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Inducers and Inhibitors of Laccase from *Penicillium*

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Abstract: The objectives of the present research was to enhance laccase production by *Penicillium aculeatum*, *P. digitatum* and *P. cyclopium*. The effects of different inducers such as guaiacol, caffeic acid, syringaldazine and sinapinic acid were investigated. The procedure developed for laccase purification enabled us to obtain enzymic preparations purified 250, 360 and 400 fold from *P. aculeatum*, *P. digitatum* and *P. cyclopium*, respectively. Phenylmercuric acetate (PMA), 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) and p-chloromercuribenzoate (PCMB) inhibited the activity of laccase. The inhibition by the three thiol-group reagents supports the idea that cysteinyl residues at the binding sites are important for the enzyme activity. Treatment of laccase with histidine selective reagent, diethylpyrocarbonate (DEPC) resulted in simple linear pseudo-first-order kinetics. The order of the inactivation kinetics and the protection by the substrate revealed that histidine is at the active site and taking part in the enzyme activity. The results show that desferal (desferrioxamine mesylate) is an inhibitor of laccase. The inhibitory effect was not dependent on the nature of the prosthetic group. Its use as an inhibitor of laccase, is promising since desferal seems to deactivate phenoxy radicals formed by the action phenol-oxidizing enzymes.

Key words: Laccase, purification, green Penicilli, activators, inhibitors, kinetics,

INTRODUCTION

Laccases (p-diphenol: dioxygen oxidoreductase; EC 1.10.3.2) belongs to a small group of enzymes called the large blue copper proteins or blue copper oxidases, which are widely distributed in fungi (Naggi *et al*., 2003). Laccases catalyze the oxidation of a range of inorganic and aromatic substances by the removal of electrons with the concomitant reduction of O_2 to water (Xu, 1996). This reaction leads to the formation of free radicals that, as demonstrated with model lignin compounds, can undergo rearrangements leading to alkyl-aryl cleavage, oxidation of benzyl alcohol and cleavage of side chains and aromatic rings (Xu, 1999).

Laccases have an overlapping substrate range and they are remarkably non-specific as to their reducing substrate. The range of substrates oxidized also varies from one type of laccase to another. The substrate specificity of laccases can be explained by their physiological functions (Thurston, 1994; Younes *et al*., 2007).

Laccases are regulated during fungal development and show diverse functions in different fungi such as controlling pathogenicity in *Cryptococcus neofonnans* (Zhu *et al*., 2001), lignin degradation (Kim *et al*., 2002) and detoxification (Kadhim *et al*., 1999). The laccase role in lignin biodegradation is important in carbon recycling and/or recalcitrant degradation. Some attempts were reported to apply laccases to industrial uses, e.g., to Kraft pulp delignification, to the removal of phenolic wastes, or to the construction of biosensors (Smith *et al*., 1997; Ortega *et al*., 2007).

Laccases are stable, easy-to-handle enzymes, so they obviously should be used in chemoenzymatic synthesis. Surprisingly, their potential has not been explored and undeservedly so. This might be partly attributed to the fact that laccases are regarded as insufficiently specific enzymes and that they are also likely to mediate the formation of polymeric by-products (Chen *et al*., 2003; Bukh *et al*., 2006).

Nevertheless, some reports concerning the use of laccase for the bioconversion of such diverse groups of compounds as *Vinca* alcaloids (Eckenrode *et al*., 1982), steroid hormones (Lugaro *et al*., 1973), β-lactames (Agematu *et al*., 1993), triazolobenzothiazadines (Bhalearo *et al*., 1994), or mithramycin (Anyanwukatu *et al*., 1994) imply the suitability of these enzymes as regioselective reagents. Apparently, recent

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demonstrations of the redox mediating activity of some artificial reducing substrates give a promise of laccases application to novel biotechnologies and synthetic organic chemistry (Smith *et al*., 1997).

Most of laccases are extracellular and a given species may produce isoenzymes of both intracellular and extracellular types (Galhaup, 2002; Younes *et al*., 2007). Therefore, the first task was the promotion of enzyme production.

