ELSEVIER

FEMS Microbiology Letters 212 (2002) 41–45

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Effect of olive oil mill wastewater on extracellular ligninolytic enzymes produced by *Phanerochaete flavido-alba*

Juan Carlos Ruiz, Teresa de la Rubia, Juana Pérez, José Martínez Lopez *

Departamento de Microbiología, Facultad de Farmacia, Campus de Cartuja, Universidad de Granada, 18071 Granada, Spain

Received 12 November 2001; received in revised form 18 April 2002; accepted 18 April 2002

First published online 22 May 2002

Abstract

Our previous results have demonstrated that *Phanerochaete flavido-alba* decoloration, dephenolization and detoxification of olive oil mill wastewater (OMW) were associated with changes in the ligninolytic major exoenzymes accumulated in the cultures. This paper describes the effect of the two main OMW components (monomeric aromatic compounds and a major brownish polymeric pigment), on extracellular *P. flavido-alba* ligninolytic enzymes. Laccase was the sole ligninolytic enzyme detected in cultures containing monomeric aromatic compounds. Laccase and an acidic manganese-dependent peroxidase (MnPA, p$I < 2.8$) were accumulated in cultures with OMW or polymeric pigment. Also, modified manganese-dependent peroxidases were observed mainly in OMW-supplemented cultures. Laccase was more stable to the effect of OMW toxic components and was accumulated in monomeric aromatic-supplemented cultures, suggesting a more important role than manganese-dependent peroxidases in OMW detoxification. Alternatively, MnPA accumulated in cultures containing the polymeric pigment seems to be more essential than laccase for degradation of this recalcitrant macromolecule by *P. flavido-alba*. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Olive oil mill wastewater; *Phanerochaete flavido-alba*; Lignin; Phenolic; Laccase; Manganese peroxidase

1. Introduction

Traditional biological methods used to treat industrial wastewaters cannot be applied to olive oil mill wastewater (OMW) (for a review see [1]). This effluent is variable in composition but is always antibacterial and phytotoxic, because of its phenolic content. These phenolics are present in the residue as a monomeric aromatics mix and as a polymerized heterogeneous pigment, which is responsible for the recalcitrant brownish color of OMW.

The white rot fungi ligninolytic system includes lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac). This enzymatic system is unspecific and is able to degrade a wide spectrum of aromatic pollutants causing environmental problems [2].

Among the ligninolytic systems those containing MnP and Lac are the most common [3]. Different compounds are described as inducers or mediators of these enzymes [4,5].

In a previous publication we reported that *Phanerochaete flavido-alba* is able to decolor OMW with a concomitant decrease in phenolic content and toxicity [6,7]. In defined culture media *P. flavido-alba* produces two types of manganese peroxidase, MnPA and MnPB [8], LiP [9] and Lac [10]. We have also described the induction of Lac and the accumulation of unusual acidic MnP isoforms in decolored OMW cultures [11].

In this paper the results of extracellular and mycelial-bound ligninolytic enzymes during the degradation of OMW by *P. flavido-alba* are reported. The selective accumulation of ligninolytic enzyme isoforms was associated with the major aromatic components of OMW, monomeric aromatic compounds and polymeric pigment.

2. Materials and methods

2.1. Microorganism and culture conditions

P. flavido-alba FPL 106507 was inoculated into 250-ml Erlenmeyer flasks containing 16 ml of nitrogen-limited culture medium with 40 ppm MnII, according to Ben Hamman et al. [7]. After incubation for 48 h at 30°C,

* Corresponding author. Tel.: +34 (58) 243873; Fax: +34 (58) 246235.
E-mail address: jmtnez@vgr.es (J. Martínez Lopez).

0378-1097/02/$22.00 © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.
PII: S0378-1097(02)00683-3

static cultures were flushed daily with 100% O_2 (3 l min^{-1} for 1 min).

Autoclaved solutions of OMW, polymeric pigment and monomeric aromatics (see below) were added to 5-day-old cultures (10 cultures per solution). Two sets of control cultures were prepared: *P. flavido-alba* cultures with no other addition and with the addition of the solvent used in the monomeric aromatic solution preparation. OMW and OMW major pigment were prepared as described elsewhere [7]. The qualitative phenolic composition of OMW, as described by Balice and Cera [12], was reproduced as in previous studies [13]. The mixture of monomeric aromatics (Sigma) was dissolved in *N*,*N*-dimethyl formamide–water (3:7 v/v), and added to the cultures to a final concentration of 0.83 M, as described elsewhere [14].

