©Copyright 1991 by The Humana Press Inc.
All rights of any nature whatsoever reserved.
0273-2289/91/3103—0223$02.60

Olive Milling Wastewater as a Medium for Growth of Four *Pleurotus* Species

ENRICO SANJUST,[1] RAFFAELLO POMPEI,[2] ANTONIO RESCIGNO,[1] AUGUSTO RINALDI,*[,1] AND MAURO BALLERO[3]

Istituto di [1]Chimica Biologica; [2]Microbiologia; and [3]Botanica, Universita' di Cagliari, Via della Pineta, 77 09125 Italy

Received April 22, 1991; Accepted June 4, 1991

ABSTRACT

Four species of *Pleurotus* were adapted to grow on olive milling wastewater, and in certain conditions produced high yield of fruit bodies. Some biochemical transformations were observed in the olive milling wastewater owing to the growth of *Pleurotus*. In particular, the fungi actively excreted large amounts of laccase in the medium, and at the same time the concentration of phenolics and other toxic compounds significantly decreased, as revealed by HPLC analysis and toxicity tests on standard cultures of human cell lines.

Index Entries: *Pleurotus;* olive milling wastewater; laccase; phenolics; detoxification.

INTRODUCTION

Olive milling wastewater (OMW) is an important byproduct of olive oil technology, and represents up to 50% by volume of milled olives (*1*). The disposal of this waste is strongly hindered by the high content of organic matter (*2*): mainly unextracted oil, pectic substances, xylans, and similar polysaccharides, mono- and oligosaccharides. OMW also contains relatively high concentrations of potassium, magnesium, and phosphate

*Author to whom all correspondence and reprint requests should be addressed.

salts, but is rather poor in nitrogen compounds. With regard to the organic components, phenolics and related substances are present in notable concentrations, and are the major cause of the very slow and incomplete biodegradation of this waste (*1*).

Among the various classes of phenolics contained in OMW, the most typical are those derived from phenylalanine via the cinnamic acid pathway: *p*-coumaric, caffeic, ferulic, and sinapic acid, with their glycosides; *p*-hydroxybenzoic, protocatechuic, vanillic, gallic, and syringic acid. Other phenolics derive from 1,3-dihydroxybenzene or 1,3,5-trihydroxybenzene and originate metabolically from acetate units. Flavones and their derivatives are also present, as well as anthocyanins, cathechins, and tannic substances. Other phenolics, present in relatively high amounts are tyrosol (β-(4-hydroxyphenyl)-ethanol) and hydroxytyrosol (β-(3,4-dihydroxyphenyl)-ethanol). Compounds related to coumarin are also present. In conclusion, several types of phenolic substances can be detected in OMW (*3–8*).

Both green and black olives contain phenoloxidase (tirosinase) (*9*), and on milling many phenolics are then oxidized to quinones (via semiquinones); the most reactive quinones can polymerize on standing and may attack proteins and polysaccharides leading to crosslinked polymers, which are rather complex in their molecular structure and very difficult for microorganisms to degrade.

MATERIALS

All products utilized were of the best grade available, and were used without further purification. *Pleurotus ostreatus* (Jacq.: Fr.) Kummer, *Pl.* sp. *florida*, *Pl. sajor-caju* (Fr.) Singer, and *Pl. eryngii* (DC.: Fr.) Quél. came from the collection of the Istituto di Botanica, Universita' di Cagliari. HEp2 cells used for toxicity experiments were from Flow (UK).

METHODS

Instrumentation

Laccase activity, phenolics concentration, and clarification were measured with a Cary 219 spectrophotometer (Varian).

HPLC experiments were performed using a System Gold chromatography apparatus (Beckman) equipped with a C-18-RP column, 4.6×200 mm, and interfaced with a PS2/50 personal computer (IBM).

Culture Conditions

To study *Pleurotus* behavior on OMW in fully aerobic conditions, expanded perlite was wetted with OMW to obtain a OMW/Perlite ratio

of 2.40, the mixture was placed in loosely capped polystyrene boxes (35×15×15 cm internal dimensions, 980 g mixture per box) and inoculated with the mycelia, grown on millet seeds (38 g, 5.5% by weight with respect to OMW). Within a few days, luxuriant mycelial mats were evident on the surface of the mixture, and after 15 d the boxes were opened; fruit differentiation and growth quickly took place and continued for some weeks; fruit bodies were harvested when fully developed. In these experiments, small amounts of distilled water were added to the mixture when it became too dry (usually one week after the beginning of fruiting).

