Tr. J. of Biology
23 (1999) 231-240

Treatment of Olive Oil Mill Wastewater With Fungi

Özer YEŞİLADA
İnönü University, Science and Arts Faculty, Department of Biology, Malatya-TURKEY
Sibel ŞIK
İnönü University, Faculty of Education, Department of Biology, Malatya-TURKEY
Mesut ŞAM
Balıkesir University, Science and Arts Faculty, Department of Biology, Balıkesir-TURKEY

Received: 13.08.1997

Abstract:: Olive oil mills produce a liquid waste called olive black water in the olive oil production process. In this study, olive oil mill wastewater (OOMW) was analysed and then treated aerobically with fungi. Consequently, high chemical oxygen demand (COD), phenol and color reduction were obtained. High biomass yields and laccase enzyme activities were also determined.

Key Words: Olive oil mill wastewater, Fungi, Chemical oxygen demand, Phenol, Laccase.

Zeytinyağı Fabrikası Atıksuyunun Funguslarla Arıtımı

Özet: Zeytinyağı, fabrikaları, zeytin yağı üretim sürecinde zeytin kara suyu adı verilen bir sıvı atık üretmektedir. Bu çalışmada, zeytinyağı fabrikası atıksuyu (ZYFA) analiz edilmiş ve daha sonra funguslar ile aerobik olarak muamele edilmiştir. Çalışmamız sonucunda, yüksek kimyasal oksijen istemi (KOİ), fenol ve renk giderimi elde edilmiştir. Yüksek biyokütle verimi ve lakkaz enzimi aktiviteleri de ayrıca saptanmıştır.

Anahtar Sözcükler: Zeytinyağı Fabrikası Atığı, Fungus, Kimyasal Oksijen İstemi, Fenol, Lakkaz.

Introduction

Olive oil technology uses two methods for production of oil from olive fruit: the discontinuous press process or the continuous solid-liquid centrifuge system. Wastewater with a high pollution potential is produced whichever processing method is used (1). This olive oil mill wastewater is called "Kara Su" in Turkey.

The annual olive oil mill wastewater production in Mediterranean countries is estimated to be over $3x10^7$ m^3 (2). Biochemical (biological) and chemical oxygen demand of this waste may be as high as 100 and 200 g/l respectively. The organic fraction includes some sugars, tannins, polyphenols, polyalcohols, pectins and lipids (1). This waste is highly toxic because of its high content of phenolic compounds. The phenolic content of this waste causes phytotoxic and antimicrobial effects (3-5). Many phenolic and aromatic compounds have been detected in this

waste (6, 7). The dark color of this waste is caused by polyhenols (8). This color depends on the age and type of olive processed and also the type of technology used.

The presence of this waste in rivers decreases the dissolved oxygen content but increases the organic matter and K, Fe, Zn and Mn contents. Olive oil mill wastewater pollution also decreases the fish population (9). Many studies have been carried out in order to degrade and use this wastewater (10, 11).

Conventional wastewater treatment methods are relatively ineffective for removing these kind of pollutants. New methods for the treatment of this wastewater must be developed, in particular, by using the microorganisms.

White-rot fungi which produce highly oxidative enzymes, such as ligninase, phenol-oxidase and Mn-peroxidase, are able to degrade lignin, phenol, various xenobiotics and environmental pollutants (12-18).

The aim of this study was to determine the effect of various white-rot fungi and also a brown-rot fungus *Laetiporus sulphureus* for reducing the polluting characteristics and the phenol content of olive oil mill wastewater.

Materials and Methods

Organisms

Coriolus versicolor, Funalina trogii, Phanerochaete chrysosporium ME446, Pleurotus ostreatus, Pleurotus sajor-caju, Lentinus tigrinus and *Laetiporus sulphureus* (brown-not fungus) were used.

P. chrysosporium and *P. sajor-caju* were supplied by N. Kolankaya (Hacettepe University, Science Faculty, Department of Biology, Biotechnology Section) *C. versicolor, F. trogii, P. ostreatus, Lentinus tigrinus* and *Laetiporus sulphureus* were identified by M. Isiloglu (Muğla University, Department of Biology).

The cultures were maintained by subculturing on sabouraud dextrose agar and were kept at +4˚C for 2-3 weeks before use.