A considerable number of inhibitors of enzymes have been identified, including residue-specific reagents which have given clues about those amino acids that might be vital for catalytic activity (Nawloka *et al*., 2003). Thus, the present investigation aimed also to examine laccase in relation to both the possibilities of practical applications of the enzyme and investigation of the structure of the active site.

MATERIALS AND METHODS

Chemicals: All the chemicals used were AR grade and obtained from Sigma chemical Co and BDH chemicals LTD.

Fungus culture: The three organisms namely, *Penicillium aculeatum*, *P. digitatum* and *P. cyclopium* were grown on a liquid medium containing the following components: corn steep liquor 2%, $NH_4H_2PO_4$ 1.2%, KCl 0.07%, $MgSO_4$ $7H_2O$ 0.05% and $FeSO_4$ $7H_2O$ 0.01 at pH 7.5. The liquid cultures were usually grown for 3 days at 27°C in 100 mL medium in 250 Erlenmeyer flasks in an orbital incubator. Cultures were inoculated from stocks kept on malt extract agar plates (El-Shora and Khalaf, 2002a).

Enzyme extract: Thirty grams of mycelium collected from 500 mL of fungal culture were homogenized with an electric mixer in an extraction buffer (100 mM Na phosphate buffer pH 4.7, 5 mM dithiothreitol). Extracts were filtered through gauze and clarified by cetrifugation at 5000 rpm for 20 min at 4°C. The resulting supernatent was called the crude extract (El-Shora and Khalaf, 2002a).

Enzyme assay: Laccase activity was determined spectrophotometrically at 525 nm in 0.1 M phosphate buffer (pH 5.0) by oxidation of syringaldazine. Enzyme activity was expressed in the following units: 1U= 1 μmol of syringaldazine oxidized to quinone per min at 25°C (Leonowicz and Grzywnowicz, 1983). Each value is the mean of three assays±standard error.

Laccase purification: The purification process was carried out according to El-Shora and Khalaf (2002b).

Protein determination: The protein concentration was measured by the method of Lowry *et al.* (1951).

Chemical modification reaction: Chemical modification was carried out according to El-Shora (1995).

RESULTS

Because one of the objectives of this study was to enhance laccase production, the effects of different inducers were examined. All of the inducers investigated showed a dose-dependent influence on laccase synthesis by the three *Penicillium* species (Table 1). The mean laccase activity in control flasks (without the inducer) was taken as 100%. Sinapinic acid was the most efficient inducer for laccase biosynthesis by the three *Penicillium* species, providing a 280% increase in laccase activity in case of *P. cyclopium*. Other enzyme substrates, guaiacol, caffeic acid and syringaldazine at 0.5 mM were less efficient inducers and increased the level of laccase activity by 162, 186 and 255%, respectively.

Laccase was purified from the three *Penicillium* species (Table 2) using ammonium sulphate, DEAE-Sephacel and Sephacryl S-100 column. The obtained specific activities were 500, 576 and 600 U mg^{-1} protein from *P. aculeatum*, *P. digitatum* and *P. cyclopium*, respectively. The values of the purification-fold were 250, 360 and 400 for the three species mentioned previously in the same order.

The enzyme activity was assayed at pH 5.0 in the presence of chloroacetamide, iodoacetate, PMA, DTNB and p-CMB. The five compounds are known as-SH reagents (Nawloka *et al*., 2003). Chloroacetamide and iodoacetate have no appreciable effect on laccase activity from the three *Penicillium* species. However, the last three compounds inhibited laccase activities from the three *Penicillium* species (Table 3). Under the conditions of the inactivation experiment by PMA, the rate of activity loss exhibited pseudo-first-order kinetics and was proportional to the inhibitor concentration (Fig. 1a). The values of the second order rate constant for PMA inactivation of laccase isolated from *P. aculeatum*, *P. digitatum* and *P. cyclopium* were calculated from the slope of (Fig. 1b) as 0.470, 0.035 and 0.019 μM^{-1} min^{-1}, respectively.