For cultures in stationary conditions, OMW was sterilized by autoclaving for 15′ at 121°C. Fifty mL of OMW were poured into sterile 100-mL Erlenmeyer glass flasks. The fungi were previously adapted to grow on agar-solidified OMW in 9-cm Petri dishes, before collecting mycelia to inoculate the flasks. Cultures were kept in stationary conditions at room temperature (22–27°C) for the scheduled times.

Toxicity of OMW

The toxicity of OMW used as a culture medium for the four species of *Pleurotus* or of untreated OMW, has been studied on in vitro monolayers of HEp2 cells (Flow), grown in EMEM with 2% bovine serum. HEp2 cells (10^5) were seeded in 35-mm culture Petri dishes. After 24 h, dilutions of either untreated or *Pleurotus*-treated OMW were added to each dish (samples were previously centrifuged and sterilized by filtration, and all the experiments were performed in duplicate). After a further 48 h of incubation, the cells were detached from the dishes by a trypsin solution (0.25% w/v in the standard buffer) and counted in a Burker glass chamber at 400X magnification.

The OMW toxicity was checked after 25 d of culture of *Pleurotus sp.*, grown at room temperature (22–27°C) and in stationary conditions.

Clarification of OMW

Clarification of OMW by the four *Pleurotus* species was detected after 25 d of growth. Erlenmeyer glass flasks (100 mL) were filled with 50 mL of autoclave-sterilized OMW. Mycelia grown on plates containing agar-solidified OMW were collected by sterile cotton swabs and dispersed into the OMW. To facilitate a rapid growth of mycelia, either sucrose (0.5% w/v) or lactose (0.5% w/v) was added to the OMW.

The cultures were left at room temperature (22–27°C) in stationary conditions, and after the indicated time, OMW was centrifuged and the supernatant was read for absorbance at 400 nm.

Analytical Procedures

For the determination of protein and phenolics, fruit bodies were lyophilyzed and ground to a fine powder in a mortar.

For protein determination, this powder was extracted with 20 m*M* Tris/HCl buffer, pH 7.2, for 6 h; the fluid suspension was then centrifuged and filtered and the protein concentration in the clear filtrate was determined by the Bradford method (*10*).

For phenolics estimation, the lyophilized powder was extracted for 6 h with a 1:1 mixture of acetonitrile and methanol; the fluid suspension was then centrifuged and filtered and the phenolics concentration in the clear filtrate was determined by means of the Folin method (*11*). Suitable dilutions of vanillic acid served as the standard phenol.

The same procedure was followed for the determination of phenolics in OMW; in this case, lyophylized OMW was of course the starting material.

For HPLC analysis, the methanol/acetonotrile extracts were passed through C-18 reverse-phase filters to remove pigments before loading on to the HPLC column.

An increasing water/methanol linear gradient (methanol concentration rising from 0 to 80% in 12 min) was used, and the column was washed with water/methanol 20/80 for another 17 min before reconditioning it for further analysis. The detector was set for reading absorbances at 280 nm.

For laccase activity measurements, OMW samples were first treated with insoluble polyvinylpyrrolidone, then the mixture was filtered and diluted with 0.1*M* potassium phosphate buffer, pH 6.0. The activity measurements were performed following a photometric method (*12*), using syringaldazine as the chromogenic substrate (*13*).

RESULTS AND DISCUSSION

One important result obtained from this work was that, by adapting some species of *Pleurotus* for fast growth on OMW alone, the latter being simply sterilized by autoclaving, the resulting mycelia were morphologically indistinguishable from those grown on conventional substrates.

Pl. ostreatus and in particular *Pl. floridae* excreted into the medium large amounts of soluble laccase in both the chosen culture conditions; *Pl. eryngii* and *P. sajor caju* produced extracellular laccase only in fully aerobic conditions (*see* Tables 1 and 2). It is interesting to note that laccase production in stationary cultures followed a curve, characterized by a lag time of about 10 or 15 d and with a maximum at about 25 d of growth; after this time enzyme production slowly decreased.

In contrast with these findings, mycelia grown on perlite (fully aerobic conditions) all excreted laccase, even if *Pl. eryngii* was the slowest in growth and the worst laccase producer. This fact concurs with the similar behavior of this fungus when grown on conventional media.

