Discovery of two low redox potential, novel laccases *Pc*Lac1 and *Pc*Lac2 from *Pleurotus citrinopileatus.*

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The technological utilization of lignocellulosic biomass and, in particular, its bioconversion towards biofuels and other high value-added products attract the scientific interest in the field of Industrial Biotechnology. Especially in terms of circular economy, such bioprocesses become more advantageous when a process of sustainable waste management is involved. White-rot Basidiomycetes posses an intricate enzymatic system which is able to efficiently decompose lignocellulosic biomass through synergistic interactions. More specifically, laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2), belonging to the family of multicopper oxidases, catalyze the breakdown of covalent carbon-carbon or carbon-oxygen bonds in complex lignin polymers (Leonowicz et al., 2001). Due to their high E_0 values, varying between +500 and +800 mV vs NHE (normal hydrogen electrode) (Riva, 2006), laccase substrate spectrum usually includes mono-, di-, polyphenols, methoxy-substituted phenols, aromatic compounds and amines, such as acrylamines or aminophenols, therefore, their substrate specificity is low. Fungal laccases are extracellular glycoproteins, with a monomeric structure, approximately 60-70 kDa with acidic isoelectric points around pH 4.0, however, there is great heterogeneity among them. Their biological functions involve lignin degradation, morphogenesis, stress response, pigment and melanin formation, plant pathogenesis and others (Baldrian, 2006). The aim of the present study is the characterization of two novel laccases, isolated from the culture supernatants of the Basidiomycete *Pleurotus citrinopileatus* LGAM 28684 grown on olive oil mill wastewater (OOMW), with potential for biotechnological application in the field of phenolic oligomer synthesis.

*Pc*Lac1 and *Pc*Lac2 were isolated from the supernatant of liquid culture of *P. citrinopileatus*. *P. citrinopileatus* was cultivated in an olive mill wastewater- based liquid medium. The purification process of the isolated laccases was accomplished through ion-exchange chromatography columns Q-Sepharose and DEAE–cellulose HiPrep DEAE FF 16/10 (GE Healthcare). Protein concentration of the enzymes was quantified by the Lowry method. Molecular weights were defined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis SDS-PAGE electrophoresis while pI determination was accomplished through IEF-PAGE electrophoresis. The optimum pH and temperature conditions were defined using ABTS (2 mM) as the substrate, after incubation for 10 min at 35˚C, over the pH range 2-9 using buffers 0.1 M citrate-posphate (pH 2-5), 0.1 M phosphate (pH 5-6) and 0.1 M Tris-HCl (pH 8-9) and over the range 20-70˚C for 10 min respectively. In addition, qualitive measurements were performed for the oxidative potential of *Pc*Lac1 and *Pc*Lac2 on a wide spectrum of phenolic substrates (2 mM) at room temperature for 24 h, while substrate specificity assays were performed by measuring laccase activity against ABTS, 2,6-dimethoxyphenol, catechol, pyrogallol, guaiacol and hydroquinone (2mM), at 40˚C, 950 rpm and pH 3 and 4 for *Pc*Lac1 and *Pc*Lac2 respectively. Kinetic measurements of laccase activity were performed for ABTS and 2,6-DMP, in 0.1 M phosphate-citrate buffer pH 3 for *Pc*Lac1 and pH 4 for *Pc*Lac2. Michaelis-Menten constants were calculated from Lineweaver-Burk plots using the software GraphPad Prism 5 (GraphPad Software, Inc., U.S.A.). The inhibition effect of methanol, ethanol, acetone, 1,4-dioxane (10 and 50% v/v), DMSO (10% v/v), NaCl (10 and 100 mM), NaN_3 (0.01 and 0.1 mM), EDTA (1 and 10 mM), SDS (0.1 and 1 mM), Cu (0.25 and 2.5 mM) and H_2O_2 (1 and 5 mM) was also studied. Redox potential E_0 was determined through large amplitude Fourier transform alterating current cyclic voltammetry (FTacV). The oligomer synthesis ability of the laccases was examined on ferulic acid, sinapic acid, catechol, gallic acid and caffeic acid, in reactions at optimum conditions for each enzyme and enzyme load 0.16 Unit mL^{-1}.