2.2. Enzymatic activity assays

The activity of both the extracellular and mycelial-bound enzymes was assayed. Extracellular Lac, MnP and LiP activities were determined as in Niku-Paavola et al. [15], Paszczynski et al. [16] and Tien and Kirk [17]. Extracellular enzyme activity was expressed in nmol min^{-1} substrate oxidized per ml. Mycelial-bound enzymatic activity was determined in broken-mycelium suspensions in 50 mM sodium malonate buffer (pH 4.5), obtained according to Périé and Gold [18]. The reaction mixtures were kept for 1 h at 30°C and activity was expressed as nmol of substrate oxidized per mg mycelium per hour. MnP was assayed as 2,6-dimethoxyphenol oxidation (0.2 mM in 0.2 mM manganese sulfate), according to Périé and Gold [18]. LiP and Lac substrates were the same as in the extracellular LiP and Lac assays.

2.3. Enzyme characterization

Extracellular enzymes in concentrated extracellular fluids were characterized by SDS–PAGE and isoelectric focusing (IEF). The mycelia were discharged after filtration and the extracellular fluids from each set of cultures were pooled. 60-ml aliquots of extracellular fluid were concentrated by ultrafiltration, semi-purified through an anion exchange column and further concentrated up to 1500-fold as described by Pérez et al. [11]. SDS–PAGE and IEF gels were revealed for protein, MnP and Lac activity as described elsewhere [11].

3. Results and discussion

3.1. Enzymatic activities

Extracellular (in culture fluids) and mycelial-bound enzymatic activities were determined. After the addition of the OMW, monomeric aromatics or polymeric pigment substrates (Fig. 1), the time course of both MnP and Lac activity changed in a substrate-dependent manner. After the addition of either OMW or polymeric pigment, MnP activity was lower than in the controls cultured without substrate (Fig. 1A). The solvent used in the monomeric aromatics solution was too toxic: MnP activity was not detected either in the cultures with aromatics added or in the cultures with the solvent used in the aromatic solution preparation. The accumulation of Lac activity (Fig. 1B) increased after the addition of either substrate. No extracellular LiP activity was detected.

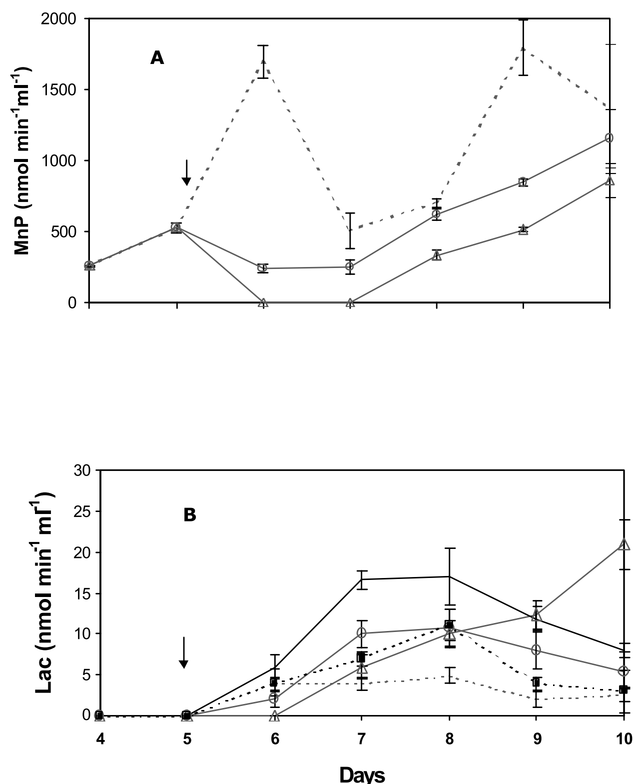

Fig. 1. Extracellular enzymatic activities of *P. flavido-alba* cultures. Control cultures (dashed line), cultures with OMW (△), cultures with polymeric pigment (○), cultures with monomeric aromatic compounds (solid line), cultures with *N*,*N*-dimethyl formamide–water) (■). Arrows: day of substrate addition. Values are means of triplicate cultures.