The copper-containing, blue protein laccase has attracted much interest since it was discovered in lacquer trees by Yoshida (*14*). This enzyme,

Table 1
Laccase Production by *Pleurotus* spp. Growing on OMW in Stationary Conditions[a]

	Time, d						
Species	0	5	10	15	20	25	30
Pl. ostreatus	0	0	0	1	5	19	15
Pl. eryngii	0	0	0	0	traces	traces	traces
Pl. sajor-caju	0	0	0	0	traces	traces	traces
Pl. floridae	0	0	0	2	27	64	42

[a]Enzymic activity is given as U/mL.

Table 2
Laccase Production by *Pleurotus* spp. Growing on OMW/Perlite Mixture[a]

	Time, d					
Species	0	5	10	15	20	25
Pl. ostreatus	0	1	9	35	31	27
Pl. eryngii	0	traces	1	5	16	14
Pl. sajor-caju	0	traces	2	7	21	19
Pl. floridae	0	traces	4	8	20	14

[a]Enzymic activity is given as U/mL.

found later in many ligninolytic fungi as an extracellular enzyme, is defined as being a *p*-diphenol:oxygen oxidoreductase (EC 1.10.3.2), oxidizing a wide range of polyphenols and aromatic amines, with concomitant reduction of molecular oxygen to water. The enzyme is present in white rot fungi and absent from the soft rot ones (*15–17*). The ability of the white rot fungi to degrade lignins appears to be strictly related to the excretion of a soluble laccase in the growth medium, even if laccase alone cannot depolymerize lignins. Nevertheless, it should be considered as being a very important digestive enzyme for lignin-degrading organisms, perhaps further oxidizing small fragments released by the action of ligninases (*18*). Fungal laccases are very often inducible enzymes, and this is also the case for the *Pleurotus* laccase. Several compounds were found to be laccase inducers in white rot fungi; they are usually phenolics or aromatic amines, and are also commonly substrates for laccase. Much work has been done about laccase induction in some white rot fungi by ferulic acid (*19–22*), and by other compounds metabolically related to lignin (*23*). In fact, in the presence of an inducer, many white rot fungi excrete large amounts of laccase into the medium.

Pleurotus laccase has been purified and characterized (*24*); it is very similar to other fungal laccases in its spectral and kinetic properties.

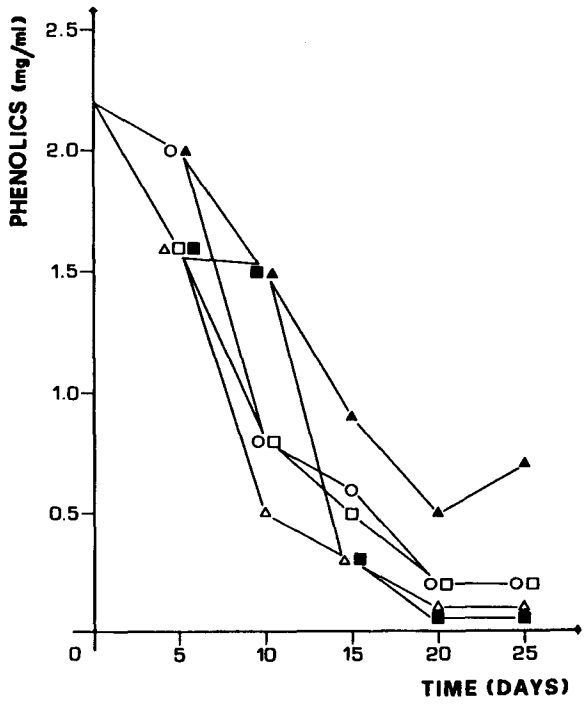


Fig. 1. Phenolics concentration pattern vs time for the four strains of *Pleurotus* growing on OMW in stationary conditions. Values are expressed as mg phenolics/mL OMW; the standard phenol was vanillic acid. *Pl. ostreatus* (△); *Pl. eryngii* (○); *Pl. sajor-caju* (□); *Pl. floridae* (■); Autoclaved OMW (▲).

Figure 1 shows the phenolics concentrations vs time for the four strains grown in stationary conditions; Figure 2 shows the HPLC chromatograms of phenolics during the growth of *Pleurotus floridae,* the best phenol metabolizing species among those tested.