DEPC is known as a regent for histidyl group of enzymes (Nawloka *et al*., 2003). In the present results laccase was inactivated by the various concentrations of DEPC tested in 0.1 M borate buffer, pH 6.2 (Fig. 2). The inactivation of laccase with DEPC was dependent on time and modifier concentration. Incubation of laccase with DEPC resulted in the loss of enzymatic activity. The plots of logarithmic values of residual enzyme activity versus

Table 1: Effect of different inducers on laccase biosynthesis

	P. aculeatum		*P. digitatum*		*P. cyclopium*	
Inducer	Activity (U mL^{-1})	Control (%)	Activity (U mL^{-1})	Control (%)	Activity (U mL^{-1})	Control (%)
Control	8.0	100	4.0	100	6.0	100
Caffeic acid						
0.1 mM	9.7±0.3	121	5.2±0.4	130	8.5±0.3	141
0.5 mM	12.2±0.6	153	6.5±0.7	162	11.2±0.5	186
Syringaldazine						
0.1 mM	11.3±0.7	141	5.8±0.4	144	9.7±0.5	162
0.5 mM	13.9±0.4	174	7.0±0.3	176	15.3±0.3	225
Sinapinic acid						
0.1 mM	14.8±0.2	185	7.7±0.5	192	14.8±0.4	246
0.5 mM	20.0±0.6	250	8.8±0.6	220	48.3±0.6	280
Guaiacol						
0.1 mM	10.0±0.3	115	4.5±0.6	113	7.9±0.6	131
0.5 mM	11.0±0.5	138	5.6±0.4	141	9.7±0.8	162

Table 2: Purification of laccase from different *Penicillium* species

Penicillium species	Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1} protein)	Yield	Fold of purification
P. aculeatum	Crude extract	400.00	800	2.0	100.0	1.0
	Amm. Sulphate	46.30	722	15.6	90.3	7.8
	DEAE-sphacel	4.00	310	77.5	38.8	38.8
	Sephacryl S-100	0.20	100	500.0	12.5	250.0
P. digitatum	Crude extract	300.00	480	1.6	100.0	1.0
	Amm. Sulphate	31.00	210	6.8	43.8	4.3
	DEAE-sphacel	3.00	120	40.0	25.0	25.0
	Sephacryl S-100	0.11	64	576.0	13.3	360.0
P. cyclopium	Crude extract	433.00	650	1.5	100.0	1.0
	Amm. Sulphate	62.00	410	6.6	73.2	4.4
	DEAE-sphacel	5.00	310	62.0	47.7	41.3
	Sephacryl S-100	0.30	180	600.0	27.7	400.0

