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Bioremediation of olive oil mill wastewater and production of microbial biomass

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Abstract Olive oil mill wastewater (OMWW) was used as a substrate for the culture of a mixture of edible fungi in order to obtain a potentially useful microbial biomass and to induce a partial bioremediation of this fastidious waste. Before fermentation, the OMWW underwent an alkaline-oxidative treatment with the aim of decreasing the polyphenolic content which is the main cause of its toxicity. The fungal mixture grew fairly well in the treated OMWW and reached a maximum of biomass production within about 14 days of fermentation at room temperature. Up to 150–160 g of wet biomass was obtained per liter of OMWW. Analysis of the partially dehydrated biomass revealed a protein content of about 13 g% and 6 g% of row fiber. A relevant presence of unsaturated fatty acids was found, as well as the presence of significant amounts of vitamins A and E, nicotinic acid, calcium, potassium and iron. The possibility of using the microbial biomass produced from OMWW as an additive to animal feed is discussed.

Keywords Biodegradation · Bioproteins · Microbial biomass · *Pleurotus* · Olive oil mill wastewater · Yeasts

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Introduction

Olive oil mill wastewaters (OMWWs), also known as vegetation waters, are an important by-product of olive oil technology. The disposal of OMWW, which represents up to 50% by volume of milled olives (Di Giovacchino 1989), is strongly hindered by its high concentration of organic matter (B.O.D. higher than 100.0 g O_2 l^{-1}), mainly unextracted oil, aromatic compounds, such as polyphenols (from 2 to 6 g l^{-1}), sugars (up to 35 g l^{-1}) and pectic substances, and inorganic salts such as potassium, magnesium and phosphate (Amat di Sanfilippo et al. 1987; Handy et al. 1992).

Often the OMWW are poured into the soil or disposed of in sewage, causing soil and water pollution. In fact untreated OMWW are able to change the microbial composition of the soil through their antibacterial activity (Borya et al. 1995; Tardioli et al. 1997) and to produce phytopathogenic effects due to their high toxicity (Bressan et al. 2004). In recent years, some

methods have been proposed for OMWW bioremediation, such as chemical or microbiological treatment (Fountoulakis et al. 2002; Isadori et al. 2004; Kissi et al. 2001; Maullu et al. 1999; Rigoni-Stern et al. 1988; Sayady and Ellouz 1993). Some systems were also patented (Brueggemann and Huebner 2002; Catalano and De Nobili 2002; Reverso 1999). However, simple chemical or microbiological treatment cannot completely reduce OMWW pollution; furthermore the costs are too high and up to 85% of the organic substances, which could be recycled, are destroyed. Combining both chemical and biological treatment is believed to produce the best results (Bressan et al. 2004). Untreated OMWW and other agricultural wastes have been proposed for the production of animal feed, although digestibility and nutritional value were found to be somewhat unsatisfactory (Cabiddu et al. 2004; Oliveira et al. 2006).

The aim of the present study is to use the OMWW, produced by a pressure system and with an organic material content of about 120 g l^{-1}, as a culture medium for growing a cocktail of microbial strains. The novelty of this system is the possibility to degrade the high polyphenolic concentration of this material and, at the same time, produce microbial biomasses potentially useful as animal feed integrators.

Materials and methods

Microbial strains

Four strains of the ligninolytic basidiomycete genus of *Pleurotus* (namely *P. floridae*, *P. eryngii*, *P. ostreatus*, *P. sajor-caju*) were used, together with the yeast strains *Saccharomyces cerevisiae* and *Kluyveromyces lactis*; the species of filamentous fungi *Oidodendron* spp. and *Penicillum* spp. were also employed as inocula in treated OMWW (Table 1). These strains were chosen because they were all edible fungi, grew fairly well in treated OMWW and produced a good quality biomass. Some of these strains and their properties have been described elsewhere (Maullu et al. 1999; Pompei et al. 1994; Sanjust et al. 1991). The fungal strains were grown in potato dextrose agar (Oxoid, Hampshire, UK) at room temperature and were previously adapted to grow on agar solidified OMWW added with different percentages of Sabouraud medium (Difco Laboratories, Detroit, MI, USA).

Culture media and OMWW treatment

Olive oil mill wastewater was supplied by mills from the province of Cagliari (Italy) and was obtained from a plant with an olive pressure system, which produces a more abundant organic matter concentration than that obtained from plants employing a centrifugation system. The OMWW stock used in this work contained 3.2 g l^{-1} of polyphenols, 18.4 g l^{-1} of reducing sugars, with a total B.O.D. of 101.7 g l^{-1}, which is about twice the amount found in the OMWW produced by the centrifugation system (Di Giovacchino 1989).

