chrysosporium* extracts to diluted $ABTS^{\bullet+}$ solution.

2.4. Reactive oxygen radical scavenging activity assay

$^{\bullet}OH$ scavenger activity was measured according to Jeong et al. [19], with minor modification. The assay was performed by orderly mixing 1.5 mM $FeSO_4$, 6 mM H_2O_2, 20 mM sodium salicylate and *P. chrysosporium* extracts. The mixture was incubated for 30 min in dark, and then the absorbance of the mixture was measured at 562 nm. $O_2^{\bullet-}$-scavenger ability was conducted by comparing the percentage of oxidation inhibition of pyrogallic acid at the presence of *P. chrysosporium* extracts. The inhibition ratio between autoxidation and oxidation rate with the addition *P. chrysosporium* extracts was detected to determine the $O_2^{\bullet-}$-scavenger ability. H_2O_2 decomposition activity was determined by measuring the reduction of H_2O_2 by *P. chrysosporium* extracts after incubation for 1 h in dark at 30 °C.

2.5. Antioxidants assays

Briefly, catalase (CAT) was measured with 50 mM PB solution (pH 7.0) and 100 mM H_2O_2 at 240 nm [20]. The CAT assays were calculated as velocity constant of absorbance decrease through H_2O_2 consumption. One unit of activity was defined as the amount of enzyme that decomposed 1 μmol of H_2O_2 in 1 min under these standard conditions. Superoxide dismutase (SOD) activity was assayed by quantifying the inhibition of superoxide-dependent pyrogallol (PAPG) self-oxidation by spectrophotometer at 320 nm. The reaction mixture contained 100 mM Tris–HCl (pH 8.2) and 10 mM PAPG, and measured the absorbance at 320 nm. One SOD unit is defined as the enzyme quantity that inhibits the autoxidation of PAPG by 50%. Activity of the glutathione peroxidase (GSH-Px) was monitored was described as Aydin et al. [21]. The decrease of absorbance due to the oxidation of NADPH was measured on a spectrophotometer at 340 nm for 2 min. One unit was defined as 1 mmol of GSH oxidized/min.

The reduced glutathione (GSH) content was determined by the method of Ellman with minor modifications [22]. 0.25 ml of *P. chrysosporium* extracts were mixed with 0.5 ml of Tris–HCl buffer solution (0.25 M, pH 8.0), and then added 0.25 ml methanal (3%) for 20 min. Then the mixture was allowed to react with 3 ml 5,5-dithiobis-2-nitrobenzoic acid (DTNB) (1 mM). The solution

was then kept at room temperature for 5 min and the extinction was read at 412 nm. Total phenolics were determined by Folin–Ciocalteau method according to the previous literature [23], using gallic acid as a standard. Flavonoids content was estimated according to a literature procedure [24].

In addition, antioxidants under Cd exposure have also been investigated, in order to determine the antioxidant response of *P. chrysosporium* to heavy metal exposure. 0.5 g wet biomass of *P. chrysosporium* was added to 100 ml Cd-containing ultrapure water (50 mg/l), vibrated at 30 °C for 24 h. Thereafter, the biomass was collected and homogenized after addition of 5 ml PB solution (50 mM, pH 7.0), filtered for antioxidants analysis as stated above.

2.6. Statistical analysis

The analysis of experimental samples was made three replications, and results were expressed as the mean value with the standard deviation. Correlation and regression analyses of ROS scavenging activity (Y) versus the antioxidant enzymes and components (X) were carried out using the regression program in SPSS software (SPSS 18.0, Germany). Differences at $p < 0.05$ were considered to be significant.

3. Results and discussion

3.1. Total antioxidant activity and radical scavenging activity analysis

The total antioxidant activities of *P. chrysosporium* extracts were evaluated by $ABTS^{\bullet+}$ radical. The decreases in absorbance of $ABTS^{\bullet+}$ versus time (ΔA/min) at the presence of various concentrations of *P. chrysosporium* extracts are shown in Fig. 1A. Consequently, the *P. chrysosporium* extracts showed increasing progressively total antioxidant activity with increasing *P. chrysosporium* concentrations. Specifically, the TAA was considered to determine the dose/response curve at concentrations of 10–100 mg/ml *P. chrysosporium* extracts. A linear dose response was found for PWE ($R^2 = 0.9870$), and a similar linear dose-response was also found for PPE ($R^2 = 0.9629$), given that the *P. chrysosporium* extracts exhibited a dose-dependent TAA at all of the tested concentrations.

Radical scavenging activity (RSA) is an important mechanism involving in antioxidant activities [19]. Reactive oxygen RSA results are given in Fig. 1B. Exceptionally high H_2O_2 decomposition activity, over 95%, was observed in PPE system, over a concentration range of 10–100 mg/ml. The extract at 10 mg/ml PPE and PWE could decompose almost 95.27% and 77.66% of the H_2O_2, whereas the 100 mg/ml extracts decomposed over 97.28% and 88.38%, respectively. This difference reflected the small increase in the H_2O_2 decomposition ability, even the considerably large rise in level and activity of several antioxidant components. These results suggested that *P. chrysosporium* could decompose H_2O_2 efficiently and further prevent H_2O_2-induced cell damage. Simultaneously, $^{\bullet}OH$ scavenging activities were found to be significant different between PPE and PWE, with relatively higher $^{\bullet}OH$ scavenging activities (beyond 80%) in PPE. Instead, the *P. chrysosporium* extracts exhibited relative lower $O_2^{\bullet-}$RSA of the two kind of extracts. Indeed, those two types of $O_2^{\bullet-}$and $^{\bullet}OH$ radical scavenging activity differed greatly in PPE and PWE, probably in terms of the variability of soluble antioxidant concentrations.

3.2. Antioxidant enzymes and low-molecular-weight antioxidants analysis

In our study, significant variability in the antioxidant enzymes of PPE and PWE was observed, and the levels of enzymes in the PPE