It is interesting to note that the best laccase producers degraded phenolics more quickly and more completely than the worst ones. Since at least one phenolic substance in OMW must be a strong laccase inducer, the lag time in enzyme production could be owing to the high content of readily metabolizable substances in the medium, namely soluble sugars and partly hydrolyzed glycerides; another possibility could be that the high content of quinones (already present as the product of phenoloxidase activity in olives or generated by laccase action) inhibit the enzyme itself at the early stages of excretion. In any case, all the strains tested destroyed at least 90% of the Folin-detectable phenolics within 25 d; *Pl. ostreatus* and *Pl. floridae* were even more efficient in their action. A similar behavior pattern was confirmed for the perlite-grown strains, as shown in Fig. 3.

Figure 4 reports the toxicity of OMW used as a culture medium for *Pleurotus spp.* and of untreated OMW. Data are referred as percentages of the control.

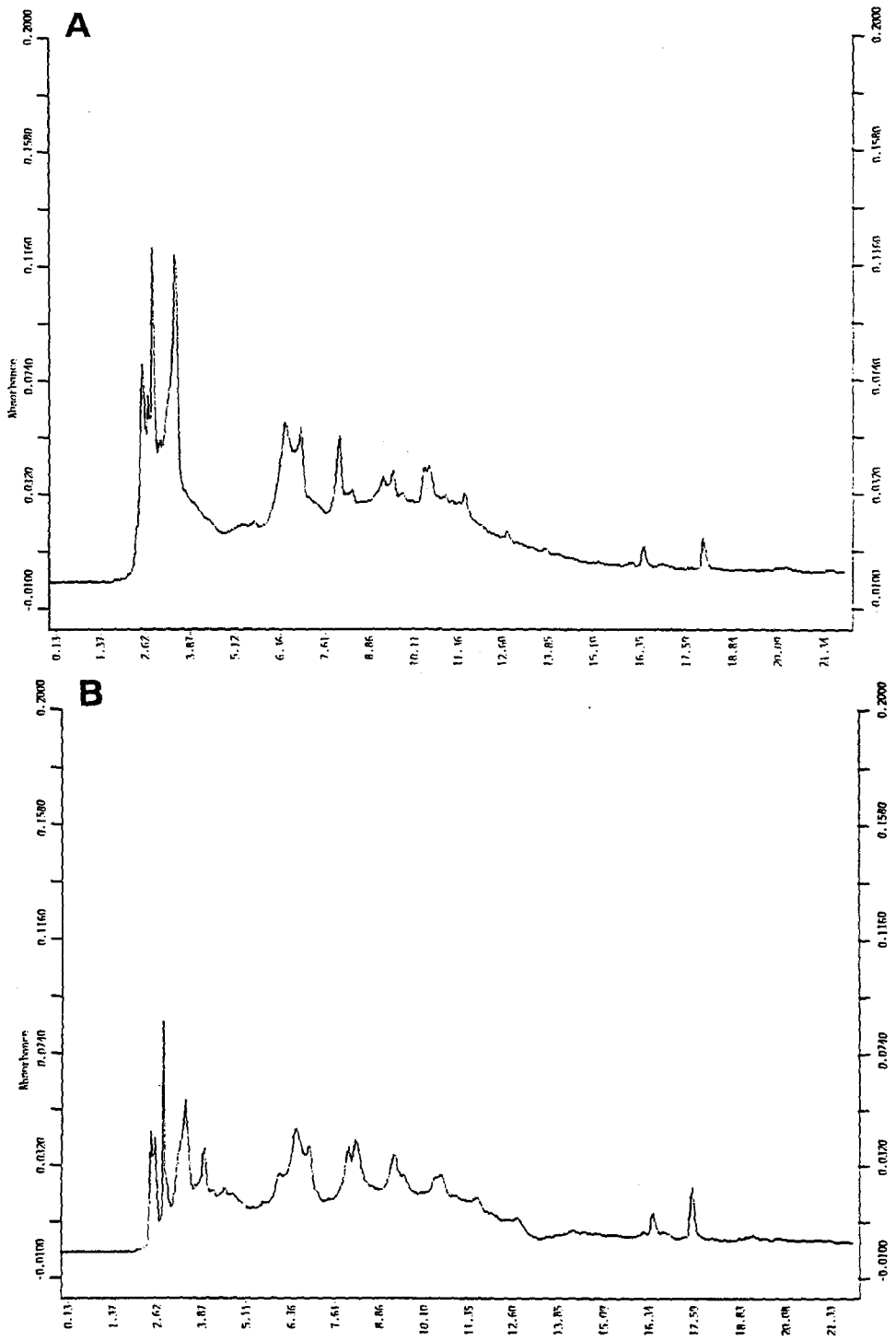
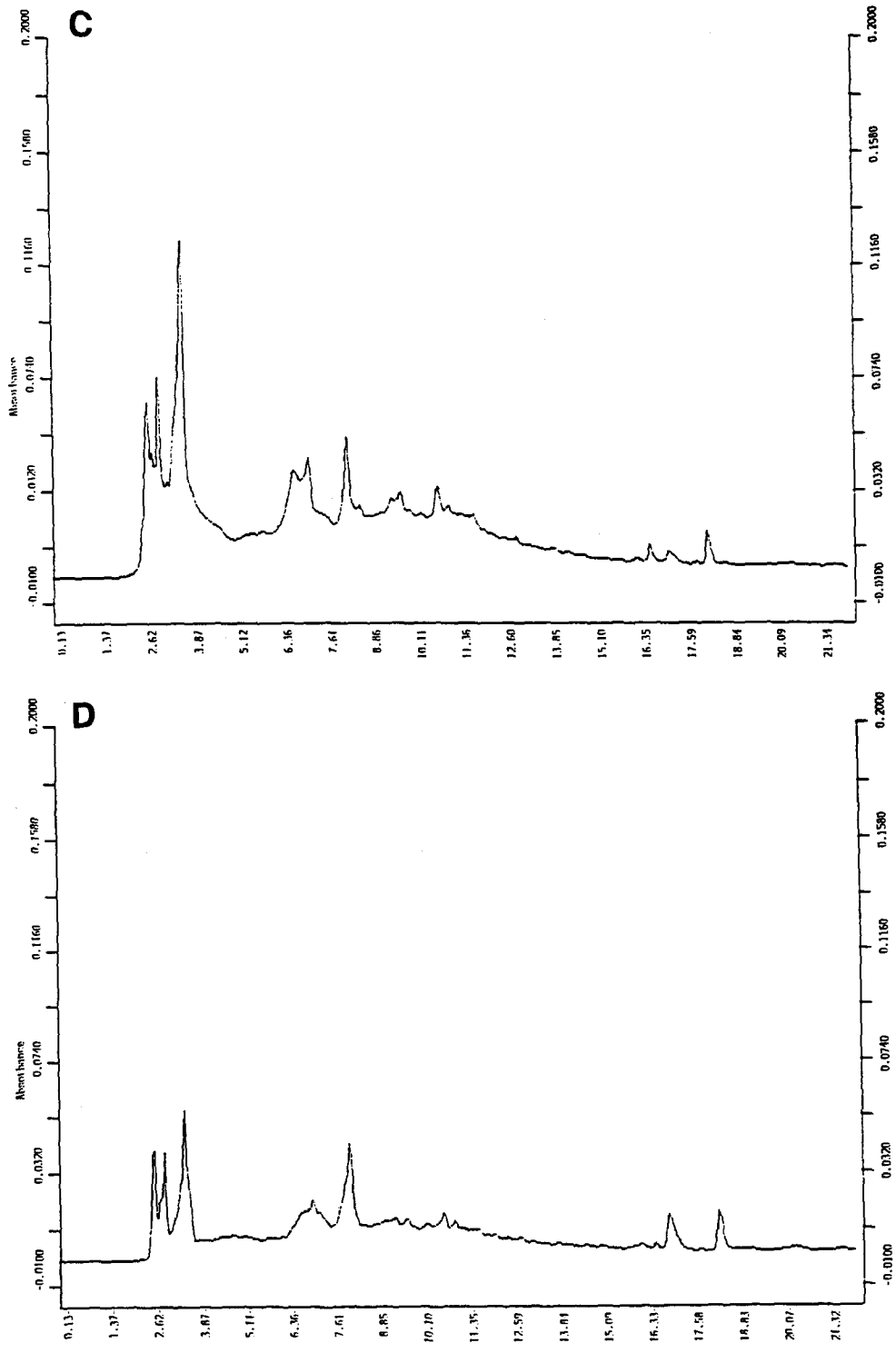


