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ORIGINAL ARTICLE

# Enzymatic extract containing lignin peroxidase immobilized on carbon nanotubes: Potential biocatalyst in dye decolourization

**Sabrina Feliciano Oliveira [a], José Maria Rodrigues da Luz [b], Maria Catarina Megumi Kasuya [b,*], Luiz Orlando Ladeira [a], Ary Correa Junior [a]**

[a] *Department of Microbiology, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627 – Pampulha, 31270-901 Belo Horizonte, Minas Gerais, Brazil*
[b] *Department of Microbiology, Universidade Federal de Viçosa, Av. Peter Henry Rolfs, s/n° – Campus Universitário, Viçosa, Minas Gerais, Brazil*

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**Abstract** The majority of the textile dyes are harmful to the environment and potentially carcinogenic. Among strategies for their exclusion, the treatment of dye contaminated wastewater with fungal extract, containing lignin peroxidase (LiP), may be useful. Two fungi isolates, *Pleurotus ostreatus* (PLO9) and *Ganoderma lucidum* (GRM117), produced the enzymatic extract by fermentation in the lignocellulosic residue, *Jatropha curcas* seed cake. The extracts from PLO9 and GRM117 were immobilized on carbon nanotubes and showed an increase of 18 and 27-fold of LiP specific activity compared to the free enzyme. Also, LiP from both fungi extracts showed higher Vmax and lower Km values. Only the immobilized extracts could be efficiently reused in the dye decolourization, contrary, the carbon nanotubes became saturated and they should be discarded over time. This device may offer a final biocatalyst with higher catalytic efficiency and capability to be reused in the dye decolourization process.


## 1. Introduction

Dyes are an important class of macromolecules which are an integral part of our lives and used by, textile, paint and plastic, solar cells, optics, metal extraction and sensor industries, among others (Rauf and Ashraf, 2012). Due to the extensive use, they have become a part of industrial effluent. It is estimated that up to 50% of dye contents are lost after dyeing textiles and about 10–15% are discarded in effluents (Papadoulou et al., 2013). Further to esthetic pollution, the colored effluent

* Corresponding author at: Av. P. H. Rolfs, s/n, Campus UFV, Viçosa, Minas Gerais 36570-000, Brazil. Tel.: +55 31 3899 2970; fax: +55 31 3899 2573.
E-mail address: mkasuya@ufv.br (M.C.M. Kasuya).
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promotes the inhibition of light penetration along the depth of water bodies, affecting biological cycles, particularly the photosynthesis processes. Most of these dyes are harmful and potentially carcinogenic in nature and their exclusion from wastewaters is a major environmental challenge (Chequer et al., 2011).

The use of enzymes for environmental remediation purposes has increased due to peculiar properties of this class of proteins. These molecules operate in a wide range of contaminant concentrations, pH, temperature and salinity (Rao et al., 2014). The ligninolytic enzyme system (lignin peroxidase, manganese peroxidase and laccase) produced by the white rot fungi is of particular interest because it has a low substrate specificity, no steric selectivity and strong oxidative abilities (Ashger et al., 2014). Besides, due to the high cost of biotechnology processes, the current challenge is to increase the production of these enzymes, using low-cost substrates. Therefore the use of lignocellulosic residues, such as the *Jatropha curcas* seed cake, as substrate for the synthesis of enzymes is an alternative for cost reduction and at the same time solves an environmental problem regarding the disposal of these agro-industrial wastes. Among those enzymes, lignin peroxidase (LiP) stands out as a relatively nonspecific enzyme, able to mineralize various recalcitrant aromatic and halogenated phenolic compounds, with high redox potential compared with the classical peroxidases, therefore being a stronger oxidant (Wong, 2009). Regarding the biotechnological potential, this enzyme has been successfully applied in food and pharmaceutical industries, wastewater treatment of pulp and paper and textile industry, bioremediation and biomass delignification (Wong, 2009; Ashger et al., 2014).

Although the natural catalysts are sustainable, selective and efficient, they are often not perfectly adapted for industrial applications. To promote the use of enzymes in industrial processes, its stability and reuse ability should be considered (Gardossi et al., 2010). Hence enzyme immobilization emerges as a key enabling technology (Liese and Hilterhaus, 2013). Enzyme immobilization on various solid supports offers a number of advantages such as easy handling and a possible increase in the thermal stability and pH range supported by the protein. An additional benefit is greater stability under storage and operating conditions, for example, to heat denaturation, organic solvents or autolysis (Gardossi et al., 2010).