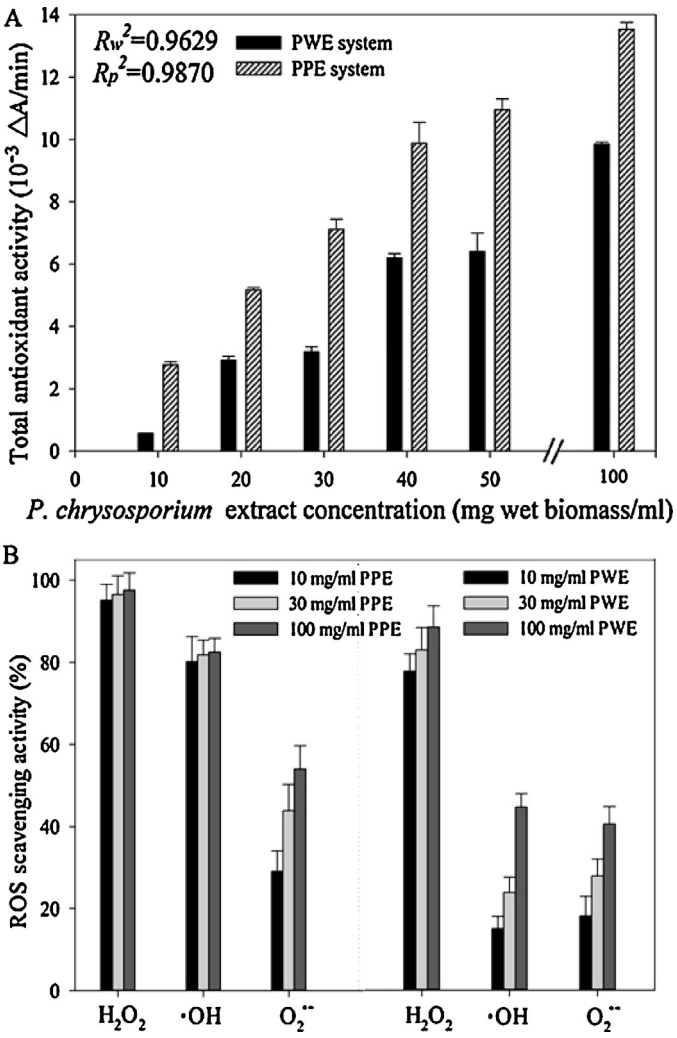


Fig. 1. Antioxidant activity analysis of the *P. chrysosporium*: (A) total antioxidant activity of *P. chrysosporium* PB solution extracts (PPE) and *P. chrysosporium* water extracts (PWE); (B) three kinds of reactive oxygen radical scavenging activity of PPE and PWE.

system were higher than in the PWE system (Fig. 2). High production levels of CAT activity were observed, then followed by SOD, and moderate GSH-Px levels were found in *P. chrysosporium* extracts. A significant link between antioxidant molecules and *P. chrysosporium* biomass content was suggested (Fig. 2A, $R^2 > 0.75$), which indicated the stable secretion of antioxidants in *P. chrysosporium*. The dose/activity curve and the increase of antioxidant enzyme activity with increasing *P. chrysosporium* extracts are likely to depend on a defense mechanism leading to a great yield of antioxidant enzymes by *P. chrysosporium* fungi, accounting for apparent inherent ROS scavenging activity contained in the tested samples.

Although the enzymatic antioxidants are considered the most efficient and specific regulators in the antioxidant system, several other low-molecular-weight antioxidant compounds also exist. The antioxidants were distinguished from the two extracts (Fig. 2B). Higher amounts of flavonoids and total phenolic were observed in PPE than that in PWE. The amounts of total phenolics in PPE ranged from 0.24 to 0.94 μmol, while PWE presented at lower concentrations at the scope of 0.18–0.54 μmol. At all the tested extracts, significant relationships have been found between antioxidant contents and *P. chrysosporium* extract dosage, indicating the stability existence of antioxidants. The contents of the GSH, flavonoids and total phenolic of PPE was 1.4, 0.8 and 1.6 μmol/g. Differences in

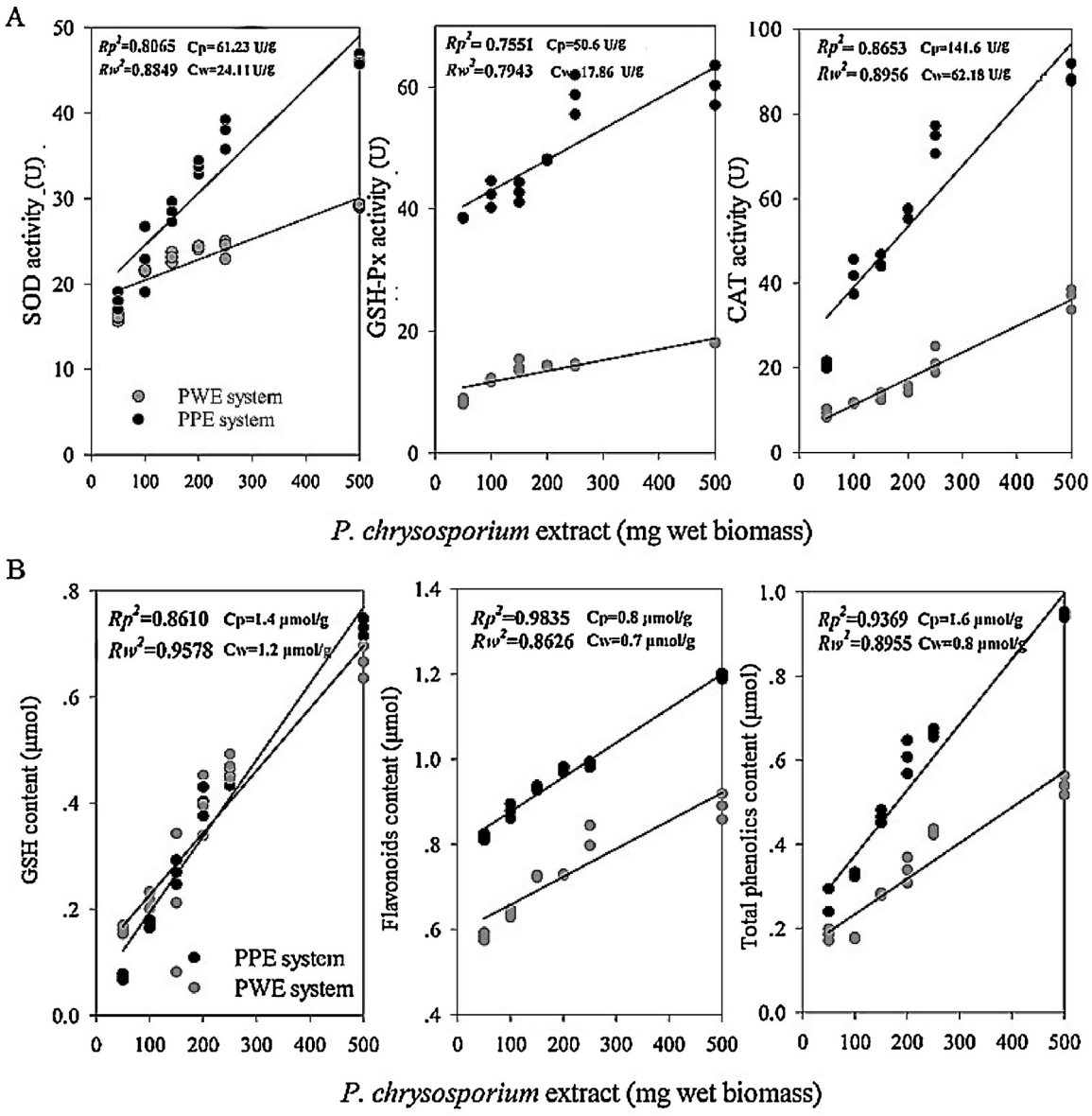


Fig. 2. Antioxidants analysis of the *P. chrysosporium* extracts. (A) Antioxidantenzymes in *P. chrysosporium* PB solution extracts (PPE) and *P. chrysosporium* water extracts (PWE); (B) low-molecular-weight antioxidant compounds in PPE and PWE.

the yields of the extracts might be attributed to the solubility and availability of various extractable components in *P. chrysosporium* extracts.