Very low activity was detected for mycelial-bound LiP (lower than 2.0 nmol mg^{-1} h^{-1}), MnP (lower than 2.5 nmol mg^{-1} h^{-1}) and Lac (lower than 3.0 nmol mg^{-1} h^{-1}). Two days after the addition of monomeric aromatics or polymeric pigment into the cultures (when maximum decoloration occurs) [7] we observed different effects on mycelial-bound Lac and LiP: monomeric aromatics enhanced the mycelial-bound activities of the three enzymes but pigment increased MnP activity alone. The different effect of monomeric aromatics and polymeric pigment on mycelial-bound enzymatic activities suggests a differential effect of both OMW components on fungal metabolism in the hyphal vicinity. The low enzyme activities hindered our efforts to characterize these changes.

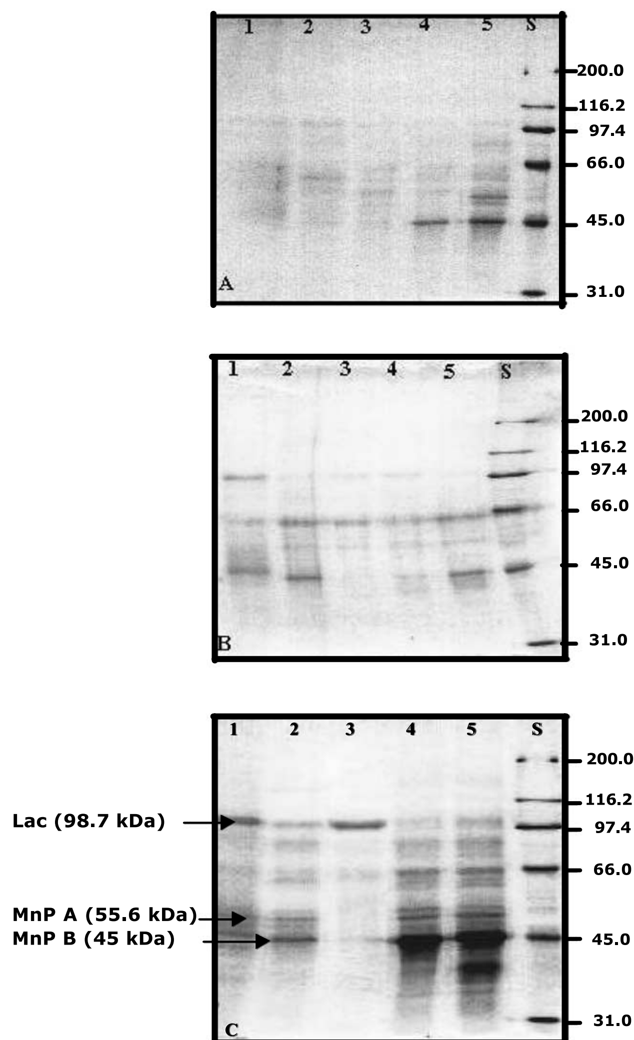

Fig. 2. SDS–PAGE of extracellular fluids from *P. flavido-alba* cultures semi-purified by anion exchange chromatography. Cultures immediately after the addition of substrates on day 5 (A), 48 h (B) and 120 h (C) after substrate addition. Cultures with OMW (lane 1), polymeric pigment (lane 2), aromatic compounds mix (lane 3), *N*,*N*-dimethyl formamide–water (lane 4). Lane 5: control cultures. Lane S: standards.

3.2. Enzymatic characterization

SDS–PAGE and IEF gels revealed that extracellular enzyme loss occurred on the addition of the substrates (Figs. 2 and 3). This enzyme loss is probably due to the precipitation of the proteins because no other additional bands were detected in the SDS–PAGE gels and there was a decrease in the protein content. Fig. 2A shows the protein profiles of the two sets of controls on day 5 (lanes 4 and 5). Immediately after the addition of OMW, pigment or monomer aromatic compounds, very weak and diffuse bands could be seen in the molecular mass ranges of the MnPs (Fig. 2A, lanes 1–3), at about 55 kDa for MnPA and 44 kDa for MnPB. Thus, monomeric aromatics mix and polymeric pigment, the major components of OMW, modified the extracellular protein profile in *P. flavido-alba* cultures in a similar way to whole OMW.

In the concentrated extracellular fluids from cultures with OMW, *P. flavido-alba* MnPs had different p*I*s from those from OMW-free cultures: after IEF an unresolved smear of MnP (referred to as MnPI) with an intermediate p*I* was detected (Fig. 3-I, panel A, lanes 1 and 2). Similar modifications in MnP enzymes were observed in the cultures with polymeric pigment (Fig. 3-I, panel A, lane 3).