SDS-PAGE analysis confirmed the purity of both enzymes, concluding that *Pc*Lac1 is a 60 kDa protein, while *Pc*Lac2 is closer to 75 kDa. Isoelectric point pI for *Pc*Lac1 was detected around 3.5 by IEF-PAGE electrophoresis. Optimum reaction conditions for *Pc*Lac1 were pH 4.5 and 55˚C whereas for *Pc*Lac2 are pH 4 and 55˚C. The substrate oxidation study revealed that the two laccases differ considerably in their substrate specificity. *Pc*Lac1 shows preference towards catecholic structures and hydroxycinnamic acids with hydroxyls at ortho-position, Also, it oxidizes dimethoxy-substituted compounds but not phenolic alcohols, amines, aldehydes or hydroxybenzoic acids. *Pc*Lac2 oxidizes mainly phenolic compounds with catecholic structure. The results of substrate specificity study are shown in Table 1.

Table 1. Substrate specificity of *Pc*Lac1 and *Pc*Lac2.

Enzyme	ABTS (U mg^{-1})	2,6 DMP (U mg^{-1})	Catechol (U mg^{-1})	Pyrogallol (U mg^{-1})	Guaiacol (U mg^{-1})	Hydroquinone (U mg^{-1})
*Pc*Lac1	1.48 ± 0.09	0.08 ± 0.00	0.06 ± 0.01	0.020 ± 0.001	0.003 ± 0.000	0.079 ± 0.003
*Pc*Lac2	4.78 ± 0.33	0.019 ± 0.003	0.04 ± 0.01	0.0390 ± 0.0003	0.0006 ± 0.0002	0.09 ± 0.02

Both enzymes show a high affinity for ABTS, similarly to other fungal laccases, but a significantly lower affinity for 2,6-DMP. However, *Pc*Lac1 shows much higher affinity for both substrates than *Pc*Lac2. The turnover number is low in the case of 2,6-DMP oxidation, but considerably higher in the case of ABTS oxidation. *Pc*Lac2 shows a very high turnover number for ABTS oxidation in comparison with *Pc*Lac1. The same trend is followed for catalytic efficiency. As to the inhibition effect analysis, *Pc*Lac1 is rather unstable in the presence of organic solvents, maintaining only around 50% of its activity in 10% v/v solvent. *Pc*Lac2 shows higher stability in organic solvents, maintaining more than 80% of its activity in 10% v/v solvent. *Pc*Lac1 seems to be more active in presence of EDTA, H_2O_2 and Cu, achieving up to 1.3 times higher activity in the presence of 0.25 mM Cu. A slight inhibition of 10 % is detected in the presence of SDS 0.1 mM or H_2O_2 5 mM. On the contrary, *Pc*Lac2 shows a 20% activity decrease in the presence of NaCl 10 mM, EDTA, SDS 0.1 mM, H_2O_2 1 mM and Cu.

Table 2. E_0 redox potential for *Pc*Lac1 and *Pc*Lac2 vs. NHE

T (°C)	E_o'corrected vs NHE (mV)	Standard Deviation	E_o'corrected vs NHE (mV)	Standard Deviation
	*Pc*Lac1		*Pc*Lac2	
30	453	1.2	374	3.9
33	454	0.7	379	2.8
39	452	1.0	380	1.8
43	462	7.8	374	10.1

According to the UV-Vis spectrum analysis, *Pc*Lac1 does not demonstrate the characteristic peak of blue laccases at 610 nm, while it absorbs around 330 nm, which indicates it is a yellow laccase. *Pc*Lac2 shows two peaks, at 300 and 600 nm, indicating it probably belongs to blue laccases. The synthetic potential of laccases was confirmed by the presence of insoluble products in the reaction mixtures when catechol, gallic acid, sinapic acid and ferulic acid were used as substrates.

In the last decades, interest has risen for the development of modern biorefineries, aiming at lignocellulosic biomass processing for the production of new generation biofuels as well as other commercial chemical compounds. Laccases are of great importance due to their ability to modify lignin and a variety of other pollutants and substrates (Roth and Spiess, 2015). Hence, these enzymes are an important tool for designing green processes in the field of Industrial Biotechnology. In the present work, two novel laccases, *Pc*Lac1 and *Pc*Lac2, were purified and characterized. Despite the fact that these two enzymes are secreted from the same microorganism, they possess different biochemical, catalytic and redox properties. Even though they have similar optimal reaction conditions, they exhibit different redox potential patterns as well as different substrate specificity. Therefore, they have the potential to be utilized on a variety of customized biotechnological applications in which the required reaction conditions may vary. In addition, the synthetic potential of *Pc*Lac1 and *Pc*Lac2 has been highlighted, by using phenolic acids as substrates. The presence of insoluble products indicates the potential of both laccases for use in organic synthesis of novel compounds as innovative products with new properties.

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