Indeed, considerable efforts have been expended in screening various natural sources as antioxidant components. Potential sources of antioxidant compounds have been searched in several types of organisms and the protective biochemical function of naturally occurring antioxidants in biological systems. For example, Oke and Aslim [25] characterized the individual profiles of phenolic compounds and evaluated the antioxidant activity of the *Pleurotus eryngii* and *Auricularia auricula-judae* extracts against H_2O_2 induced cell damage. Likewise, biological potentials of various natural sources as antioxidant components, such as glutathione, flavonoids and phenolics, are widely evaluated [26–28]. A comparison of low-molecular-weight antioxidant components between *P. chrysosporium* and some of the other studied biomaterials has been made in Table 1 [7,29–36]. It was obviously that scarcely study has focused on the inherent production of those antioxidants in environmental microbiology as a integrated antioxidant system. By comparison with the existing studies, it is apparent

Table 1
A comparison of low-molecular-weight antioxidant compounds of *P. chrysosporium* extracts and some studied biomaterials.

Antioxidants	Biomaterials	Content	References
GSH	Scots pine seedlings	~0.43 μmol/g	[29]
	poplars	~0.4 μmol/g	[30]
	Sedum alfredii	~0.1 μmol/g	[31]
	Ceratophyllum demersum	~0.85 μmol/g	[32]
	P. chrysosporium	1.4 μmol/g	This study
Flavonoids	*Phaseolus vulgaris* L	~28 μg/g	[33]
	Brassica campretis L.	~10 μg/g	[33]
	P. chrysosporium	488.8 μg/g	This study
Total phenolics	Solin seed	4.73 mg/g	[11]
	Pisum savitum	0.4 mg/g	[34]
	Triticum aestivum	0.2 mg/g	[34]
	Non-mycorrhizal pine	~2.6 mg/g	[35]
	Fistulina hepatica	0.37 mg/g	[36]
	P. chrysosporium	0.27 mg/g	This study

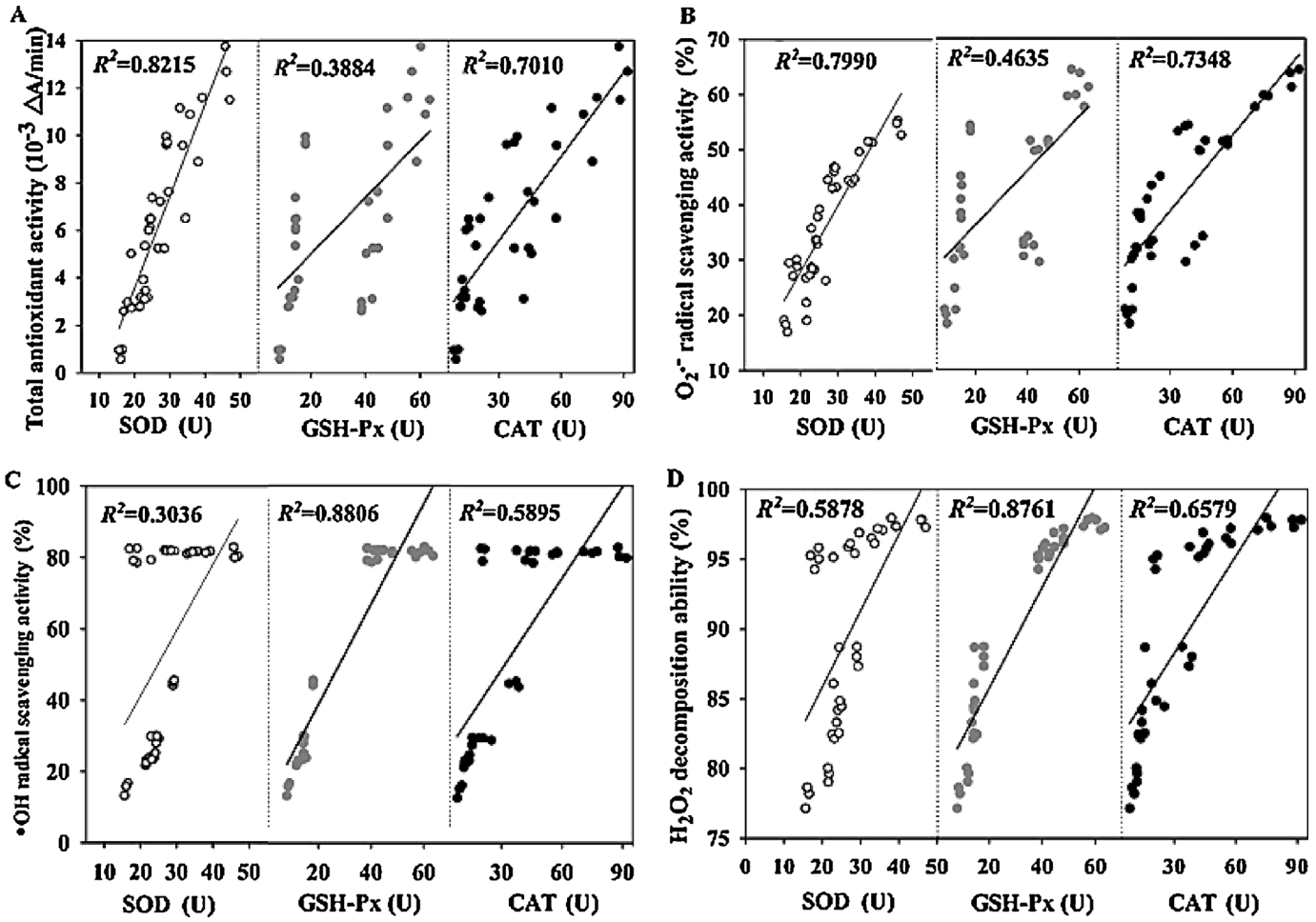


Fig. 3. Enzymes–activity relationship (EAR) analysis. (A) The relationship between the total antioxidant activity and antioxidant enzymes. (B–D) The relationship between three kinds of reactive oxygen radical scavenging activity and antioxidant enzymes.

that *P. chrysosporium* show a multiple and high yield production of low-molecular-weight antioxidant components, donating to the remarkable total antioxidant activity and ROS scavenging activity.

3.3. Enzyme–activity relationship analysis

Positive associations between TAA and those enzymes were determined by enzyme–activity relationship (EAR) analysis (Fig. 3A), indicating that the higher level of TAA could be caused by coaction with those internal antioxidant enzymes. Linear regression analysis showed that $O_2^{\bullet-}$ scavenging activity was significantly related to SOD activity, with the highest correlation coefficient (Fig. 3B, $R^2 = 0.7990$), then followed by CAT activity (Fig. 3B, $R^2 = 0.7348$). Accordingly, SOD, a class of metalloproteins, is the dominant biological agents for $O_2^{\bullet-}$ decomposition, with a superoxide decomposition rate constant of $1.3 \times 10^9\ M^{-1}\ s^{-1}$ [1]. These initial observations confirmed that the $O_2^{\bullet-}$ scavenging activity is associated with the presence of SOD in *P. chrysosporium*.