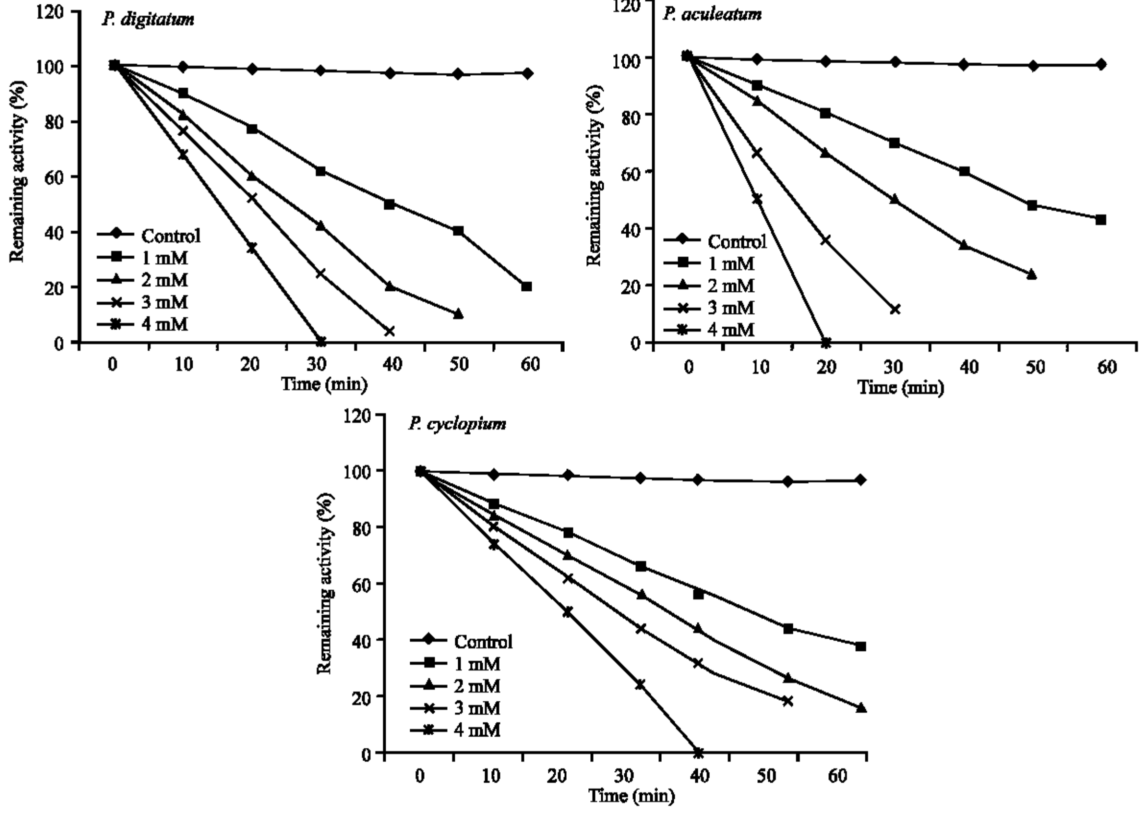

Fig. 1a: Inactivation of laccase by phenylmercuric acetate

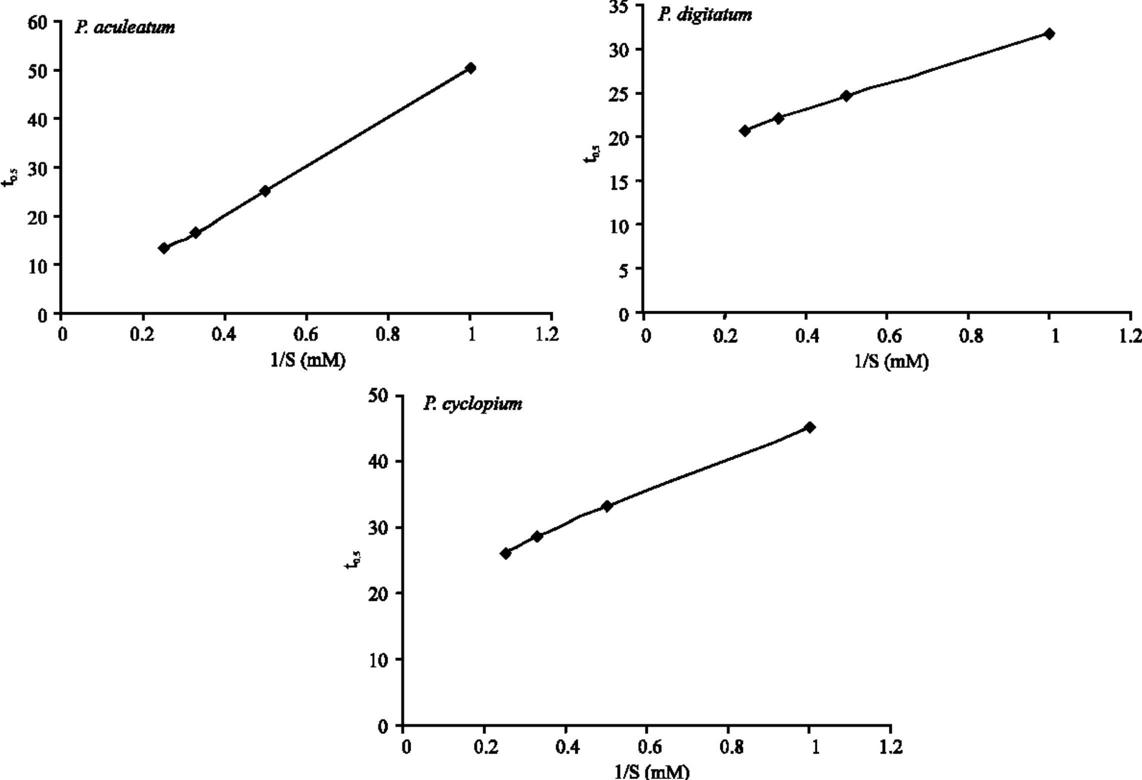

Fig. 1b: Relation between $t_{0.5}$ and reciprocal of inhibitor concentration

Table 3: Effect of sulfhydryl group reagents on laccase activity from *Penicillium* species at pH 5.0

	Inhibition (%)		
Reagents	*P. aculeatum*	*P. digitatum*	*P. cyclopium*
Control	-	-	-
p-Chloromercuribenzoate	87.4±0.4	55.6±0.5	71.7±0.6
5,5'-Dithiobis (2-nitrobezoate)	71.2±0.3	44.2±0.4	60.2±0.5
Iodoacetate	0.6±0.6	0.3±0.8	0.6±0.9
Phenylmercuric acetate	53.3±0.5	33.4±07	49.0±0.7
Chloroacetamide	0.9±0.3	0.7±0.2	0.4±0.3

Table 4: Recovery of activity by 0.5 M hydroxylamine of DEPC treated laccase

Time of addition of NH_2OH after 100% inhibition (min)	Recovery activity (%)		
	P. aculeatum	*P. digitatum*	*P. cyclopium*
10	88±0.8	93±0.2	68±0.4
30	72±0.7	87±0.9	52±0.9
60	64±0.3	74±0.9	48±0.7
90	59±0.1	70±1.1	34±0.9
120	51±0.9	65±1.2	21±0.6

The activity of each sample was determined and expressed as % of the activity of a control sample (without DEPC) to which a NH_2OH was added

incubation time were linear (data not shown), suggesting that the inactivation obeyed pseudo-first-order kinetics.