Fig. 2. HPLC chromatograms of OMW phenolics during the cultivation of *Pleurotus floridae* at different stages of growth. The conditions of analysis are given in the text, and the chromatographic patterns are expressed as absorbance at 280 nm vs time (min). (**a**) Autoclaved OMW; (**b**) autoclaved OMW after 25-d incubation at room temperature;


C
D

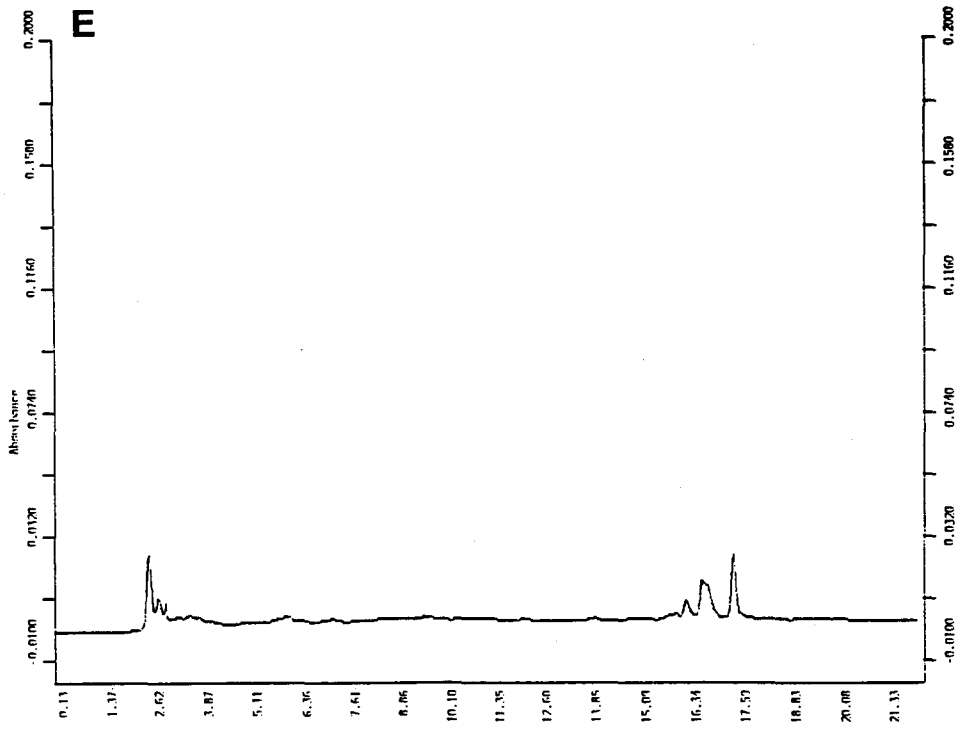


Fig. 2. (*cont'd*). (c) autoclaved OMW after 5-d growth of *Pl. floridae;* (d) autoclaved OMW after 15-d growth of *Pl. floridae;* (e) autoclaved OMW after 25-d growth of *Pl. floridae.*

Different stocks of OMW were found to have a wide range of toxicity, depending on the dilution and on the quality of the product treated. The most toxic samples had a minimum nontoxic dilution (MNTD) of 1:320, the least toxic a MNTD of 1:80. In every case, *Pleurotus spp.* induced a remarkable decrease in toxicity. The best results were obtained with the species *Pl. floridae* and *Pl. eryngii,* with a loss in toxicity of about 90%; *Pl. ostreatus* and *Pl. sajor-caju* induced a decrease in OMW toxicity of about 75%. The results refer to two different samples of OMW, obtained from different olive oil plants and at different times.

Another macroscopic property of OMW, after growth of mycelia in both the cultural conditions, was of course the color, which became brighter as the mycelia grew. This phenomenon was checked by measuring absorbance of OMW at 400 nm; results are shown in Table 3.