Hydroxyl radicals are major active oxygen species causing lipid peroxidation and enormous biological damage. Hydroxyl radical scavenging capacity of *P. chrysosporium* extract is directly related to its antioxidant activity. A significant inter-individual variation in the correlation of •OH scavenging activity between three tested enzymes has been obtained. High linear correlation is observed between •OH scavenging activity and GSH-Px (Fig. 3C, $R^2 = 0.8806$). The admirable activity of GSH-Px would, in any case, assure good protection against •OH.

H_2O_2 decomposition showed the highest correlation with GSH-Px (Fig. 3D, $R^2 = 0.8761$), while CAT showed the moderate correlation (Fig. 3D, $R^2 = 0.6579$), which were consistent with the previous conclusion that H_2O_2 decomposition was catalyzed by CAT and GSH-Px. GSH-Px are capable of participating in cellular antioxidant defense via enzymatic reduction of reactive cellular hydroperoxides. Such a reaction is probable due to the relatively high reactivity of GSH-Px using GSH as substrate (Eq. (1)). The thiol group of cysteine on GSH first donated a reducing equivalent ($H^+ + e^-$) to reactive oxygen species, initiating the decomposition reaction. And then the formed reactive glutathione radicals readily reacted with another glutathione molecular with the generation of glutathione disulfide (GSSG), by the catalysis of GSH-Px [37]. On the basis of our research, we conclude that high levels of CAT and GSH-Px bring about a higher rate of H_2O_2 decomposition at all the tested concentrations, suggesting that those antioxidant enzymes acted as efficient antioxidants in protecting cells from oxidative damage.

$$2H_2O_2 + GSH \xrightarrow{GSH\text{-}Px} GSSG + 2H_2O \quad (1)$$

3.4. Compounds–activity relationship analysis

Compounds–activity relationship (CAR) between antioxidant activities and each of the three kind of principal low-molecular-weight antioxidant compounds can be seen from the loading plot (Fig. 4). A positive and highly significant ($p < 0.001$) relationship was observed from GSH ($R^2 = 0.6845$), total phenolic ($R^2 = 0.6998$) and

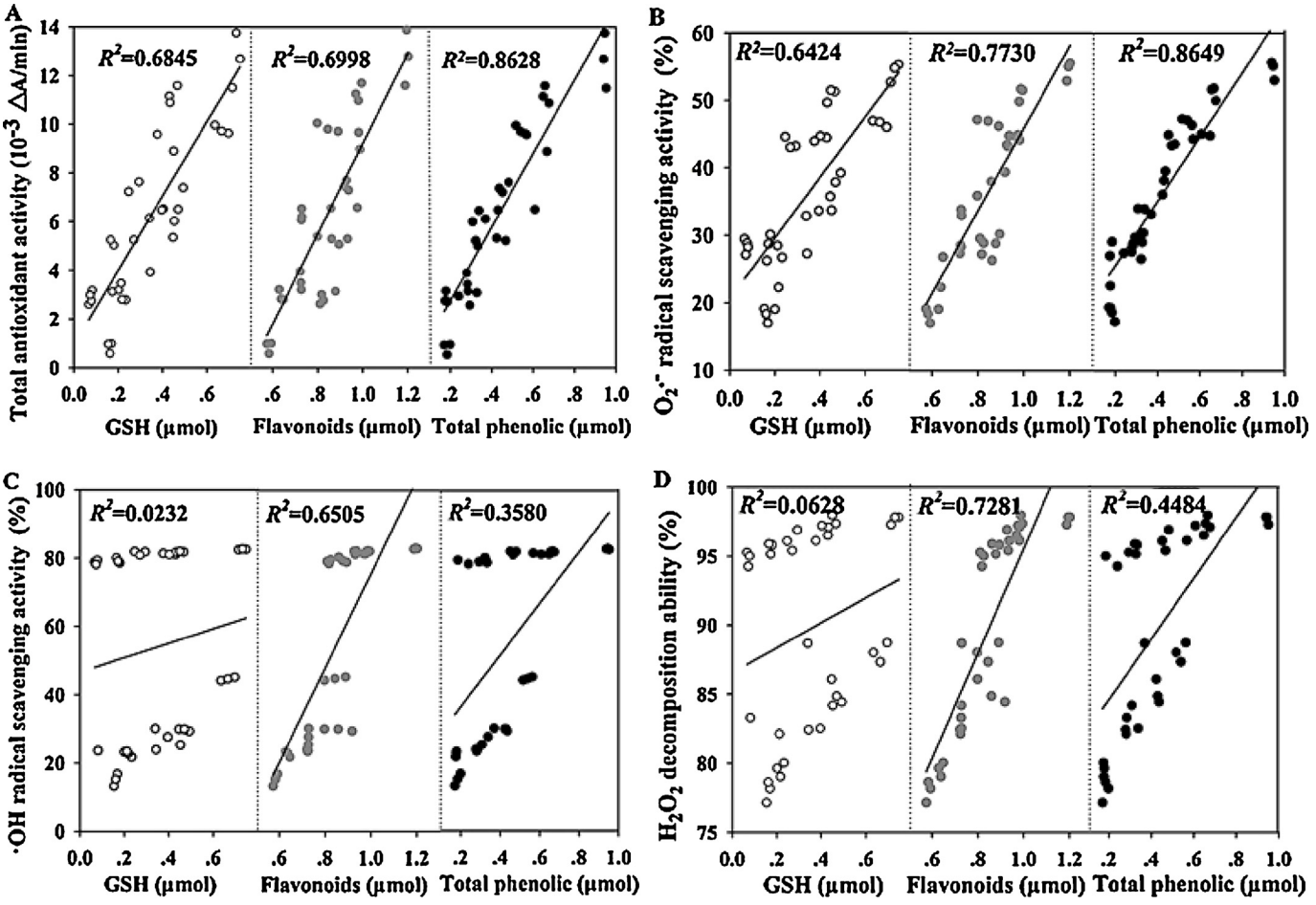
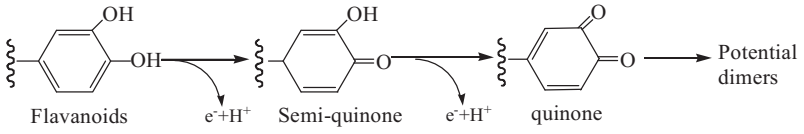


Fig. 4. Compounds–activity relationship (CAR) analysis. (A) The relationship between the total antioxidant activity and low-molecular-weight antioxidant compounds. (B–D) The relationship between three kind of reactive oxygen radical scavenging activity (RSA) and low-molecular-weight antioxidant compounds.

flavonoids (R^2 = 0.8628), suggested for the antioxidant properties of the tested components. Apparently, total phenolic was positively correlated to TAA (Fig. 4A, R^2 = 0.8628) and also to $O_2^{\bullet -}$RSA (Fig. 4B, R^2 = 0.8649).