Incubation of inactivated laccase with 0.5 M hydroxylamine in 0.1 M borate buffer, pH 6.2 resulted in partial restoration of the enzyme activity (Table 4). These results indicated that the histidine residues in the enzyme were modified with DEPC and that the resulting bonds linking carbethoxyls and histidine residues were relatively unstable and can be broken by hydroxylamine.

Laccase activity from the three different *penicillium* species was protected in the presence of 3 mM of various substrates such as syringaldazine, p-phenylenediamine, pyrogallol, guaiacol, 2,6-dimethoxyphenol, catechol, ferulic acid (Table 5). Syringaldazine was the best protector.

Desferrioxamine mesylate is a powerful iron chelator which is commercially available as desferal. Desferal chelates iron into a catalytically inactive form (Palmer, 1985). Apart from iron, desferal can bind several other transition metals. Desferal is known as a powerful inhibitor of peroxidases and a powerful activator of catechol oxidase (De Pinto and Barcelo, 1996). Therefore, the effect of desferal as a transition metal chelator, on the catalytic activity of laccase was studied.

The obtained results (Fig. 3a and b) showed that desferal is an inhibitor of laccase activity from the various *Penicillium* species. At 50 mM, desferal totally blocks the laccacse activity. The inhibition is mainly manifested as a reduction in the steady-state oxidation rate.

