PRODUCTION OF LIGNINOLYTIC ENZYMES BY DYE-DECOLORIZING MICROORGANISMS ISOLATED FROM A COMPOSTING ENVIRONMENT

Guisado G., López M.J., Vargas-García M.C., Suárez-Estrella F., Moreno J.
Unit of Microbiology. Department of Applied Biology. University of Almería. CITE-II-B. La Cañada de San Urbano s/n. 04120 Almería. Spain. Tel:+34 950015891. gmaria@ual.es

1 INTRODUCTION

Ligninolytic enzymes are a non-specific enzymatic system capable of degrading a broad range of chemical structures apart from lignin. Therefore, lignin-modifying microorganisms and enzymes can be applied, not only in the biotransformation of lignocellulose into value-added products, but also in the decontamination of pesticides, dyes and many other recalcitrant pollutants (Rodríguez Couto, 2009).

The most efficient lignin-modifying microorganisms are white rot fungi, which produce a ligninolytic system mainly comprised of laccases and peroxidases (lignin, manganese or versatile peroxidases). Laccases and peroxidases are assisted in the degradation of lignin moieties by auxiliary enzymes and mediators: low-molecular weight compounds that improve lignin biotransformation by readily diffusing into the lignocellulosic matrix and by providing high redox potentials that enhance the variety of substrates that laccases and peroxidases are able to degrade (Wesenberg *et al*., 2003). These enzyme-mediator systems have been considered a useful tool for the improvement of several biotechnological processes where ligninolytic enzymes are involved, such as biopulping and biobleaching techniques. Direct use of ligninolytic microorganisms for delignification often entails extended practices and application of crude ligninolytic enzymes for that purpose is frequently hindered by prompt inactivation (Hamid and Khalil-ur-Rehman, 2009). Unfortunately, synthetic mediators have also a restricted use owing to their high cost and potential toxicity (Da Re *et al*., 2008). A way to overcome these drawbacks could be the development of new screening and selection protocols, in order to achieve ligninolytic microorganisms and enzymes better adapted to operational conditions; hence an optimization of their industrial and environmental applications is desired (Camassola and Dillon, 2009).

The aim of the present study was to select microorganisms capable of producing ligninolytic enzymes (laccase, lignin peroxidase and/or manganese peroxidase) from a collection of environmental strains isolated from composting materials. A selection protocol based on the ability of ligninolytic microorganisms to decolorize synthetic dyes was developed and production of ligninolytic enzymes by selected strains was assessed under the presence of different ligninolytic inducers.

2 MATERIALS AND METHODS

2.1 Environmental strains

A collection of 20 environmental strains (19 fungi and 1 bacterium) was employed in this study. Strains were isolated from vegetal wastes at different stages of the composting process. In previous studies, these microorganisms had demonstrated *in vivo* decolorization of three industrial dyes: polymeric dyes Poly B-478 and Poly S-119, and anthraquinonic dye Remazol Brilliant Blue R (RBBR) (López *et al*., 2006).

All strains were stored at 4ºC on Nutrient Agar (Cultimed-Panreac, Barcelona, Spain) slants, except for thermophilic fungi, for which YMPG Agar was used (pH=4.5, in g/l: 10 glucose, 10 malt extract, 2 bacteriological peptone, 1 yeast extract, 1 $MgSO_4.7H_2O$, 1 L-asparagine, 20 agar).

2.2 Selection protocol based on dye decolorization

Mechanisms involved in *in vivo* decolorization of synthetic dyes were analysed. For that purpose, previously reported *in vivo* decolorization values were compared to those exclusively related to dye sorption on microbial biomass. Decolorization due to enzymes and/or metabolites was estimated as the difference between *in vivo* and biosorption decolorization values and the best dye-decolorizing microorganisms by means of this mechanism were selected for further study of their ligninolytic potential.