Likewise, high positive correlations have been found among flavonoids and three kind of radical scavenging activity (Fig. 4B–D, R^2 > 0.65), due to the catabolism ability of flavonoids to scavenging reactive oxygen radicals. Reactive oxygen radical scavenging capacity of flavonoids is primarily ascribed to configuration of hydroxyl on B-ring, which stabilize the free radicals by donating hydrogen and/or electron to hydroxyl and/or peroxyl radicals [38]. During the antioxidant process, flavonoids donate an electron to the exoteric radical, resulting in the formation of a semi-quinone, and then donate a further electron to form the stable quinone (Eq. (2)) [39]. Consequently, the reactive oxygen scavenging activity high relative to low-molecular-weight compounds was the best predictor of the important role of the tested compounds and appeared to strongly confirming the scavenging mechanism of antioxidant system. No correlation was found between GSH and •OH decomposition (Fig. 4C and D). It was mainly because the complicated GSH metabolism process and nonselective electron donating capabilities of GSH. GSH is one of the best known non-enzymatic defense components in the detoxification of a variety of peroxides. However, the interrelations between GSH and ROS scavenging activity in *P. chrysosporium* are complex, directly participating in the neutralization of free radicals and reactive oxygen species, such as •OH, R•, DNA•, DNAOO•, LOOH and H_2O_2 [37].



(2)

3.5. Antioxidant mechanism analysis and mutual collaboration of antioxidants

Indeed, to protect against the noxious effects of ROS, microorganisms will develop different kinds of defensive or reparative mechanisms to eliminate the excrescent ROS intracellularly and also extracellularly [40]. According to Cadenas [41], three types of molecular mechanisms underlying antioxidant activities may be distinguished: (i) a process encompassed by a redox transition yielding the 'reactive' antioxidant-derived radical. (ii) A similar process in which transfer of the radical character with formation of a 'stable' or 'inert' antioxidant-derived radical. (iii) A enzyme-mimetic activity with antioxidant enzymes scavenging of reactive oxygen species, such as superoxide dismutase and glutathione peroxidase [42]. Therefore, an antioxidant-mimetic activity with

Table 2
Pearson correlation coefficients among total antioxidant activity (TAA), radical scavenging activity (RSA) and antioxidants.

	TAA	$O_2^{\bullet-}$RSA	•OH RSA	H_2O_2 RSA	SOD	GSH-Px	CAT	Phenolic	GSH	Flavonoid
TAA	1									
$O_2^{\bullet-}$RSA	0.931**	1								
•OH RSA	0.774	0.881*	1							
H_2O_2 RSA	0.991**	0.956**	0.773	1						
SOD	0.927**	0.994**	0.896*	0.943**	1					
GSH-Px	0.915*	0.917*	0.632	0.953**	0.884*	1				
CAT	0.784	0.836*	0.548	0.820*	0.847*	0.859*	1			
Phenolic	0.940**	0.923**	0.677	0.951**	0.928**	0.939**	0.943**	1		
GSH	0.929**	0.919**	0.672	0.942**	0.926**	0.930**	0.957**	0.999**	1	
Flavonoid	0.866*	0.888*	0.645	0.882*	0.906*	0.879*	0.982**	0.980**	0.987**	1

* Correlation is significant at the 0.05 level (two-tailed).
** Correlation is significant at the 0.01 level (two-tailed).

antioxidants scavenging of ROS might be a molecular mechanism underlying antioxidant activities. Studies on involvement of ROS scavenging activity in interaction between TAA elucidate that $O_2^{\bullet-}$RSA, •OH RSA and H_2O_2 decomposition ability were involved in antioxidant activity in with acceptable correlation coefficients (Fig. 5). The most significant relationship between TAA and $O_2^{\bullet-}$RSA (Fig. 5, $R^2 = 0.8835$) was then obtained, followed by H_2O_2 decomposition ability (Fig. 5, $R^2 = 0.6217$). SOD is the dominant biological agents for $O_2^{\bullet-}$decomposition, therefore the TAA was found to be significantly related to the SOD activity with the highest correlation coefficient. Indeed, H_2O_2 decomposition is catalyzed by a variety of enzymes, such as CAT and GSH-Px, and even related to some non-enzymatic components, such as flavonoids and phenolics. As a result, high correlation coefficients have also been found between TAA and CAT, flavonoids, phenolic, followed as SOD (Figs. 3 and 4). While •OH showed the lowest correlation to TAA (Fig. 5, $R^2 = 0.5464$), due to the formation of •OH in the presence of reduced transition metals via the Fenton reaction during H_2O_2 decomposition process, leading to the strongest ROS and triggers extensive cellular damage to cells [43]. The ordination diagram obtained by the Pearson analysis shown in Table 2 clearly performed that the total antioxidant activity are quite related with the three kind of oxygen radical scavenging activities (Table 2). Actually, the high dispersion of three kind of correlation coefficient can be related to the intrinsic variability of the extract composition, the variability of the physiological composition and specialty of the test microorganisms, determining their scavenging activity to ROS and antioxidant activity.

Contemporary, by simple analysis of concentrations of antioxidants and activities of defense enzymes in *P. chrysosporium*, it will not be possible to understand the regulation in the network of interacting antioxidant reactions. Tests performed with *P. chrysosporium* extracts via Pearson analysis was therefore considered to understand if the observed antioxidant activities are related to the reactive oxygen scavenging activity or to the reactions occurred between some components of the *P. chrysosporium* extracts (Table 2). As expected, due to the reasons outlined above, significant correlations between ROS scavenging activities and the tested antioxidants were obviously observed. Antioxidants, which act as the first-line defense against ROS intracellularly and also extracellularly, avoid or delay oxidation by scavenging free radicals, inactivating peroxides and other ROS, quenching singlet oxygen and chelating proxidant metal ions [6]. In our study, the main antioxidant activity mechanisms can be ascribed to scavenging of superoxide anions, peroxyl, or synthetic radicals by antioxidant enzymes, such as CAT, SOD and GSH-Px, or stabilize the free radicals by donating hydrogen and/or electron to hydroxyl and/or peroxyl radicals by phenolics and flavonoids. Actually, H_2O_2 RSA showed significant correlations with SOD, CAT, GSH and phenolics. Therefore high *P. chrysosporium* extracts with high levels of antioxidants showed favorable total antioxidant activity and ROS scavenging activities. While •OH RSA showed the most significant correlation with phenolics. The antioxidant mechanism of phenolic might probably be via the formation of a relatively stable phenoxyl radical by reaction of a phenolic antioxidant with a free radical [33]. Then the formed phenoxyl radical is further stabilized by delocalization of unpaired electrons around the aromatic ring. The whole antioxidant process was shown in Scheme 1. The combined results showed that antioxidants presented different antioxidant responses, which indicates that the antioxidant system may be associated with a portion of components with a more specific chemical structure, such as CAT and flavonoids.