Lac activity was detected in IEF gels, in that cultures with the substrates (Fig. 3). No apparent modifications were observed in the Lac band in SDS–PAGE gels immediately after the addition of substrates (Fig. 2A, lanes 1–3). This result suggests that Lac is more stable than MnP against the inactivating effects of the three substrates.

In summary, OMW inactivated both the extracellular MnPs and Lac produced by *P. flavido-alba* and there was a decrease in the protein content in the extracellular fluid. IEF and SDS–PAGE showed that MnP seems to be more sensitive than Lac to the inactivating effect of OMW. The inactivation of these enzymes by OMW can be attributed to its aromatic content, both as a polymeric pigment and as monomeric aromatics.

We have described the antibacterial effect of OMW phenolics in a previous publication [19] and furthermore Cowan [20] has reported that the antimicrobial capacity of phenolics and polyphenolics derives from their capacity to inactivate extracellular enzymes. Thus we might hypothesize that the antimicrobial effect of OMW could also be due to such enzyme inactivation.

To demonstrate that *P. flavido-alba* MnPIs are modified derivatives of typical MnPs we performed an experiment in which OMW was added to semi-purified MnP solutions [11]. Fig. 4 shows that OMW modified the extracellular proteins in MnP solutions, yielding MnPs with altered p*I*s.

Once OMW and pigment decoloration reached about 60% (after 48 h incubation) MnPI and MnPA were detected by IEF analysis in cultures with either OMW or polymeric pigment (Fig. 3-II, panel A). After SDS–PAGE of cultures with OMW the MnPB protein band was not observed, but the band of MnPA and a band showing an intermediate molecular mass (45–55 kDa) were detected (Fig. 2B). In the cultures with monomeric aromatics MnP bands were not be seen, either in IEF or in SDS–PAGE gels. However, Lac activity was detected in IEF gels of samples from control and aromatic-supplemented cultures. This result suggests that in *P. flavido-alba* cultures, Lac (being more stable than MnP) participates more actively in the removal of phenolics from OMW than MnP does.

At the end of the experiment MnP activity in the cultures with polymeric pigment was mainly due to MnPA (Fig. 3-III, panel A, lane 3). In those with OMW, MnP activity was the result of MnPA, MnPB and MnPI (Fig. 3-III, panel A, lane 1).

In cultures with monomeric aromatics only one band

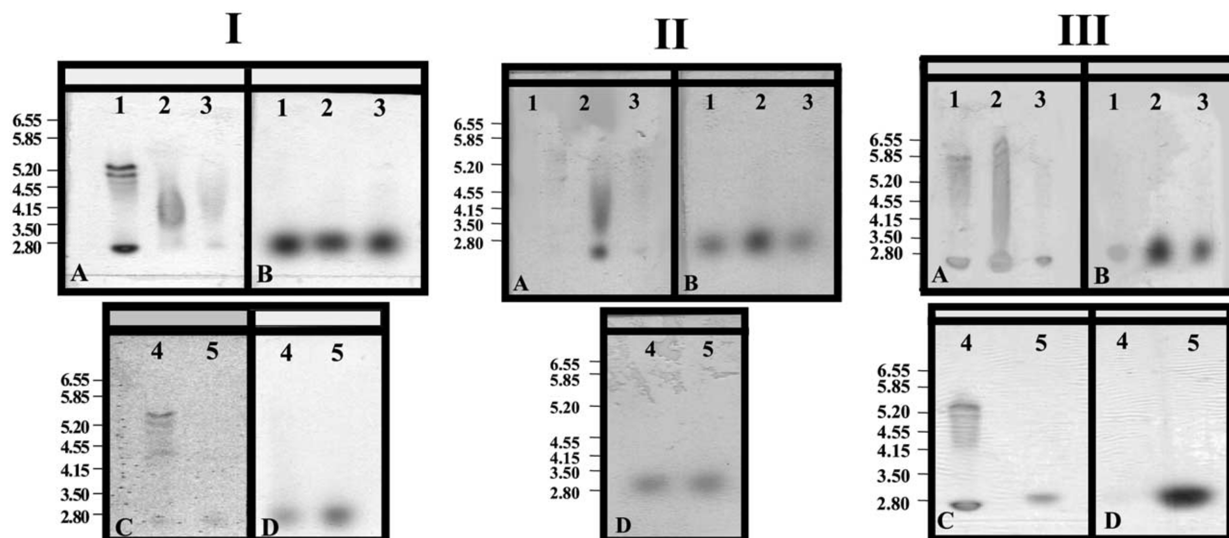

Fig. 3. IEF of anion-exchange semi-purified extracellular *P. flavido-alba* culture fluids. Cultures immediately after the addition of substrates (I), and 48 h (II) and 120 h (III) after substrate addition. Gels A and C: MnP activity stain; gels B and D: Lac activity stain. Lanes 1: Control cultures; lanes 2: cultures with OMW; lanes 3: cultures with polymeric pigment, lanes 4: cultures with *N,N*-dimethyl formamide–water; lanes 5: cultures with aromatic compounds mix. No MnP bands were detected in aromatics mix added cultures on 48 h.