Preparation of Growth Medium

Filtered and sterilized (by autoclaving for 12 minutes at 120˚C) olive oil mill wastewater was used as the growth medium with a pH of 4.9.

Preparation of Inoculum and Growth

Fungi, except *P. chrysosporium*, were cultured at 30˚C on sabouraud dextrose agar slants. After 1 week of incubation, conidial suspensions were prepared and used for the preparation of inoculum. 10 ml of the suspension was transferred into a 240 ml flask containing 100 ml sabouraud dextrose broth + 2ml OOMW and agitated on a rotary shaker at 150 rpm for 4 days at 30˚C. After incubation, the cultures were homogenized (Kinematic Polytron Homogenizer) and used to inoculate fresh media.

5 ml of homogenate was transferred into 250 ml Erlenmeyer flasks containing 50 ml of OOMW media. Triplicate cultures were then incubated at 30˚C with agitation (150 rpm) for 6 days. The same experimental protocol was carried out for *P. chrysosporium* but at 40˚C.

Assay

Decolorization: Cultures were harvested and filtered through Whatman No:1 filter paper. Filtered media were diluted 50 fold and their absorbance was recorded at 395 nm (Spectrophotometer, UV/Visible, Phillips) (12).

Determination of total phenol content: Total phenol content was determined by colorimetric assay as described by Yesilada et al. (1995) (12).

The amount of total and reducing sugars in the medium was measured using Anthrone and DNS methods respectively (19, 20).

The determination of chemical oxygen demand (COD), total solid and volatile solid was performed acoording to the standard methods (21).

The dry weight of fungal mass was determined by filtering the contents of each flaks through preveighed Whatman No:1 filter paper and drying it to a constant weight at 65˚C (12). Yields were expressed as g of biomass per 50 ml of culture.

Enzymatic assay: Laccase (O_2: p-diphenol oxido-reductase E.C.1.10.3.2) activity was determined by the oxidation of guaiacol and absorbance was read at 465 nm. The enzyme activity was expressed in relative terms as colorimetric units (CU/ml) (15, 22).

Results

In this study, fresh OOMW without adding any organic or inorganic compounds was used. Its chemical composition is shown in Table 1.

Figure 1 shows COD, total phenol and color removal obtained in liquid cultures of *P. sajor-caju.* This fungus reduced COD, total phenol and color proportional to biomass production. After

Table 1. Composition of OOMW used in this study.

pH		4.91
Color (A 395)		32.5
COD	(g/1)	28.2
Total phenol	(g/1)	1.9
Total sugar	(g/1)	12.6
Reducing sugar	(g/1)	8.32
Total solid	(g/1)	27.40
Volatile solid	(g/1)	20.72

*Results are the mean of three replicates.

Table 2. The total sugar, reducing sugar, pH and laccase activity change in P. sajor-caju growth medium.

DAYS	Total sugar (g/l)	Reducing sugar (g/l)	Laccase (CU/mL)	pH
0	12.62±0.127	8.32±1.4	0	4.91±0.05
3	5.98±0.567	2.19±0.026	4.22±0.04	5.78±0.15
6	4.64±0.177	1.97±0.038	2.15±0.12	5.45±0.05
9	3.64±0.045	1.93±0.049	1.43±0.032	5.48±0.08
12	2.44±0.087	1.76±0.044	0.53±0.11	6.01±0.3
15	1.93±0.134	1.76±0.076	0.22±0.09	6.03±0.11