In addition, certain antioxidants are able to regenerate or affect other antioxidants and thus restore their original function, which is so-called as an "antioxidant network". However, too little is known about the interactions and in situ concentrations of the antioxidant systems to include these factors at present into the model assumptions. Interestingly, strong correlations were also observed between those tested antioxidants, which confirmed that

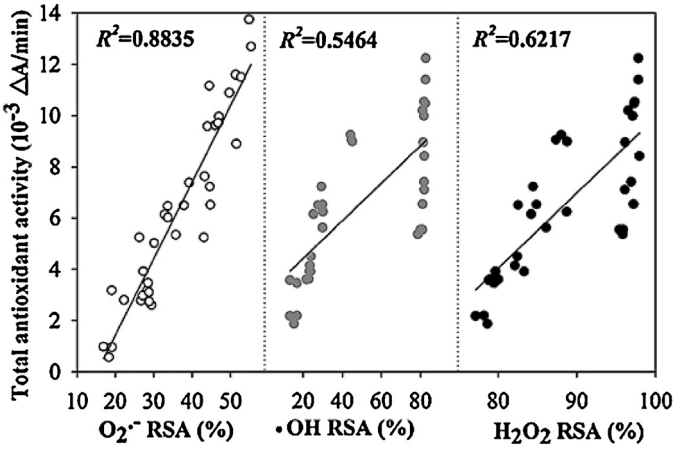
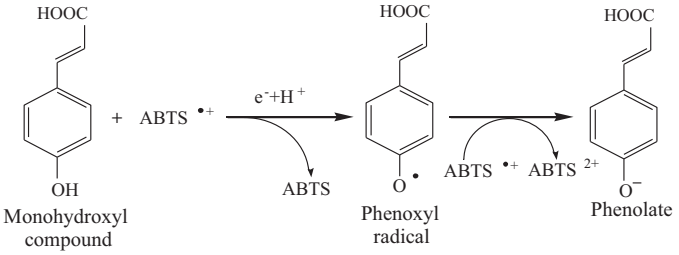


Fig. 5. The relationship between the total antioxidant activity and reactive oxygen ($O_2^{\bullet-}$, •OH and H_2O_2) radical scavenging activity (RSA).



Scheme 1. Possible mechanism of action of phenolic compounds.

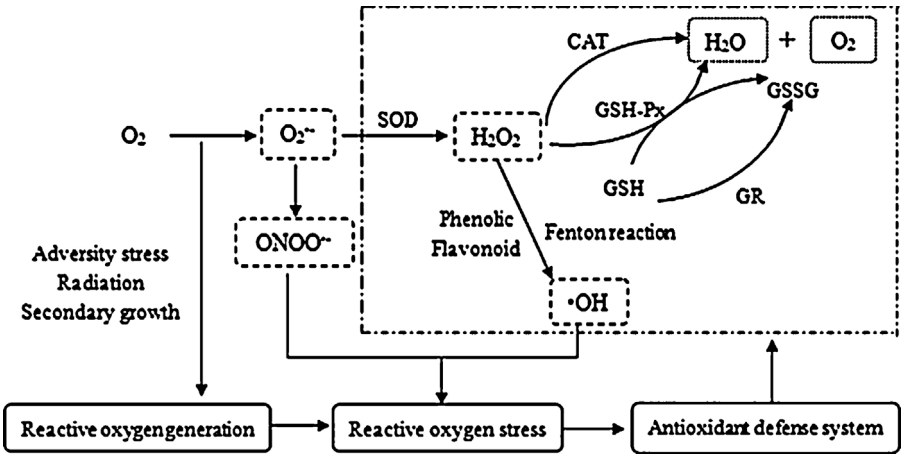
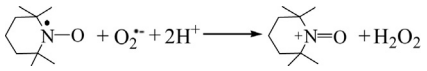
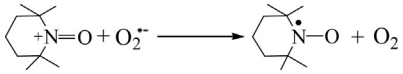


Fig. 6. Antioxidant defense mechanism and mutual collaboration of antioxidants as antioxidant network.

antioxidants acted as "antioxidant network" in *P. chrysosporium*. The mutual collaboration of antioxidants as "antioxidant network" is shown in Fig. 6. As examples, SOD activity showed a significant positive correlation with CAT and GSH-Px activities, mainly due to their interactional roles in ROS scavenging activity. In fact, the overall reaction of $O_2^{\bullet-}$decomposition is usually a second-order process via the participation of numerous enzymes. The first step is dismutation of $O_2^{\bullet-}$ to H_2O_2 and oxygen which was controlled by SOD (Eqs. (3) and (4)), one superoxide molecule transfers an electron to another superoxide molecule with the generation of H_2O_2, and then CAT and/or GSH-Px catalyzed H_2O_2 to H_2O (Eq. (5)) [40]. Thus the $O_2^{\bullet-}$RSA activity presented a significant positive correlation with the SOD activity, however, it also presented a positive correlation with GSH-Px, CAT and phenolic levels, based the fact that SOD enzymes work in conjunction with H_2O_2-removing enzymes, such as CAT and GSH-Px. Likewise, the scavenging activity of GSH also accompanied with. GSH could scavenge H_2O_2 and detoxify $^{\bullet}OH$ by the catalytic action of GSH-Px, confirming by the significant correlation between GSH and GSH-Px.


(3)


(4)

$$2H_2O_2 \rightarrow 2H_2O + O_2 \quad (5)$$

3.6. Antioxidant response of *P. chrysosporium* under Cd exposure

Heavy metals are well known to be toxic to most organisms when present in excessive concentrations. It has been found that Cd is a common consequence of most heavy metals and can result in an oxidative stress by inducing a pro-oxidant state in biological systems. Cd can react with polythiol groups on cellular macromolecules and substitute for zinc in Zn-containing enzymes, e.g., carboxypeptidases and metallothioneins, causing oxidative stress indirectly [44]. A tightly controlled metal homeostasis network to adjust to fluctuations in heavy metals is therefore a necessity for all organisms, to maintain the overall redox/oxidative balance. In order to investigate the antioxidant response of *P. chrysosporium* under heavy metal exposure, *P. chrysosporium* under the exposure of 50 mg/l Cd for 24 h was further studied. As shown in Fig. 7, antioxidant enzymes (GSH-Px, SOD and CAT), which are involved in ROS remission, generally increased in contents in the presence of Cd compared with controls regardless of the Cd. Commonly, superoxide anion ($O_2^{\bullet-}$), is considered the primary ROS, and can further interact with other molecules to generate secondary ROS, such as H_2O_2 and $^{\bullet}OH$. Although there are no known direct scavengers of singlet oxygen (O_2) or the hydroxyl radical ($^{\bullet}OH$), SOD is believed to function in their elimination by biochemical reaction. In *P. chrysosporium*, treatment with Cd resulted in 2.78-fold of stimulation of SOD activities (Fig. 7, $p < 0.05$), most probably in consequence of the direct participation of SOD in enzymatically converting $O_2^{\bullet-}$ to O_2. Similarly, Cd exposure also stimulated the biosynthesis of low-molecular-weight antioxidants intracellularly (Fig. 7, $p < 0.05$). GSH and total phenolics were most sensitive to the Cd exposure, a 1.81-fold and 2.35-fold of stimulation has been found in GSH and total phenolics, respectively. The result is consistent with the previous conclusions that Cd-exposure frequently contributes to the high levels of GSH [29,30,45]. GSH is the most abundant cellular SH in most living organisms and involved in alleviating metal stress as a first, rapidly-responsive, thiol-based line of defense against Cd exposure [31]. In this case, a prompt induction of antioxidant response, via up-regulation of ROS scavenging enzymes and a network of low-molecular-weight antioxidants, is critical to control the steady-state levels of ROS maintaining redox/oxidative balance. Actually, antioxidant defense