During biosorption experiment, production of microbial biomass was carried out in 250 ml flasks with 25 ml of Liquid Basal Medium (LBM) comprised of 10 g/l glucose, 5 g/l bacteriological peptone, 2 g/l yeast extract and 70 ml/l trace element solution (Kirk *et al*., 1986). Flasks were inoculated with a 1 cm^2 plug of a 4-day-old fungal culture on Nutrient Agar (Cultimed) or YMPG Agar (for thermophiles), or with 0.25 ml of an overnight bacterial culture on Nutrient Broth (Cultimed). After 7 days of incubation at 120 rpm and 30 or 40º C, for mesophiles and termophiles respectively, biomass was harvested by centrifugation (10,000 rpm, 4ºC, 5 min). Subsequently, recovered biomass was grinded (only for fungal biomass) to increase surface area, disposed in 250 ml Erlenmeyer flasks and autoclaved at 121º C during 20 minutes. Dead biomass from each strain was incubated during 48 hours in 25 ml of sterilized LBM amended with 200 mg/l of Poly R-478, Poly S-119 or RBBR, at the appropriate temperature and 120 rpm. Aliquots of 3 ml were taken from each flask at the beginning and end of the incubation period. These samples were centrifugated and the absorbance of supernatants was measured in a Shimadzu UV-160A spectrophotometer (Shimadzu Corporation, Kyoto, Japan) at the corresponding maximum wavelength (595 nm for RBBR, 473 nm for Poly S-119 and 520 nm for Poly R-478). Biosorption decolorization efficiency was expressed as the percentage of absorbance which was lost as a result of the treatment with dead biomass, considering initial absorbance as 100%. Triplicate flasks were used for each isolate and dye.

2.3 Production of ligninolytic enzymes

Production of laccase (Lac), lignin peroxidase (LiP) and manganese peroxidase (MnP) by selected microorganisms was assessed in LBM amended with the following ligninolytic inducers: 1 mM ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)), 2.5 mM veratryl alcohol, 2.16 mM ferulic acid, 3.38 mM guaiacol, 0.05% Tween 80 and 0.1% Kraft lignin. Lac, LiP and MnP activity values obtained in these media were compared to those achieved in LBM without supplementation of inducers. Triplicate flasks were employed for each isolate and inducer.

Selected mesophilic fungal strains were inoculated in 25 ml of each autoclaved medium (250 ml Erlenmeyer flasks) with a 1 cm^2 plug of the corresponding fungal culture. Flasks were incubated during 15 days at 30º C and 120 rpm. Aliquots (3 ml) of culture supernatants were centrifuged (10,000 rpm, 4º C, 5 min) prior to the quantification of ligninolytic enzymes. Lac and LiP were spectrofotometrically estimated (Shimadzu UV-160A spectrophotometer, Shimadzu Corporation) according to the methods described by Orth *et al*. (1993), whereas MnP was measured as stated by Camarero *et al*. (1999). Enzyme activities were expressed in units defined as 1 nmol of product formed per minute and ml.

2.4 Chemicals

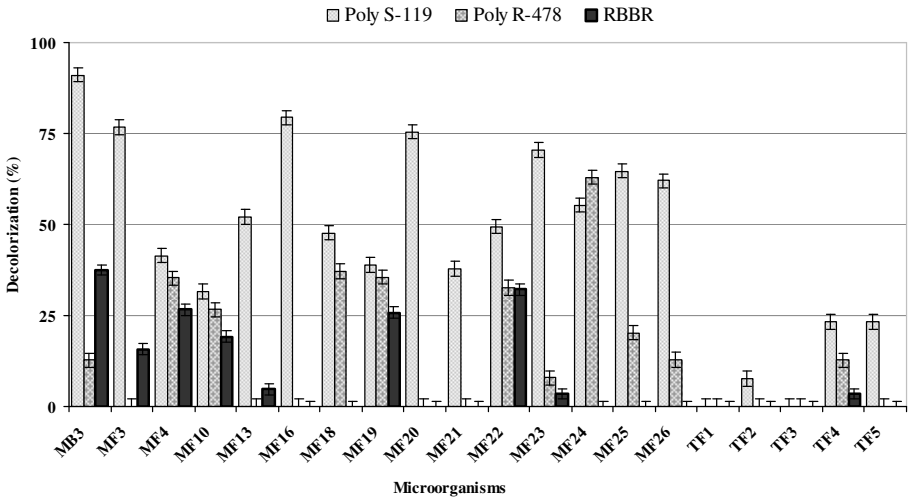
Dyes, inducers and substrates for enzymatic assays were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