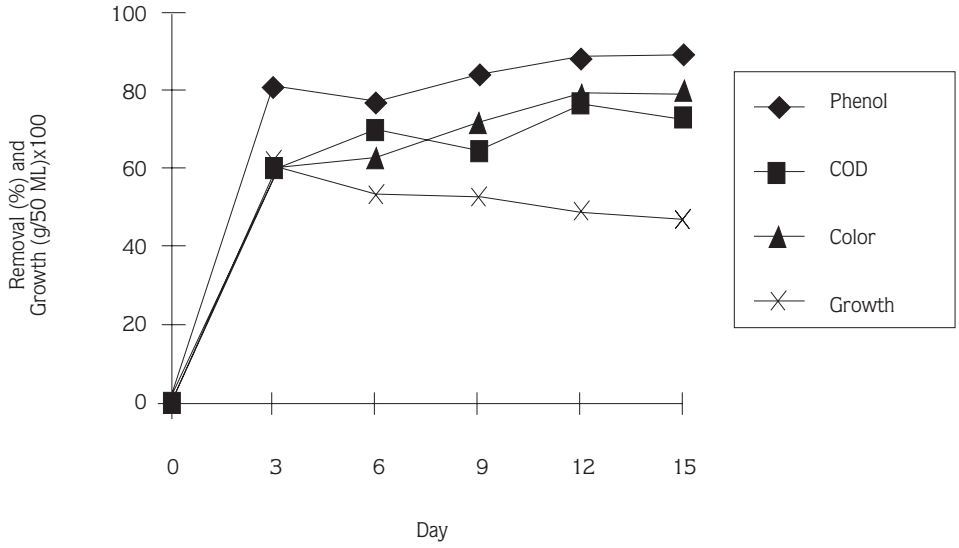

Figure 1. Typical culture profile of *P. sajor-caju* on OOMW during the agitated incubation.

3 days of growth 60% COD, 81% phenol and 60% color removal were detected but later the removal rates declined. High biomass yield was obtained after 3 days of incubation (0.616 g/ 50 ml). 53% total and 74% reducing sugar was removed in 3 days (Table 2). The most relevant reduction in total phenol content was observed in the early phases of growth and then declined, as shown in Figure 1. This could be ascribed to the organic compound depletion of the liquid

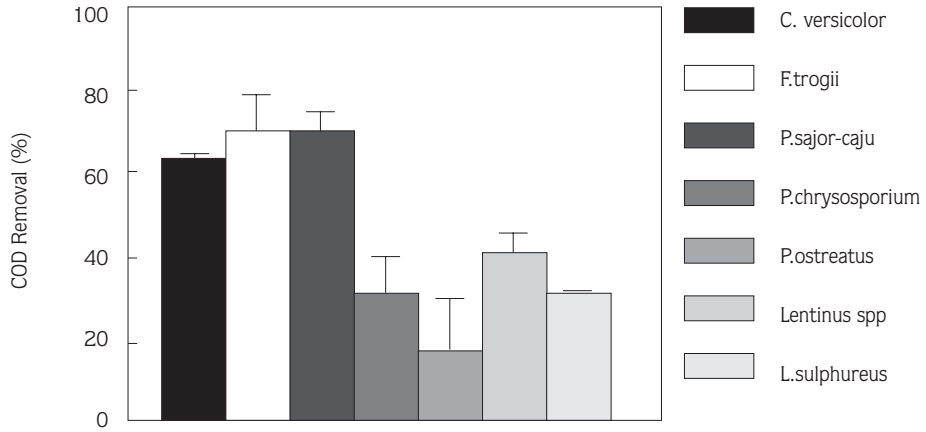

Figure 2. COD, Phenol, color, total and reducing sugar removal (%), biomass yields and laccase activity of various fungi after agitated incubation for 6 days (All symbols areresented in figure 2).

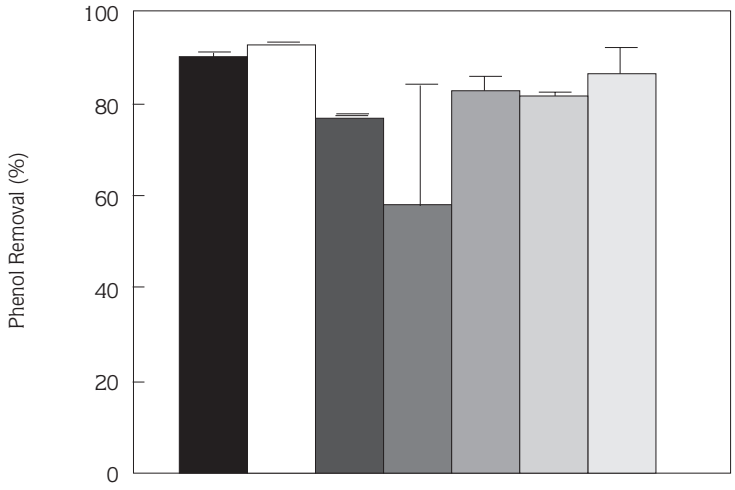

Figure 3. COD, Phenol, color, total and reducing sugar removal (%), biomass yields and laccase activity of various fungi after agitated incubation for 6 days (All symbols areresented in figure 2).

medium, which occurred within the same period (Figure 1). This result is similar to previous studies (12, 13, 23).