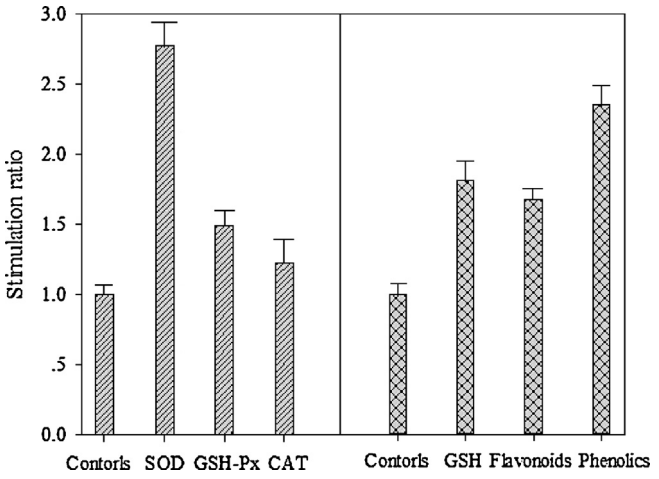


Fig. 7. Stimulation of antioxidants in *P. chrysosporium* under the exposure of 50 mg/l Cd at 30 °C for 24 h. Data are means of three replicates (±SD). Results indicate significant differences at $p \leq 0.05$ as determined by ANOVA followed by a multiple range test (LSD).

system, which is controlled by the antioxidants of the microbiology, could enhance the viability of cells challenged by a subsequent oxidative stress. The paper proposes a new validation procedure to specifically validate the admirable tolerance of *P. chrysosporium* in environmental treatment application related to its antioxidant processes.

4. Conclusions

On the basis of these results obtained in the present study, it is concluded that *P. chrysosporium* extract showed remarkable antioxidant activity and admirable in vitro free radical scavenging capacity, containing abundant antioxidants. Significantly positive correlations between antioxidant activity and antioxidants were found by EAR and CAR analysis. Furthermore, stimulation of antioxidants in *P. chrysosporium* extracts under Cd exposure was found, which provided a novel dimension to the involvement of antioxidants in the antioxidant defense system of *P. chrysosporium*. A 2.78-fold and 2.35-fold of increase has been found in SOD and total phenolics, respectively. To summarize, such data are important in elucidating the roles of these antioxidants in the physiological mechanisms of antioxidant activity, which provides a depth of new insight into the defense mechanisms of *P. chrysosporium* adapting to reactive oxygen stress.