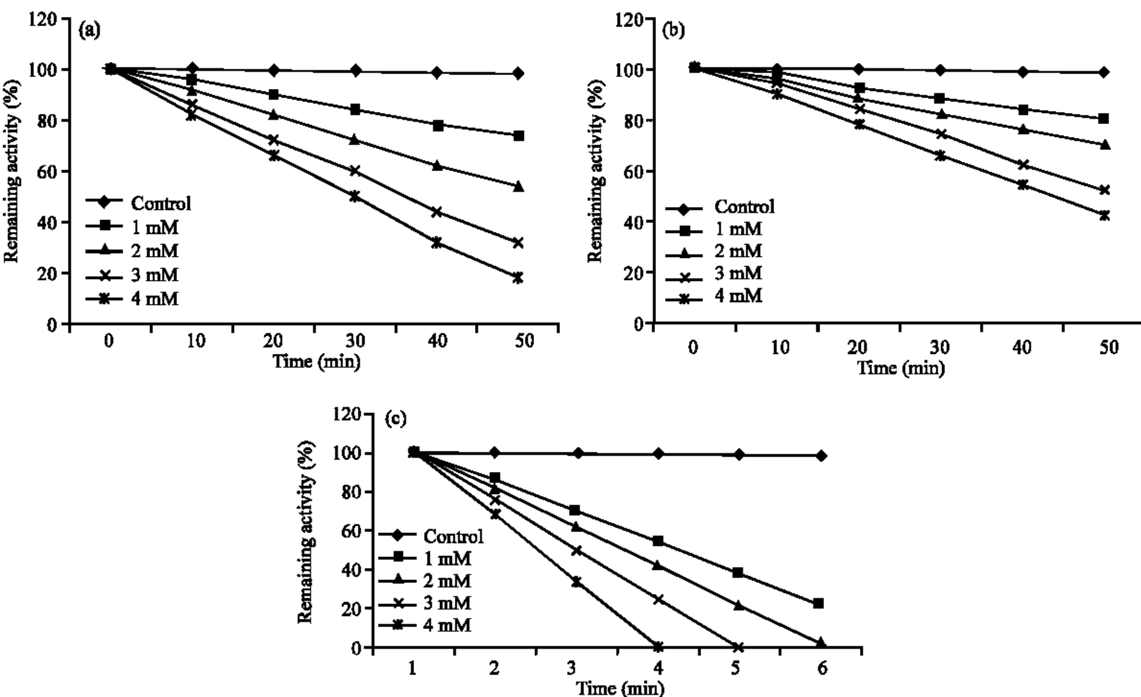

Fig. 2: Inactivation of laccase by DEPC. (a): *P. aculeatum*; (b): *P. digitatum* and (c): *P. cyclopium*

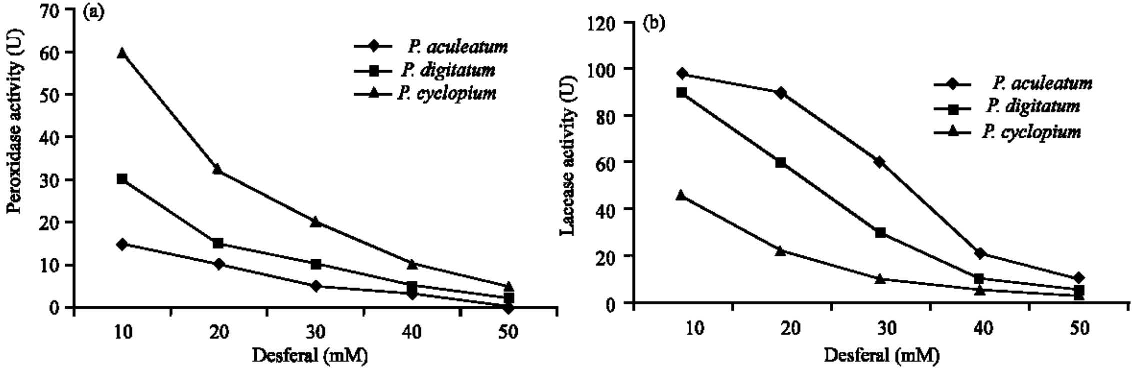

Fig. 3: Effect of desferal concentration on laccase activity; (a) peroxidase activity; (b) laccase activity