P.sajor-caju produced high laccase enzyme proportional to biomass production. The peak of enzyme activity was reached 3 days after the inoculation (Table 2). This enzyme is an extracellular enzyme (p-diphenol: oxygen oxidoreductase (E.C.1.10.3.2) which oxidizes a wide

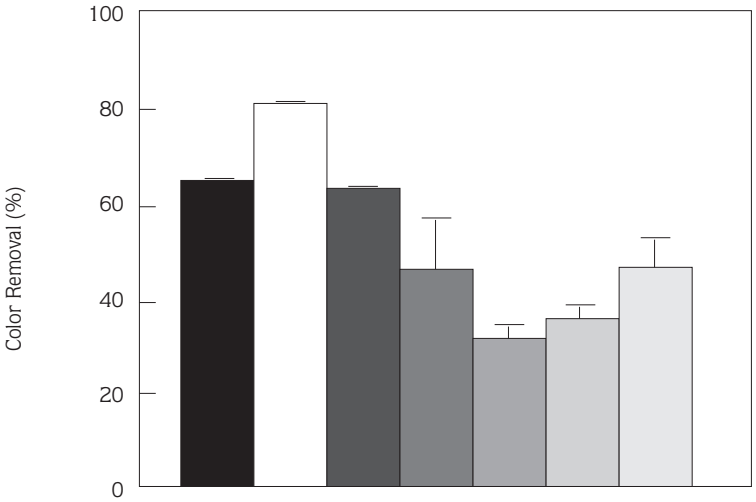

Figure 4. COD, Phenol, color, total and reducing sugar removal (%), biomass yields and laccase activity of various fungi after agitated incubation for 6 days (All symbols areresented in figure 2).

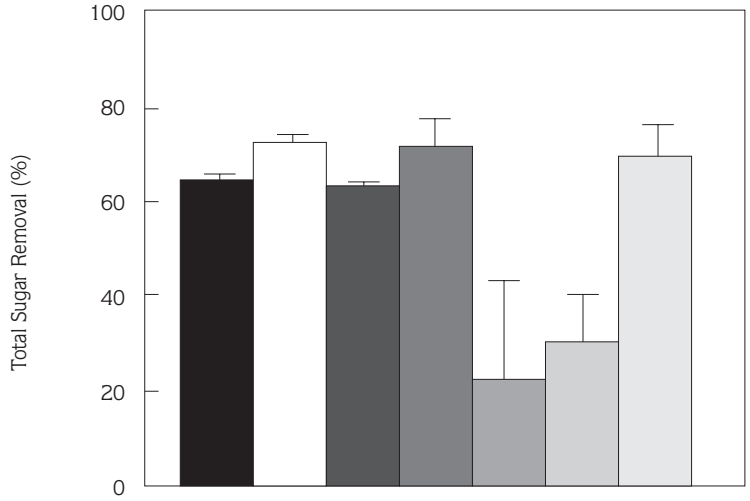

Figure 5. COD, Phenol, color, total and reducing sugar removal (%), biomass yields and laccase activity of various fungi after agitated incubation for 6 days (All symbols areresented in figure 2).

range of phenols and aromatic amines (6). In the presence of an inducer, many white-rot fungi excrete large amounts of laccase into the medium. Vinciguerra et al. (23) reported that olive oil mill wastewater addition greatly enhances phenol-oxidase produciton.