was detected after IEF. This band showed the electrophoretic mobility of the purified Lac, which was also able to oxidize 4-chloro-1-naphthol, the reagent used in MnP zymograms (Fig. 3-III, panels C and D, lanes 5). As can be observed, the Lac p*I* was slightly higher than the MnPA p*I*, although both of them were unusually low. Lac was the only ligninolytic enzyme detected in SDS–PAGE gels and zymograms on IEF gels in cultures with monomeric aromatics (Figs. 2C and 3-II, panel D and III, panel D). From these results we may conclude that Lac is the main ligninolytic enzyme responsible for the degradation of OMW phenolics by *P. flavido-alba*. In fact, D'Annibale et al. [21] propose *Lentinula edodes* Lac as being responsible for OMW dephenolization.

Lac activity was detected in all samples throughout the experiments (Figs. 2 and 3) and was more stable than MnP against the inactivating effects of OMW components. Furthermore, both Lac activity and intensity, as detected by zymograms, confirm our previous observation concerning the induction of Lac by OMW components [11]. The production of MnP by *P. flavido-alba* seems to be affected mainly by monomeric aromatics (Fig. 2C).

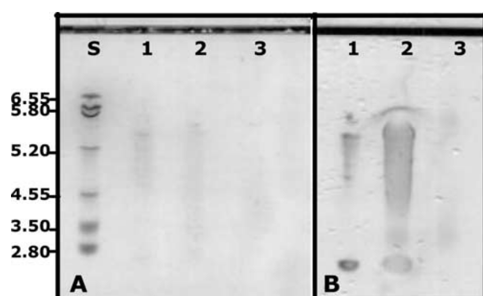

Fig. 4. IEF of concentrated OMW (lane 3), concentrated semi-purified MnP solutions from control cultures (lane 1), and concentrated semi-purified MnP solutions from control cultures with OMW (lane 2) and OMW (lane 3). Gel A: protein stain. Gel B: MnP activity stain.

In summary, during *P. flavido-alba* decoloration, dephenolization and detoxification, Lac plays an important role in monomeric aromatic degradation, whilst MnP does not. A selective accumulation of MnP (MnPA) is involved in the polymeric pigment degradation along with Lac. The MnPI observed in cultures to which OMW is added seems to represent a modified derivative of the extracellular MnPs accumulated in *P. flavido-alba* cultures. The accumulation of Lac and an acidic MnPA in *P. flavido-alba* cultures occurred upon the addition of either whole OMW or its main components into the cultures.

The MnPA N-terminal amino acid sequence is typical of white rot fungal MnPs, but the Lac N-terminal amino acid sequence does not appear to be homologous with that other fungal Lacs (unpublished data). *P. flavido-alba* Lac has a higher molecular mass than other white rot fungal Lacs [6]. MnPA has an unusually high molecular mass (about 55.5 kDa) and a very low p*I* (<2.8), which is only comparable to *Ceriporiopsis subvermispora* MnP [22]. The involvement of the Lac and MnP produced by *P. flavido-alba* in the degradation of OMW has prompted us to continue our research with this fungus in an attempt to characterize the proteins.

Acknowledgements

We are grateful to the Spanish CICYT for its financial support (Projects Bio 96-0393 and 1FD97-1071). We thank our colleague Dr. J. Trout for revising our English text.

References

[1] Martínez, J., Pérez, J. and de la Rubia, T. (1998) Olive oil mill waste water degradation by ligninolytic fungi. In: Recent Research Developments in Microbiology (Pandalai, S.G., Ed.), pp 373–403. Research Signpost, Trivabadrum.

[2] Barr, D.P. and Aust, S.D. (1994) Mechanism white rot fungi use to degrade pollutants. Environ. Sci. Technol. 28, A78–A87.

[3] Thuor, U., Winterhalter, J. and Fietcher, A. (1995) Enzymes of white rot fungi involved in lignin degradation and ecological determinants of wood decay. J. Biotechnol. 41, 1–17.