Clarification of plain OMW was not very efficient. The addition of either sucrose or lactose, which enhanced mycelial growth and decreased the pH of the medium at the later stages of cultivation, induced a remarkable brightening of OMW within 25 d of cultivation. The best results were obtained with *Pl. floridae,* which induced a clarification of about 40% in

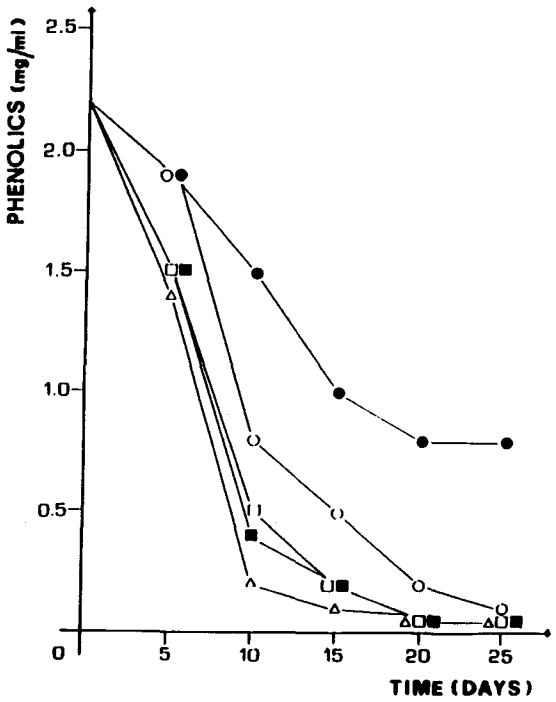


Fig. 3. Phenolics concentration pattern vs time for the four strains of *Pleurotus* growing on OMW/perlite mixture. Values are expressed as mg phenolics/mL OMW; the standard phenol was vanillic acid. *Pl. ostreatus* (△); *Pl. eryngii* (○); *Pl. sajor-caju* (□); *Pl. floridae* (■); OMW on perlite (●).

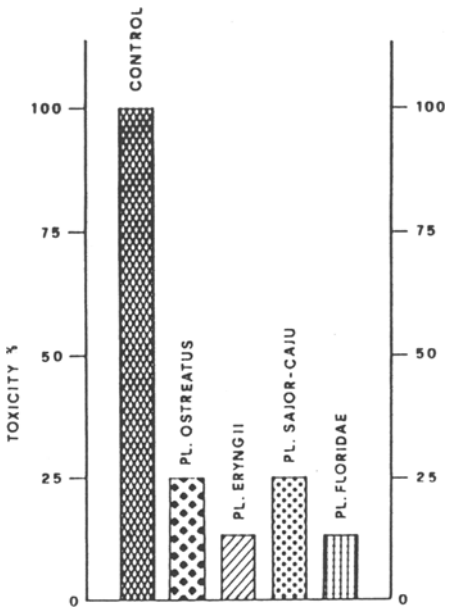


Fig. 4. *Pleurotus sp.*: residual toxicity of OMW after 25 d of growth, stationary conditions. Values are expressed as percentages of control (autoclaved OMW).

Table 3
Clarification of OMW by *Pleurotus* spp.
Growing in Stationary Conditions, After 25 d of Culture[a]

	Plain OMW		OMW+0.5% Sucrose		OMW+0.5% Lactose	
Species	Abs 400	%	Abs 400	%	Abs 400	%
Autoclaved OMW	1.327	100	1.375	100	1.686	100
Pl. ostreatus	1.308	98	1.180	85	1.528	90
Pl. eryngii	1.332	100	1.415	102	1.836	108
Pl. sajor-caju	1.280	96	1.115	81	1.363	80
Pl. floridae	1.276	96	0.848	61	0.712	42

[a]Values are expressed as the absorbances at 400 nm.

Table 4
Overall Yield of OMW/Perlite-Grown Mushrooms[a]

Species	Yield	%
Pl. ostreatus	1180	17.1
Pl. eryngii	580	8.4
Pl. sajor-caju	1150	16.7
Pl. floridae	1210	17.5

[a]About 6920 g of OMW were employed for each experiment. Yields are expressed in grams and as a percentage of the OMW weight.

the presence of sucrose and almost 60% in the presence of lactose. *Pl. ostreatus* and *Pl. sajor-caju* were not so effective in their clarifying action, whereas *Pl. eryngii*, under the test conditions, seemed to induce no significant reduction of OMW pigmentation.

A further observation can be made regarding the odor of the growth media: in fact, in the later stages of cultivation, the disagreeable smell of OMW was replaced by a pleasant anise aroma.

It was not possible to obtain fruit bodies from mycelia grown in stationary conditions, even if the organism grew rather vigorously. On the contrary, perlite-grown mycelia produced a very interesting yield of well-shaped fruit bodies, normal in both dimensions and color. Mushroom production started after about 15 d from the inoculum and continued for another 20 d. Overall yields are summarized in Table 4. The harvested mushrooms were analyzed in order to check their phenolic content; the results demonstrated that no accumulation of phenolics took place in OMW-grown mushrooms in comparison with commercial ones.