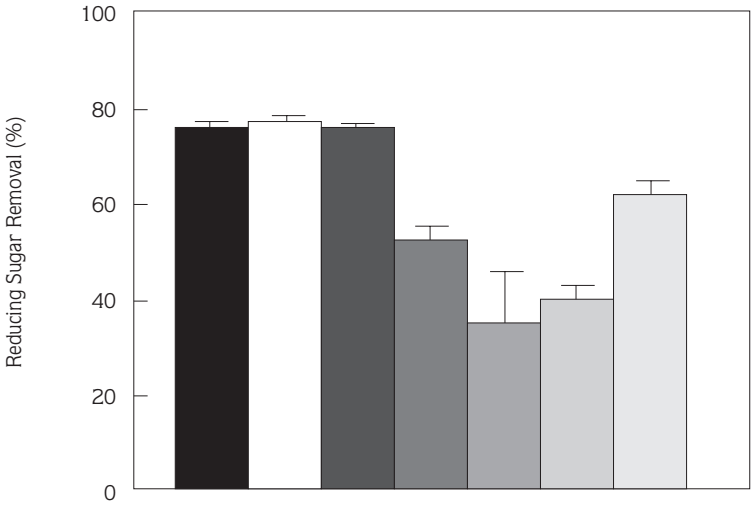

Figure 6. COD, Phenol, color, total and reducing sugar removal (%), biomass yields and laccase activity of various fungi after agitated incubation for 6 days (All symbols areresented in figure 2).

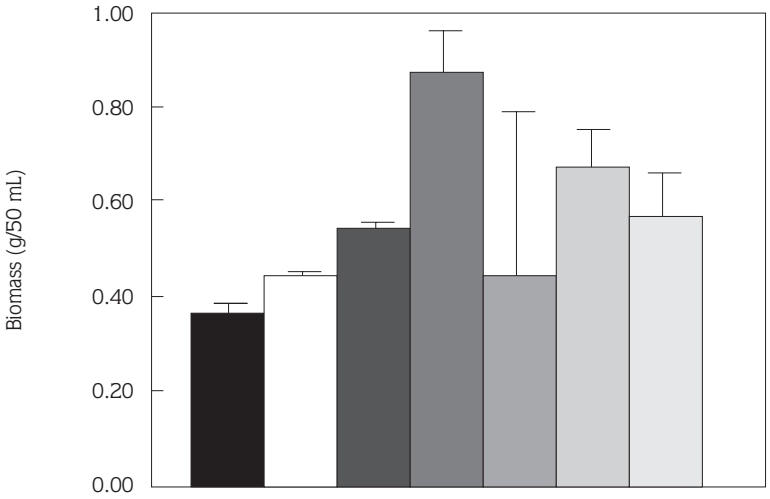

Figure 7. COD, Phenol, color, total and reducing sugar removal (%), biomass yields and laccase activity of various fungi after agitated incubation for 6 days (All symbols areresented in figure 2).

COD, phenol, color, total, and reducing sugar removal ability and laccase acivity of fungi other than a *P. sajor-caju* were also studied.

Attempts to find fungi able to reduce more COD, showed that the best results can be

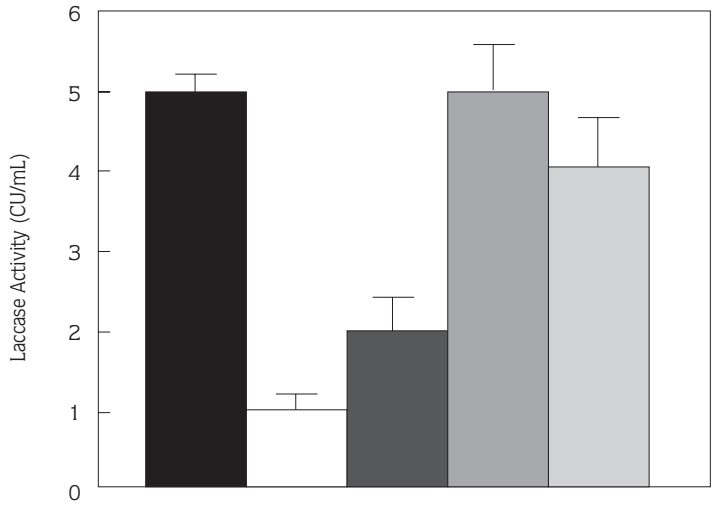

Figure 8. COD, Phenol, color, total and reducing sugar removal (%), biomass yields and laccase activity of various fungi after agitated incubation for 6 days (All symbols areresented in figure 2).

obtained with *C. versicolor, F. trogii* and *P.sajorcaju* (63-70%). A relatively low level of COD removal was obtained with *P. ostreatus* (17%) (Figure 2).

In every case, fungi induced a remarkable decrease in total phenol content (58-93%). The lowest rate of removal was obtained with *P. chrysosporium* ME446 (58%) (Figure 3).