Acknowledgments

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References

[1] L. Khachatryan, E. Vejerano, S. Lomnicki, B. Dellinger, Environmentally persistent free radicals (EPFRs). 1. Generation of reactive oxygen species in aqueous solutions, Environ. Sci. Technol. 45 (2011) 8559–8566.
[2] Y.L. Shi, A.E. James, I.F. Benzie, J.A. Buswell, Mushroom-derived preparations in the prevention of H_2O_2-induced oxidative damage to cellular DNA, Teratogen. Carcin. Mut. 22 (2002) 103–111.
[3] M.J. Koivula, M. Kanerva, J.P. Salminen, M. Nikinmaa, T. Eeva, Metal pollution indirectly increases oxidative stress in great tit (*Parus major*) nestlings, Environ. Res. 111 (2011) 362–370.
[4] K.K. Ferguson, R. Loch-Caruso, J.D. Meeker, Exploration of oxidative stress and inflammatory markers in relation to urinary phthalate metabolites: NHANES 1999–2006, Environ. Sci. Technol. 46 (2012) 477–485.
[5] W. Takabe, E. Niki, K. Uchida, S. Yamada, K. Satoh, N. Noguchi, Oxidative stress promotes the development of transformation: involvement of a potent mutagenic lipid peroxidation product, acrolein, Carcinogenesis 22 (2001) 935–941.
[6] N. Fagali, A. Catala, The antioxidant behaviour of melatonin and structural analogues during lipid peroxidation depends not only on their functional groups but also on the assay system, Biochem. Biophys. Res. Commun. 423 (2012) 873–877.
[7] Y.S. Velioglu, G. Mazza, L. Gao, B.D. Oomah, Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products, J. Agric. Food Chem. 46 (1998) 4113–4117.
[8] C.Y. Chen, Y.G. Zu, Y.J. Fu, M. Luo, C.J. Zhao, W. Wang, B.S. Zhao, J. Li, T. Efferth, Preparation and antioxidant activity of *Radix Astragali* residues extracts rich in calycosin and formononetin, Biochem. Eng. J. 56 (2011) 84–93.
[9] Y. Noda, C. Asada, C. Sasaki, S. Hashimoto, Y. Nakamura, Extraction method for increasing antioxidant activity of raw garlic using steam explosion, Biochem. Eng. J. 73 (2013) 1–4.
[10] H. Zielinski, H. Kozlowska, Antioxidant activity and total phenolics in selected cereal grains and their different morphological fractions, J. Agric. Food Chem. 48 (2000) 2008–2016.
[11] F.C. Yang, Y.H. Yang, H.C. Lu, Enhanced antioxidant and antitumor activities of *Antrodia cinnamomea* cultured with cereal substrates in solid state fermentation, Biochem. Eng. J. 78 (2013) 108–113.
[12] S. Šeatović, L. Gligić, Ž. Radulović, R.M. Jankov, Purification and partial characterization of superoxide dismutase from the thermophilic bacteria *Thermothrix* sp., J. Serb. Chem. Soc. 69 (2004) 9–16.
[13] X.Q. Xu, J.W. Zhu, Enhanced phenolic antioxidants production in submerged cultures of *Inonotus obliquus* in a ground corn stover medium, Biochem. Eng. J. 58 (2011) 103–109.
[14] P. Xu, G.M. Zeng, D.L. Huang, C. Lai, M.H. Zhao, Z. Wei, N.J. Li, C. Huang, G.X. Xie, Adsorption of Pb (II) by iron oxide nanoparticles immobilized *Phanerochaete chrysosporium*: equilibrium, kinetic, thermodynamic and mechanisms analysis, Chem. Eng. J. 203 (2012) 423–431.
[15] D.L. Huang, G.M. Zeng, C.L. Feng, S. Hu, X.Y. Jiang, L. Tang, F.F. Su, Y. Zhang, W. Zeng, H.L. Liu, Degradation of lead-contaminated lignocellulosic waste by *Phanerochaete chrysosporium* and the reduction of lead toxicity, Environ. Sci. Technol. 42 (2008) 4946–4951.
[16] P. Xu, G. Zeng, D. Huang, S. Hu, C. Feng, C. Lai, M. Zhao, C. Huang, N. Li, Z. Wei, Synthesis of iron oxide nanoparticles and their application in *Phanerochaete chrysosporium* immobilization for Pb(II) removal, Colloid. Surf. A 419 (2013) 147–155.
[17] M.H. Zhao, Z.T. Zeng, G.M. Zeng, D.L. Huang, C. Feng, C. Lai, C. Huang, Z. Wei, N.J. Li, P. Xu, Effects of ratio of manganese peroxidase to lignin peroxidase on transfer of ligninolytic enzymes in different composting substrates, Biochem. Eng. J. 67 (2012) 132–139.
[18] R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans, Antioxidant activity applying an improved ABTS radical cation decolorization assay, Free Radical Biol. Med. 26 (1999) 1231–1237.
[19] J.B. Jeong, J.H. Park, H.K. Lee, S.Y. Ju, S.C. Hong, J.R. Lee, G.Y. Chung, J.H. Lim, H.J. Jeong, Protective effect of the extracts from *Cnidium officinale* against oxidative damage induced by hydrogen peroxide via antioxidant effect, Food Chem. Toxicol. 47 (2009) 525–529.
[20] S. Nemmiche, D. Chabane-Sari, P. Guiraud, Role of α-tocopherol in cadmium-induced oxidative stress in Wistar rat's blood, liver and brain, Chem. Biol. Interact. 170 (2007) 221–230.
[21] A. Aydin, H. Orhan, A. Sayal, M. Ozata, G. Sahin, A. Işimer, Oxidative stress and nitric oxide related parameters in type II diabetes mellitus: effects of glycemic control, Clin. Biochem. 34 (2001) 65.
[22] G.L. Ellman, Tissue sulfhydryl groups, Arch. Biochem. Biophys. 82 (1959) 70–77.
[23] L.M. Cheung, P.C.K. Cheung, V.E.C. Ooi, Antioxidant activity and total phenolics of edible mushroom extracts, Food Chem. 81 (2003) 249–255.
[24] Y. Yang, Study to assaying of total flavonoids in compositus acanthopanax tablet with spectrophotometry, China Modern Doctor 50 (2012) 90–91.
[25] F. Oke, B. Aslim, Protective effect of two edible mushrooms against oxidative cell damage and their phenolic composition, Food Chem. 128 (2011) 613–619.
[26] M.A. Soobrattee, V.S. Neergheen, A. Luximon-Ramma, O.I. Aruoma, T. Bahorun, Phenolics as potential antioxidant therapeutic agents: mechanism and actions, Mut. Res. 579 (2005) 200–213.
[27] N. Lu, W. Chen, Y.-Y. Peng, Effects of glutathione, trolox and desferrioxamine on hemoglobin-induced protein oxidative damage: anti-oxidant or pro-oxidant? Eur. J. Pharmacol. 659 (2011) 95–101.
[28] A. Sekher Pannala, T.S. Chan, P.J. O'Brien, C.A. Rice-Evans, Flavonoid B-ring chemistry and antioxidant activity: fast reaction kinetics, Biochem. Biophys. Res. Commun. 282 (2001) 1161–1168.
[29] A. Schützendübel, P. Schwanz, T. Teichmann, K. Gross, R. Langenfeld-Heyser, D.L. Godbold, A. Polle, Cadmium-induced changes in antioxidative systems, hydrogen peroxide content, and differentiation in Scots pine roots, Plant Physiol. 127 (2001) 887–898.
[30] A. Arisi, B. Mocquot, A. Lagriffoul, M. Mench, C. Foyer, L. Jouanin, Responses to cadmium in leaves of transformed poplars overexpressing γ-glutamylcysteine synthetase, Physiol. Plantarum 109 (2001) 143–149.
[31] Q. Sun, Z.H. Ye, X.R. Wang, M.H. Wong, Cadmium hyperaccumulation leads to an increase of glutathione rather than phytochelatins in the cadmium hyperaccumulator *Sedum alfredii*, J. Plant. Physiol. 164 (2007) 1489–1498.
[32] S. Mishra, S. Srivastava, R. Tripathi, R. Kumar, C. Seth, D. Gupta, Lead detoxification by coontail (*Ceratophyllum demersum* L.) involves induction of phytochelatins and antioxidant system in response to its accumulation, Chemosphere 65 (2006) 1027–1039.
[33] M.G. Hertog, P.C. Hollman, M.B. Katan, Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in the Netherlands, J. Agric. Food Chem. 40 (1992) 2379–2383.
[34] M.P. Kähkönen, A.I. Hopia, H.J. Vuorela, J.P. Rauha, K. Pihlaja, T.S. Kujala, M. Heinonen, Antioxidant activity of plant extracts containing phenolic compounds, J. Agric. Food Chem. 47 (1999) 3954–3962.
[35] A. Schützendübel, A. Polle, Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization, J. Exp. Bot. 53 (2002) 1351–1365.
[36] B. Ribeiro, P. Valentão, P. Baptista, R.M. Seabra, P.B. Andrade, Phenolic compounds, organic acids profiles and antioxidative properties of beefsteak fungus (*Fistulina hepatica*), Food Chem. Toxicol. 45 (2007) 1805–1813.
[37] P.M. Kidd, Glutathione: systemic protectant against oxidative and free radical damage, Altern. Med. Rev. 1 (1997) 155–176.
[38] T. Brembu, M. Jorstad, P. Winge, K.C. Valle, A.M. Bones, Genome-wide profiling of responses to cadmium in the diatom *Phaeodactylum tricornutum*, Environ. Sci. Technol. 45 (2011) 7640–7647.

[39] C.A. Rice-evans, N.J. Miller, P.G. Bolwell, P.M. Bramley, J.B. Pridham, The relative antioxidant activities of plant-derived polyphenolic flavonoids, Free Radical Res. 22 (1995) 375–383.
[40] P. Karihtala, Y. Soini, Reactive oxygen species and antioxidant mechanisms in human tissues and their relation to malignancies, APMIS 115 (2007) 81–103.
[41] E. Cadenas, Basic mechanisms of antioxidant activity, Biofactors 6 (1997) 391–397.
[42] J.É.M. MatÉs, C. Pérez-Gómez, I.N. De Castro, Antioxidant enzymes and human diseases, Clin. Biochem. 32 (1999) 595–603.
[43] Y. Shang, C. Chen, Y. Li, J. Zhao, T. Zhu, Hydroxyl radical generation mechanism during the redox cycling process of 1,4-naphthoquinone, Environ. Sci. Technol. 46 (2012) 2935–2942.
[44] N.G. Howlett, S.V. Avery, Induction of lipid peroxidation during heavy metal stress in *Saccharomyces cerevisiae* and influence of plasma membrane fatty acid unsaturation, Appl. Environ. Microb. 63 (1997) 2971–2976.
[45] C. Xiang, D.J. Oliver, Glutathione metabolic genes coordinately respond to heavy metals and jasmonic acid in Arabidopsis, Plant Cell Online 10 (1998) 1539–1550.