3 RESULTS AND DISCUSSION

3.1 Selection of ligninolytic microorganisms

In vivo decolorization of synthetic dyes is often associated to ligninolytic potential (Wesenberg *et al*., 2003), although other mechanisms, such as biosorption on microbial biomass or production of biofloculants, can be involved (Fu and Viraraghavan, 2002; Deng *et al*., 2005). For that reason, *in vivo* decolorization values described by López *et al.* (2006) for the collection of environmental strains tested in this study were considered to be caused by several decolorization mechanisms, including the activity of ligninolytic enzymes. When biosorption efficiency of these microorganisms was assessed, decolorization values were significantly different depending on the dye, but did not exceed 70% in any case (data not shown). Quantification of the extent of decolorization by biosorption led to determine the contribution of this mechanism to *in vivo* decolorization values and hence, decolorization owing to the action of enzymes (presumably with ligninolytic activity) and/or metabolites could be estimated. High decolorization levels of Poly S-119, Poly R-478 and RBBR (from approximately 30 to 50%; Figure 1) were achieved using this latter mechanism by mesophilic fungal strains MF4 and MF22, which are similar to those obtained by D'Souza *et al.* (2006) (46 and 43% decolorization values for RBBR and Poly R-478, respectively) employing an *in vitro* treatment of dyes with a purified fungal laccase. Furthermore, mesophilic fungus MF24 was the best decolorizing strain for Poly R-478, showing 63% decolorization efficiency for that dye due to enzymes and/or metabolites (Figure 1). These isolates, which were preliminary identified as *Pseudallescheria angusta* MF4, *Penicillium chrysogenum* MF22 and *Penicillium* sp. MF24, were therefore considered to have certain ligninolytic potential. However, since production of ligninolytic enzymes can be particularly correlated with Poly R-478

decolorizing ability (Wesenberg *et al*., 2003; Kiiskinen *et al*., 2004; Dritsa *et al*., 2007), *P. angusta* MF4 and *Penicillium* sp. MF24 (the best Poly R-478 decolorizing strains amongst the above mentioned microorganisms) were finally selected to further evaluate their ability to produce Lac, LiP and/or MnP.



Error bars represent Fisher's Least Significant Difference (LSD) ($p<0.05$). **MB:** mesophilic bacterium; **MF:** mesophilic fungus; **TF:** thermophilic fungus. **Decolorization (%):** *In vivo* decolorization (%)-Biosorption decolorization (%)

FIGURE 1 **Decolorization (%) of industrial dyes due to enzymes and/or metabolites**

3.2 Effect of inducers on the production of ligninolytic enzymes by selected strains

Selected decolorizing fungi, *Pseudallescheria angusta* MF4 and *Penicillium* sp. MF24, demonstrated ligninolytic ability and produced Lac, LiP and/or MnP under the presence of several ligninolytic inducers. Lac production was barely stimulated under the assay conditions. Although lignin model compounds have been described as strong inducers of Lac production (Koroljova-Skorobogat´ko *et al*., 1998), Lac activity was only slightly increased by effect of Kraft lignin in cultures of *P. angusta* MF4 (Table 1). This dye decolorizing strain additionally showed enhanced LiP and MnP production when cultured in the presence of guaiacol (1944 U/ml) and ABTS (1383 U/ml), respectively. Phenolic and aromatic compounds as guaiacol, ABTS, veratryl alcohol or ferulic acid, have been widely employed to improve the production of ligninolytic enzymes by several fungal species (Farnet *et al*., 2004; Pazarlioğlu *et al*, 2005; Jaouani *et al*, 2006). In this study, a stimulating effect on LiP production by *Penicillium* sp. MF24 due to veratryl alcohol (2046 U/ml) and, especially, ferulic acid (3411 U/ml), was observed. Moreover, Tween 80 is known to facilitate the secretion of ligninolytic enzymes (Rodríguez Couto *et al*., 2004) and its effect also enhanced LiP production (2509 U/ml) by this selected strain (Table 1).

The results obtained suggest that selected decolorizing microorganisms could be useful, not only for the biodecontamination of dyes, but also for many biotechnological processes in which ligninolytic enzymes are involved.

TABLE 1 **Production of Lac, LiP and MnP by *Pseudallescheria angusta* MF4 and *Penicillium* sp. MF24**

	Enzyme activity (U/ml)					
	Lac		LiP		MnP	
Inducers	**MF4**	**MF24**	**MF4**	**MF24**	**MF4**	**MF24**
Without inducer	2.47 c	0.00 a	0.00 a	0.00 a	0.00 a	10.02 g
ABTS	2.06 c	0.00 a	0.00 a	2419.92 cd	1383.21 b	5.02 f
Ferulic acid	0.03 a	0.00 a	0.00 a	3410.61 e	0.36 a	2.37 d
Guaiacol	0.00 a	0.00 a	1943.68 b	1840.55 b	4.62 a	0.60 b
Veratryl alcohol	1.43 b	0.00 a	0.00 a	2046.31 bc	0.00 a	0.00 a
Tween 80	3.93 d	0.00 a	0.00 a	2509.28 d	0.00 a	3.55 e
Kraft lignin	13.13 e	0.00 a	0.00 a	1716.36 b	0.62 a	1.42 c

[1] Values in columns with same letters (a-g) are not significantly different at 95% confidence

4 CONCLUSIONS

Decolorization experiments developed in this study are based on the utilization of dyes structurally related to lignin and, according to results, constitute a useful tool for the selection of ligninolytic enzymes producers. Selected decolorizing microorganisms are stimulated in a different extent for ligninolytic enzymes production by phenolic substrates and alkaline Kraft lignin. These fungal strains have a great potential to be employed in several industrial bioprocesses, such as biopulping and biobleaching, as well as for different bioremediation techniques.