Fungi, especially, *C. versicolor, F. trogii* and *P. sajor-caju* were found to efficiently decolorize the OOMW (63-73%). *P. ostreatus* exhibited low OOMW decolorization (32%) (Figure 4). This fungus was not so effective in its clarifying action.

As shown in Figure5 and 6, fungi except *P. ostreatus* and *Lentinus tigrinus,* removed 63-73% and 52-77% total and reducing sugar, respectively. High biomass yields and laccase activities were also obtained (Figure 7 and 8).

Hence, of all the fungi assayed, *C. versicolor, F. trogii* and *P. sajor-caju* were found to be the most suitable species for OOMW treatment systems.

Discussion

Pollution by OOMW is a crucial problem in the Mediterranean area. The COD and total phenol content of this wastewater was 28.2 g/l and 1.9 g/l, respectively.

C. versicolor, F. trogii and *P. sajor-caju* showed higher removal potential than the other fungi used.

The phenol removal which was proportional to the growth of fungi indicates its use a primary carbon source (12). In the present study no carbon, energy and other, sources was

added to the OOMW. This is very important for biotechnology.

Furthermore, high laccase enzyme activity was determined in OOMW culture media with these fungi. This suggests the possibility of using such an effluent to improve the production of enzymes for biotechnological use (23). Reid and Paice (24) and Paice et al. (25) reported that kraft pulp can be demethylized and bleached by pure laccase enzyme. It was previously reported that pure laccase enzyme could be used in the application of enyzme immunoassay, laccase based biosensors and also in the production of organic materials (26). It is also possible to transform lignin-related compounds and dechlorinate a number of toxic polychorinated phenols and guaiacols by laccase (27, 28).

We found that white-rot fungi could remove up to 93% of the phenols present in OOMW. The wastewater was also clarified by this treatment. Sanjust et al. (6), Martirani et al. (29) and Yesilada and Sam (30) reported that white-rot fungi removed phenols and induced a remarkable decrease in toxicity. The high removal of phenolic compounds by the fungi used in our study was important in this respect.

L. sulphureus, which is brown-rot fungus, was also found to have a high phenol removal rate. This result showed that other brown-rot fungi may also be tested as potential waste removal organisms in future studies.

Acknowledgements

This study was supported by TUBITAK (TBAG-1277)

References

1. Hamdi, M., Future prospect and constraints of olive mill wastewater use and treatment: A Review. Bioprocess Engineering, 8, 209-214, (1993).
2. Borja, R., Garrido, E.S., Martinez, L., Cormenzana, R.A. and Martin, A., Kinetic study of anaerobic digestion of olive mill wastewater previously fermented with Aspergillus terreus. Process Biochem., 28, 397-404, (1993).
3. Gonzales, D.M., Moreno, E., Sarmiento, Q.J., Cormenzana, R.A., Studies on antibacterial activity of wastewaters from olive oil mills (Alpechin): Inhibitory activity of phenolic and fatty acids. Chemosphere, 20, 423-432, (1990).
4. Perez, D.J., Esteban, E., Gomez, M., Gallardo-Lara, F., Effects of wastewater from olive processing on seed germination and early plant growth of different vegetable species. Environ. Sci. Health, B 21, 4, 349-357, (1986).
5. Rodriquez, M.MA., Perez, J., Ramos-Cormenzana, A., Martinez, J., Effect of extracts obtained from olive oil mill waste waters on Bacillus megaterium ATCC 33085. Journal of Applied Bacteriology, 64, 219-226, (1988).
6. Sanjust, E., Pompei, R., Resciggno, A., Augusto, R., Ballero, M., Olive milling wastewater as a medium for growth of four Pleurotus species. Appl. Biochem. Biotechnol. 31, 223-235, (1991).
7. Hamdi, M., Kahdir, A., Garcia, L.J., The use of Aspergillus niger for the bioconversion of olive mill waste-waters. Applied Microbiol. 34, 828-831, (1991).
8. Hamdi, M., Garcia, L.J., Anaerobic digestion of olive mill wastewaters after detoxification by prior culture of Aspergillus niger. Process Biochemistry, 28, 155-159, (1993).
9. Cabrera, F., Toca, G.C., Diaz, E., Arambarri, D.E.P., Acid mine-water and agricultural pollution in a river skirting the Donana national park (Guardi amar river, southwest Spain). Water Res. 18, 12, 1469-1482, (1984).