Table 5: Protection of laccase activity against inhibition by DEPC

	P. aculeatum		*P. digitatum*		*P. cyclopium*	
Protectors	Activity as substrate (%)	Protection % against DEPC	Activity as substrate (%)	Protection % against DEPC	Activity as substrate (%)	Protection % against DEPC
Syringaldazine	100	100	100	100	100	100
p-Phenylenediamine	28±0.9	40±0.2	25±0.4	35±0.7	19±0.4	24±0.9
Pyrogallol	12±0.9	51±0.6	10±0.2	31±0.8	9±0.7	28±1.1
Guaiacol	37±0.7	35±0.7	28±0.3	27±0.9	21±0.8	19±0.7
2,6-Dimethoxyphenol	16±0.6	34±0.4	13±0.7	21±0.4	10±0.7	17±0.8
Catechol	18±0.5	10±0.3	15±0.8	8±0.8	11±0.7	5±0.6
Ferulic acid	25±0.3	26±0.2	17±0.3	21±0.7	14±0.5	16±0.5

The inhibition of peroxidase-mediated oxidation by desferal has been explained for a long time by its properties as a transition metal chelator, which removes the iron from the prosthetic group of peroxidases. Such properties would also explain desferal's inhibition of laccase and the necessity for higher desferal

Table 6: Inhibition by desferal of the oxidation of physiological and non-physiological substrates by both laccase and peroxidase

	Inhibition (%)					
	P. aculeatum		*P. digitatum*		*P. cyclopium*	
Substrate (10 mM)	Laccase	Peroxidase	Laccase	Peroxidase	Laccase	Peroxidase
Resveratol	86.2±0.2	80.1±0.2	72.0±0.6	75.7±0.3	64.0±0.6	58.0±0.2
Coniferyl alcohol	35.3±0.1	28.3±0.8	29.3±0.8	21.6±0.7	17.2±0.9	13.1±0.7
Resveratrol	62.4±0.8	57.1±0.5	44.0±0.6	41.4±0.9	27.0±0.7	21.0±0.6
Dihydrocapsaicn	28.0±0.3	20.0±0.9	15.3±0.4	14.3±0.7	11.0±0.7	8.0±0.8
4-Methoxy-α-naphthol	40.4±0.7	32.5±0.5	33.6±0.9	25.2±0.4	18.0±0.8	13.1±0.2
Phenol red	-	92.1±0.7	-	76.6±0.5	-	52.3±0.4
Crystal violet	-	80.3±0.3	-	68.8±0.2	-	42.0±0.3

concentrations, since desferal would also bind copper but with much lower stability constant (Palmer, 1985). If this true, the ability of desferal to inhibit phenolic oxidation by both laccase and peroxidase should be independent of the nature of the phenolic oxidized and only dependent on the nature of the prosthetic group of the enzyme.

To test this hypothesis, the inhibitory effect of desferal on the oxidation of several phenols by peroxidase and laccase was tested using putative physiological substrates of both laccase and peroxidase such as coniferyl alcohol, resveratrol and dihydrocapsaicin. The three compounds are well known substrates of both peroxidase and laccase. The obtained results (Table 6) showed surprising results as desferal inhibited the oxidation of each one of the three phenols to about the same extent, independently of the nature of the enzyme. Indeed, at 10 mM, the inhibitory effect of desferal on both peroxidase and laccase was almost identical for the same phenolic. These results show that the chelating properties of desferal are not responsible for the inhibition.

When non-physiological substrate such as 4-methoxy-α-naphthol for laccase and peroxidase was tested (Table 6), similar results were obtained. Other substrates such as crystal violet and phenol red, were only substrates of peroxidase-mediated oxidations (Table 6).

DISCUSSION

The present results indicate that *Penicillium aculeatum*, *P. digitatum* and *P. cyclopium* expressed appreciable activities of laccase. In support, laccase has been also isolated from *Penicillium chrysogenum* (Rodriguez *et al*., 1996), *Aspergillus nidulans* another member of *ascomycetes* (Scherer and Fischer, 1998) and *Pycnoporus sanguineus* (Lu *et al*., 2007).