ACKNOWLEDGEMENTS

This research was funded through project AGL 2001-2815 from the Spanish "Ministerio de Ciencia y Tecnología"

REFERENCES

Camarero S, Sarkar S, Ruíz-Dueñas FJ, Martínez MJ, Martínez AT 1999. Description of a versatile peroxidase involved in the natural degradation of lignin that has both manganese peroxidase and lignin peroxidase substrate interaction sites. Journal of Biological Chemistry 274, 10324-10330.

Camassola M, Dillon AJP 2009. Biological pretreatment of sugar cane bagasse fot the production of cellulases and xylanases by *Penicillium echinulatum*. Industrial Crops and Products 29, 642-647.

D´Souza DT, Tiwari R, Sah AK, Raghukumar C 2006. Enhanced production of laccase by a marine fungus during treatment of colored effluents and synthetic dyes. Enzyme and Microbial Technology 38, 504-511.

Da Re V, Papinutti L, Villalba L, Forchiassin F, Levin L 2008. Preliminary studies on the biobleaching of loblolly pine Kraft pulp with *Trametes trogii* crude extracts. Enzyme and Microbial Technology 43, 164-168.

Deng S, Yu G, Ting YP 2005. Production of a bioflocculant by *Aspergillus parasiticus* and its application in dye removal. Colloids and Surfaces B: Biointerfaces 44, 179-186.

Dritsa V, Rigas F, Natsis K, Marchant R 2007. Characterization of a fungal strain isolated from a polyphenol polluted site. Bioresource Technology 98, 1741-1747.

Farnet A-M, Criquet S, Cigna M, Gil G, Ferré E 2004. Purification of a laccase from *Marasmius quercophilus* induced with ferulic acid: reactivity towards natural and xenobiotic aromatic compounds. Enzyme and Microbial Technology 34, 549-554.

Fu Y, Viraraghavan T 2002. Dye biosorption sites in *Aspergillus niger*. Bioresource Technology 82, 139-145.

Hamid M , Khalil-ur-Rehman 2009. Potential applications of peroxidases. Food Chemistry 115, 1177-1186.

Jaouani A, Tabka MG, Penninckx MJ 2006. Lignin modifying enzymes of *Coriolopsis polyzona* and their role in olive oil mill wastewaters decolourisation. Chemosphere 62, 1421-1430.

Kiiskinen L-L, Rättö M, Kruus K 2004. Screening for novel laccase-producing microbes. Journal of Applied Microbiology 97, 640-646.

Kirk TK, Croan S, Tien M, Murtagh KE, Farrell RL 1986. Production of a multiple ligninase by *Phanerochaete chrysosporium*: Effect of selected growth conditions and use of a mutant strain. Enzyme and Microbial Technology 8, 27-32.

Koroljova-Skorobogat´ko OV, Stepanova EV, Gavrilova VP, Morozova OV, Lubimova NV, Dzchafarova AN, Jaropolov AI, Makower A 1998. Purification and characterization of the constitutive form of laccase from the basidiomycete *Coriolus hirsutus* and effect of inducers on laccase synthesis. Biotechnology and Applied Biochemistry 28, 47-54.

López MJ, Guisado G, Vargas-García MC, Suárez-Estrella F, Moreno J 2006. Decolorization of industrial dyes by ligninolytic microorganisms isolated from composting environment. Enzyme and Microbial Technology 40, 42-45.

Orth AB, Royse DJ, Tien M 1993. Ubiquity of lignin-degrading peroxidases among various wood-degrading fungi. Applied and Environmental Microbiology 59, 4017-4023.

Pazarlioğlu NK, Sariişik M, Telefoncu A 2005. Laccase: production by *Trametes versicolor* and application to denim washing. Process Biochemistry 40, 1673-1678.

Rodríguez Couto S 2009. Dye removal by immobilised fungi. Biotechnology Advances 27, 227-235.

Rodríguez Couto S, Rosales E, Gundín M, Sanromán MA 2004. Exploitation of a waste from the brewing industry for laccase production by two *Trametes* species. Journal of Food Engineering 64, 423-428.

Wesenberg D, Kyriakides I, Agathos SN 2003. White-rot fungi and their enzymes for the treatment of industrial dye effluents. Biotechnology Advances 22, 161-187.