The protein content of the obtained mushrooms was also estimated; the results were completely satisfactory in that the protein content of the examined mushrooms was comparable with that of commercial *Pl. ostreatus*

Table 5
Phenolics Content of OMW-Grown Mushrooms in Comparison with Commercial *Pl. ostreatus* Fruit Bodies[a]

Species	Phenolics	Soluble proteins
Pl. ostreatus (commercial)	3.48	22
Pl. ostreatus	2.64	78
Pl. eryngii	3.11	49
Pl. sajor-caju	2.89	51
Pl. floridae	2.75	63

[a]Phenolics are expressed as mg/g dry wt; the standard phenol was vanillic acid. Content of soluble proteins of OMW-grown mushrooms in comparison with commercial *Pl. ostreatus* fruit bodies. Protein concentrations are expressed as mg/g dry wt; the standard protein was bovine serum albumin.

fruit bodies. The results of both phenolics and protein concentrations are summarized in Table 5.

As a point of interest, it is worth mentioning that on cooking, the mushrooms were indistinguishable from commercial ones in both taste and flavor.

ACKNOWLEDGMENTS

This work was supported by the C.N.R. target project on biotechnology and bioinstrumentation No. 90.00091.PF70.

REFERENCES

1. Amat di San Filippo, P., Rinaldi, A., Sanjust di Teulada, E., Satta, G., and Viola, A. (1986), *Riv. Merceol.* **25,** 183–189.
2. Di Giovacchino, L. (1990), *L'Informatore Agrario* **39,** 39–46.
3. Vazquez Roncero, A. and Maestro Duran, R. (1970), *Grasas y Aceites* **21,** 208–214.
4. Vazquez Roncero, A., Maestro Duran, R., and Graciani Constante, E. (1974), *Grasas y Aceites* **25,** 341–345..
5. Inouye, H. (1974), *Tetrahedron* **30,** 201–207.
6. Cortesi, N. and Fedeli, E. (1983), *Riv. It. Sost. Grasse* **60,** 341–351.
7. Cortesi, N., Mosconi, C., and Fedeli, E. (1984), *Riv. It. Sost. Grasse* **61,** 549–557.
8. Cicchelli, A. and Solinas, M. (1984), *Riv. Merceol.* **23,** 55–69.
9. Ben-Shalom, N., Kahn, V., Harel, E., and Mayer, A. M. (1977), *J. Sci. Food Agric.* **28,** 248–255.
10. Bradford, M. M. (1976), *Anal. Biochem.* **72,** 248–254.

11. Folin, O. and Ciocalteu, V. (1927), *J. Biol. Chem.* **73,** 627–634.
12. Leonowicz, A. and Grzywnowicz, K. (1981), *Enzyme Microb. Technol.* **3,** 55–58.
13. Harkin, J. M. and Obst, J. R. (1973), *Experientia* **29,** 381–384.
14. Yoshida, H. (1983), *J. Chem. Soc.* **43,** 472–475.
15. Haider, K. and Trojanowski, J. (1976), *Arch. Mikrobiol.* **105,** 33–37.
16. Blaich, R. and Esser, K. (1975), *Arch. Mikrobiol.* **103,** 271–277.
17. Ander, P. and Eriksson, K. E. (1978), *Progr. Ind. Microb.* **14,** 1–6.
18. Ander, P. and Eriksson, K. E. (1976), *Arch. Microbiol.* **109,** 1–58.
19. Leonowicz, A., Trojanowski, J., and Nowak, G. (1972), *Microbios* **6,** 23–28.
20. Leonowicz, A. and Trojanowski, J. (1975), *Acta Biochim. Polon.* **22,** 291–295.
21. Leonowicz, A. and Trojanowski, J. (1975), *Microbios* **13,** 167–174.
22. Leonowicz, A., Trojanowski, J., and Orlicz, B. (1978), *Acta Biochim. Polon.* **25,** 369–378.
23. Gigi, O., Marbach, I., and Mayer, A. M. (1981), *Phytochemistry* **20,** 1211–1213.
24. Sannia, G., Giardina, P., Luna, M., Rossi, M., and Buonocore, V. (1986), *Biotechnol. Lett.* **8,** 797–800.