[4] Gold, M.H. and Alic, M. (1993) Molecular biology of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Microbiol. Rev. 57, 605–622.

[5] Gianfreda, L., Xu, F. and Bollag, J.M. (1999) Laccases: A useful group of oxidoreductive enzymes. Bioremed. J. 3, 1–25.

[6] Ben Hamman, O. (1998) Peroxidasas ligninolíticas de *Phanerochaete flavido-alba* y aplicación de este hongo al tratamiento del alpechín. Ph.D. Thesis, University of Granada.

[7] Ben Hamman, O., de la Rubia, T. and Martínez, J. (1999) Decolourisation of olive oil mill wastewaters by *Phanerochaete flavido-alba*. Environ. Toxicol. Chem. 18, 2410–2415.

[8] de la Rubia, T., Linares, A., Pérez, J., Romera, J. and Martínez, J. (1999) Purificación y caracterización de una manganeso peroxidasa de *Phanerochaete flavido-alba*. Proceedings 17th Congreso Nacional de Microbiología, Sociedad Española de Microbiología, Granada, p. 566.

[9] Ben Hamman, O., de la Rubia, T. and Martínez, J. (1997) Effect of carbon and nitrogen limitation on lignin peroxidase and manganese peroxidase production by *Phanerochaete flavido-alba*. J. Appl. Microbiol. 83, 751–757.

[10] Pérez, J., Martínez, J. and de la Rubia, T. (1996) Purification and partial characterization of a laccase from the white rot fungus *Phanerochaete flavido-alba*. Appl. Environ. Microbiol. 62, 4263–4267.

[11] Pérez, J., de la Rubia, T., Ben Hamman, O. and Martínez, J. (1998) *Phanerochaete flavido-alba* laccase induction and modification of manganese peroxidase isoenzyme pattern in decolorized olive oil mill waste waters. Appl. Environ. Microbiol. 64, 2726–2729.

[12] Balice, V. and Cera, O. (1984) Acidic phenolic fraction of the olive vegetation water determinated by a gas chromatographic method. Grasas Aceites 35, 178–180.

[13] Pérez, J., Ramos-Cormenzana, A. and Martínez, J. (1990) Bacteria degrading phenolic acids isolated on a polymeric phenolic pigment. J. Appl. Bacteriol. 68, 38–42.

[14] Madrid, F., de la Rubia, T. and Martínez, J. (1995) Effect of *Phanerochaete flavido-alba* on aromatic acids present in olive oil mill waste waters. Toxicol. Environ. Chem. 51, 161–168.

[15] Niku-Paavola, M.L., Karhunen, E., Salola, P. and Raunio, V. (1988) Ligninolytic enzymes of the white-rot fungus *Phlebia radiata*. Biochem. J. 254, 877–884.

[16] Paszczynski, A., Huynh, V.B. and Crawford, R. (1985) Enzymatic activities of an extracellular, manganese-dependent peroxidase from *Phanerochaete chrysosporium*. FEMS Microbiol. Lett. 29, 37–41.

[17] Tien, M. and Kirk, T.K. (1983) Lignin-degrading enzyme from the hymenomycete *Phanerochaete chrysosporium* Burds. Science 221, 661–663.

[18] Périé, F.H. and Gold, M.H. (1991) Manganese regulation of manganese peroxidase expression and lignin degradation by the white rot fungus *Dichomitus squalens*. Appl. Environ. Microbiol. 57, 2240–2245.

[19] Rodríguez, M.M., Pérez, J., Ramos-Cormenzana, A. and Martínez, J. (1988) Effect of extracts obtained from olive oil mill waste waters on *Bacillus megaterium* ATCC 33085. J. Appl. Bacteriol. 64, 219–226.

[20] Cowan, M.M. (1999) Plant products as antimicrobial agents. Clin. Microbiol. Rev. 12, 564–582.

[21] D'Annibale, A., Stazi, S.R., Vinciguerra, V., Dimattia, E. and Sermanni, G.G. (1999) Characterization of immobilized laccase from *Lentinula edodes* and its use in olive-mill waste-water treatment. Proces. Biochem. 34, 697–706.

[22] Lobos, S., Larrain, J., Salas, L., Cullen, D. and Vicuña, R. (1994) Isoenzymes of manganese-dependent peroxidase and laccase produced by the lignin-degrading basidiomycete *Ceriporiopsis subvermispora*. Microbiology 140, 2691–2698.