Laccase from the three *Penicilli* was induced by guaiacol, caffeic acid, syringaldazine and sinapinic acid. Sinapinic acid was the most efficient inducer of laccase biosynthesis. Laccase activity from the edible mushroom *Volvariella volvacea* was induced by other aromatic compounds such as ferulic acid and veratric acid (Chen *et al*., 2003).

In the present investigation laccase was purified from the three *Penicillium* species with 250 to 400 fold purification and specific activities of 500 to 600 U mg^{-1} protein. Indeed, these values are higher than those reported for laccase from rice blast fungus, *Magnaporthe grisea* (Iyer and Chattoo, 2003).

Laccase from the different tested *Penicillium* species was exposed to five different reagents of sulfhydryl group at pH 4.6, but the activity was only inhibited by PMA, p-CMB and DTNB. Each compound produced an inactivation that followed pseudo-first-order kinetics and was linear with the inhibitor concentration. The inhibition of laccase by the three-sulfhydryl reagents provides evidence that essentials cysteinyl group are present in the active site of laccase. This finding is in consistent with the results of Giardina *et al*. (1999).

The other sulfhydryl-selective reagents chloroacetamide and iodoacetate have no appreciable effect on laccase activity. This might be explained by the relatively acidic conditions of the reaction (Palmer, 1985).

The present study indicates that laccase is rapidly inactivated by DEPC. Other studies have demonstrated that DEPC can specifically react with histidine residues at a pH around 6.0 (Lee *et al*., 2001).

The modification of histidine can be reversed by hydroxylamine, which was proved to have the ability of cleaving the resulting covalent bond between carbethoxyl and the histidine residue (Carvajal *et al*., 1997). The restoration of activity of the inactivated enzyme with hydroxylamine and its protection by syringaldazine, *p*-phenylenediamine, pyrogallol, guaiacol, 2,6-dimethoxyphenol, catechol, ferulic acid support our finding that histidine residue is taking part in the enzyme catalysis.

In the present investigation desferal inhibited laccase from the various *Penicillium* species. Since desferal is a powerful inhibitor of peroxidase and a powerful activator of catechol oxidase (De Pinto and Barcelo, 1996), thus, desferal may be used to discriminate laccase and peroxidase from catechol oxidase activities.

Crystal violet and phenol red proved to be only substrates for peroxidase-mediated oxidations and these

results suggest using the two compounds for the discrimination between peroxidase and laccase activities.

The present results demonstrated that laccase activity was inhibited by desferal as in case of peroxidase. Although the mechanism of this inhibition is not understood very well, Morehouse *et al.* (1988) suggested that desferal may be oxidized by peroxidase to yield a nitroxide free radical. This property of desferal to act as a substrate of peroxidase could explain the inhibition of peroxidase by desferal since it would act as a competitive inhibitor of the enzyme. However, this would not explain the inhibition of laccase by desferal, nor the fact that its efficacy as an inhibitor depends more on the substrate employed than the oxidizing enzyme.

De Pinto and Barcelo (1996) proposed that the inhibition of phenolic oxidations catalyzed by laccase or peroxidase can be explained assuming that the phenolic radicals formed by the action of the enzyme on substrates react with desferal in accordance with the following reaction mechanism:

PhOH………laccase………….PhO* (1)

PhO* + D…………………….PhOH + D* (2)

PhO*………………..products (3)

where,

PhO* : The phenoxy radical of any phenolic oxidized by either laccase or peroxidase.

D : Desferal

D* : The nitroxide free radical.

The reaction (3) would compete with the deactivation reaction of the phenoxy radical.

This reaction mechanism would explain why inhibition by desferal is more dependent on the substrate assayed than on the oxidizing enzyme, since the inhibition would be determined by the reactivity of desferal with phenoxy radicals, according to Eq. 2.

Thus, the use of desferal as inhibitor of phenol-oxidizing enzymes, such as laccase and peoxidase, is promising, since desferal appears to deactivate phenoxy radicals, the main products of these phenolic oxidations.

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