Recent interest in nanotechnology has provided a wide variety of materials that may potentially be used as supports for immobilization. Among the various nanostructures, carbon nanotubes (CNTs) have played a key role in the nanotechnology revolution. Due to the large specific surface area of CNTs, these carbon nanoparticles have been preconized as a good biomolecules carrier, being promising scaffolds for peptides, proteins and enzymes (Feng and Ji, 2011; Marchesan and Prato, 2015). Furthermore, carbon nanotubes exhibit extraordinary mechanical properties, electrical, thermal and chemical stability (Zhang and Henthorn, 2010); hence this nanoparticle has been applied in diverse fields such as materials, electronics and nanomedicine (Oliveira et al., 2015). One of the primary fields to benefit from nanoparticle-enzyme conjugates is biocatalysis, which is becoming one of the most powerful tools in biotechnology, having a profound impact on environmental protection (Illanes et al., 2012).

The main objective of this study is the production of fungal enzymatic extract, containing LiP, and its immobilization on carbon nanotubes. We also evaluated the LiP catalytic efficiency, stability and the capability of reuse in dye decolourization. The results obtained from this study can contribute to developing a biocatalyst that can be applied in diverse environmental applications in the future, especially in the treatment of textile effluents.

## 2. Materials and methods

### 2.1. Strain and chemicals

The fungi, *Pleurotus ostreatus* (PLO9) and *Ganoderma lucidum* (GRM117) were obtained from Mycorrhizal Associations Laboratory at Universidade Federal de Viçosa (Minas Gerais, Brazil). They were stored on potato dextrose agar (PDA) slants at 4 °C and multiplied, as needed, in the same medium.

All analyses were made with analytical reagent grade. Veratryl alcohol (VA) and Remazol brilliant blue R (RBBR) dye were purchased from Sigma–Aldrich (St. Louis, MO – USA) and the commercial lignin peroxidase (LiPc) was purchased from Santa Cruz Biotechnology, Inc (Dallas, TX – USA).

### 2.2. Enzymatic extract production: solid state fermentation (SSF)

Fungal cultures were multiplied in PDA plates at 25 °C for up to 7 days for inoculum production. Four mycelial disks, containing 1 cm agar-disk, were grown in substrate containing 20 g of *J. curcas L.* seed cake, previously humidified (70% of water) and autoclaved for one hour at 121 °C. Substrates were supplemented with 2 mL of Kirk's medium (Tien and Kirk, 1983) and 1 mL of exogenous inductor Tween 80 (0.3 mmol $l^{-1}$), and incubated at 25 °C (room temperature). After 7 days of inoculation, the crude enzymatic extract (CEE) was obtained by adding 100 mL sodium tartrate buffer (100 mmol $l^{-1}$, pH 3.5) supplemented with EDTA (5 mmol $l^{-1}$), then centrifuged at 4000×*g* for 30 min and filtrated (Whatman N°. 1 filter paper).

This procedure was done for SSF of both fungal isolates in triplicates, including the control (without inoculum) and the one flask inoculated but without exogenous inductor.

### 2.3. Partial purification of enzymatic extract

The crude enzymatic extract was centrifuged at 4000×*g* for 15 min. The supernatant was firstly brought to 40% saturation by the gradual addition of solid crystals of ammonium sulfate and kept overnight at 4 °C. The precipitate was collected by centrifugation (4000×g) for 15 min at 4 °C and to the supernatant more crystals of ammonium sulfate were added to achieve 80% saturation. It was again kept overnight at 4 °C and centrifuged as described previously. After centrifugation, the sediments were dissolved in sodium tartrate buffer (100 mM $l^{-1}$, pH 3.5) and dialyzed against the same buffer. The dialysate was applied to DEAE cellulose (Sigma–Aldrich, Brazil) ion-exchange column (3 × 14 cm) which had been equilibrated with sodium tartrate buffer (100 mmol $l^{-1}$, pH 5.5). The column was washed stepwise with 50, 100, 150, 200, 300 and 500 mmol $l^{-1}$ de sodium chloride solution (1 mol $l^{-1}$). The fractions were collected with a flow rate of 1 mL $min^{-1}$ and the LiP activity and protein contents were determined

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