Firstly all the OMWW were processed by alkaline treatment with 1% $Ca(OH)_2$ for 24 h at room temperature (13–18°C) under agitation, and secondly, by oxidation treatment with 1% H_2O_2 (125%, v/v) for 48 h at room temperature under agitation (Pompei et al. 1994).

Bioreactor

The bioreactor used in this work was a cylindrical plastic tank with a 5,000 l capacity (1.5 m diameter per 3.5 m of height). It was placed in the open air (the average temperature for the months of February and March was about 13–18°C). Agitation was obtained by a simple submerged intermittent pump and the air input (about 80 l 5 m^{-3} h^{-1}) was sterilized by filtration; the pH and temperature were monitored only at the beginning and at the end of the fermentation; the initial pH was 4.2, it rose to 7.5 after the chemical treatment and resulted as 6.8 after 14 days of biological treatment. No pH and temperature corrections were made during the process of fermentation.

Fermentation conditions

The microbial strains, grown for 5–7 days at room temperature on plates containing agar solidified OMWW, were seeded in a flask containing 300 ml

Table 1 Origin and characteristics of fungal strains used in this study

Strain identification	Origin	Class	Special properties	Note
Pleurotus ostreatus Fr3	Institute of Botanics, Cagliari	Basidiomycetes	Ligninolytic	Sanjust et al. (1991)
Pleurotus floridae Fr4	Idem	Basidiomycetes	Ligninolytic	Idem
Pleurotus eryngii Fr5	Idem	Basidiomycetes	Ligninolytic	Idem
Pleurotus sajor-caju Fr8	Idem	Basidiomycetes	Ligninolytic	Idem
Saccharomyces cerevisiae SI 50	University of Siena	Ascomycetes	Probiotic	Maullu et al. (1999)
Kluyveromyces lactis WM37	Idem	Ascomycetes	From milk	Idem
Penicillum spp. M12	Institute of Microbiology, Cagliari	Ascomycetes	Cheese fermenter	Good biomass producer[a]
Oidodendron spp. M23	Idem	Ascomycetes	From food	Good biomass producer[a]

[a] On treated OMWW (personal observation)

of sterile OMWW, which had previously been treated by the alkaline-oxidative procedure.

The cocktail suspension was used for starting the fermentation in a 5 m^3 bioreactor filled with fresh pretreated OMWW. The total microbial count of microorganisms present in the OMWW before, during and after fermentation, was determined by an agar inclusion assay using plate count agar medium (Difco Laboratories, Detroit, MI, USA), and by microbial isolation on Cled, Sabouraud and McConkey media. The plates were stored at 26°C for 3–5 days. At the beginning of fermentation the microbial suspension contained 2–3 $\times 10^6$ colony forming units (CFU) of each of the four *Pleurotus* species, about 10^6 CFU of each species of yeast and about 3×10^6 CFU of *Penicillum* and *Oidodendron*.

Biomass and protein determination

The fermentation process was run for at least 21 days; at the end of this time the total microbial biomass obtained was determined as a wet weight by centrifugation (4,000 g for 15 min). The biomass was then partially dehydrated in a 55°C thermostat for 48 h and the final weight was calculated. The proteins produced were detected by the Bradford method (Bradford 1976) using the Biorad Protein Assay kit (Biorad, Richmond, CA, USA); bovine albumin was used as a standard control. Reducing sugars were determined by the Miller-method as described elsewhere (Sanjust et al. 1991). The total phenol content was determined by the Folin–Ciocalteau method using vanillic acid as a standard (Sanjust et al. 1991).

Chemical analyses

One part of the biomass was stabilized by autoclaving for 15 min at 121°C. Both the stabilized and unstabilized biomass were processed for determining the following parameters: dehydrated residue, total and insoluble proteins, ether extract, row fibers, total sugars, tannic polyphenols and biomass digestibility (IVDMD); the aminoacid composition, minerals, lipids and vitamins were determined as described elsewhere (Cabiddu et al. 2004).