10. Martin, A., Borja, R., Chica, A., Kinetic study of an anaerobic fluidized bed system used for the purification of fermented olive mill wastewater. J. Chem. Tech. Biotechnol., 56, 155-162, (1993).

11. Gharshall, N., Production of single cell protein from olive mill wastewater by yeasts. Environmental Technology, 14, 391-395, (1993).

12. Yesilada, Ö., Fiskin, K., Yesilada, E., The use of white rot fungus Funalia trogii (Malatya) for the decolorization and phenol removal from olive oil mill wastewater. Enviromental Technology, 16, 95-100, (1995).

13. Yesilada, Ö., Fiskin, K., Degradation of olive mill waste by Coriolus versicolor. Tr. J. of Biolog, 20, 73-79, (1996).

14. Yesilada, Ö., Decolorization of crystal violet by fungi. World Journal of Microbiology and Biotechnology. 11, 601-602, (1995).

15. Yesilada, Ö., Fiskin, K., The use of white rot fungi for evaluation and degradation of vinasse. Tr. J. of Biology. 19, 181-189, (1995).

16. Archibald, F., Paice, M.G. and Jurasek, L., Decolorization of kraft bleachery effluent chromophores by Coriolus (Trametes) versicolor. Enzyme Microb. Technol. 12, 846-853, (1990).

17. Hammel, K.E., Organopollutant degradation by lignolytic fungi. Enzyme Microb. Technol., 11, 776-777, (1989).

18. Chivukula, M., Renganathan, V., Phenolic azo dye oxidation by laccase from Pyricularia oryzae. Appl. Environ. Microbiol., 61, 4374-4377, (1995).

19. Rosenberg, S.L., Symp. on the Biol. Transformation of Lignocellulose, 12, 133-142, (1980).

20. Miller, G.L., Use of dinitrosalicilic acid reagent for determination of reducing sugar. Analyt. Chem. 31, 426-428, (1959).

21. Standard Methods, Standard methods for the examination of water and wastewater. Fourteenth Edition American Public Health Association. 1138, (1979).

22. Arora, S.D. and Sandhu, K.D., Laccase production and wood degradation by a white rot fungus Daedalea flavida. Enzyme Microb. Technol., 7, 405-408, (1985).

23. Vinciguerra, V., D'annibale, A., Monache, G.D., Sermanni, G.G., Correlated effects during the bioconversion of waste olive wasters by Lentinus edodes. Bioresource Technology, 51, 221-226, (1995).

24. Reid, D.I. and Paice, G.M., Biological bleaching of kraft pulps by white-rot fungi and their enzymes. FEMS Microbiological Reviews., 13, 369-376, (1994).

25. Paice, G.M., Bourbonnas, R., Reid, D.I., Archibald, S.F. and Jurasek, L., Oxidative bleaching enzymes: a review. J. of Pulp and Paper Science., 21, 280-284, (1995).

26. Yoropolov, I.A., Skorobogat'ko, V.O., Vartanov, S.S. and Vorfolomeyev, D.S., Laccase: Properties, catalytic mechanism, and applicability. Appl. Biochem. Biotech. 49, 257-280, (1994).

27. Milstein, O., Hüttermann, A., Majcherczyk, A. and Schulze K., Transformation of lignin-related compounds with laccase in organic solvents. J. of Biotech. 30, 37-47, (1993).

28. Roy-Arcand, L. and Archibald, S.F., Direct dechlorination of chlorophenolic compounds by laccase from Trametes (Coriolus) versicolor. Enzyme Microb. Technol. 13, 194-203, (1991).

29. Martirani, L., Giardina, P., Marzullo, L. and Sannia, G., Reduction of phenol content and toxicity in olive oil mill waste wasters with the ligninolytic fungus Pleurotus ostreatus. Wat. Res., 30, 8, 1914-1918, (1996).

30. Yesilada, Ö., Sam, M., Toxic effects of biodegraded and detoxified olive oil mill wastewater on the growth of Pseudomonas aeruginosa. Toxicological and Environmental Chemistry. 65, 87-94, (1998).