Results

OMWW treatment and microbial growth

In this study it was very difficult or almost impossible to obtain any growth on untreated OMWW of the majority of the fungal strains used in this study. This is consistent with data reported by other authors as regards the presence of antimicrobial substances in OMWW (Amat di sanfilippo et al. 1987; Bressan et al. 2004; Capasso et al. 1995; Isadori et al. 2004). Previous alkaline-oxidative treatment made the OMWW easily fermentable by the various seeded microorganisms. The fermentation process was run for at

least 3 weeks at room temperature. The amount of fungal cells increased until the second week, and reached the concentration of more than 10^9 CFU ml^{-1} 13–14 days after inoculation (Fig. 1). Since the OMWW was not sterilized, a relevant amount (10^3–10^4 CFU ml^{-1}) of contaminant bacteria were also present at the beginning of fermentation. Their number decreased to $<10^2$ CFU ml^{-1} after alkaline-oxidative treatment and then increased again, albeit slowly, during fermentation reaching up to 10^6 CFU ml^{-1} after 2 weeks. The total biomass was measured every 2 days as a wet weight after centrifugation. Some samples were also examined as a dehydrated weight after heating on a stove at 55°C for 48 h. In Fig. 1 the total biomass obtained during fermentation is also shown. At the end of the second week of fermentation, up to 16% of wet biomass (w/v) was obtained (up to 10% as a dehydrated weight). After this the biomass decreased very slowly, but still remained about 14% on day 21. As regards the various fungal strains; at the end of the fermentation the yeasts represented about 50–60% of the microbial biomass, whereas the Pleurotus strains and the filamentous fungi (*Penicillum* and *Oidodendron*) represented about 40% (up to 20% each).

Biodegradation

Reducing sugars, which were about 18 mg ml^{-1} in the untreated OMWW, were fully metabolized by the microbial mixture within 9–12 days (Fig. 2). The protein content of centrifuged OMWW showed a decrease of about 10% after chemical treatment and of almost 40% after fermentation. In this figure the polyphenolic content of OMWW is also reported. Polyphenols had already been reduced by about 65% (from 3.2 to 1.2 g l^{-1}) after alkaline-oxidative treatment and after fermentation decreased even further to as low as 0.2 g l^{-1} within 10–12 days. Also B.O.D. showed a dramatic decrease from 101.7 to 26.6 g l^{-1} after the chemical treatment, reaching a final concentration of 10.7 g l^{-1} after the biological fermentation.

Characterization of microbial biomass from fermentation

Table 2 shows the data on some characteristics of the biomass obtained from the microbial fermentation. One sample of biomass (A) was inactivated by heating (121°C for 15 min), whilst the other sample (B) was not; both were finally assayed as partially dehydrated material. The results showed that the organic material represented about 80% of the biomass and the ash content was more than 20%. In addition, the presence of about 13 g of proteins and 6 g of fibers per 100 g of dehydrated biomass was detected. It is important to note that most proteins were insoluble proteins (12.73 g), since they were prevalently fungal-cell-bound proteins. The polyphenols were still present in amounts of 1.8–1.9% (w/w) of the dehydrated biomass (Sanjust et al. 1991). The in vitro digestibility index (IVDMD) was found to reach about 51–55% of the biomass. The aminoacid profile (Table 3) shows a prevalence of glutamic acid, aspartic acid and leucin and lower amounts of the essential

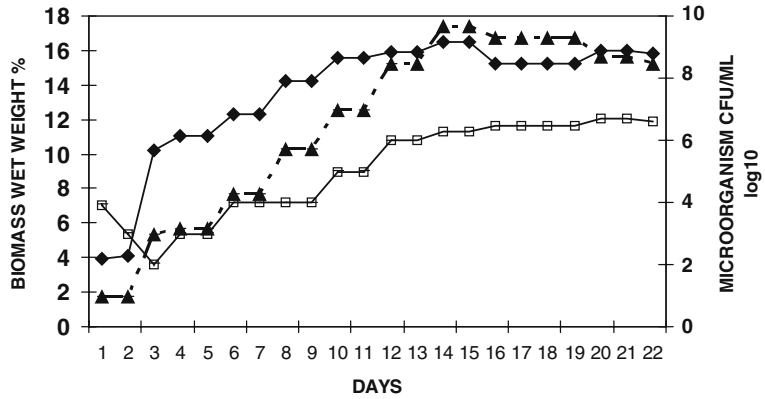

Fig. 1 Detection of fungi (▲), bacteria (□) and total biomass (■) during OMWW fermentation. The biomass is reported as a wet weight (w/v), whilst fungi and bacteria as log10 of CFU

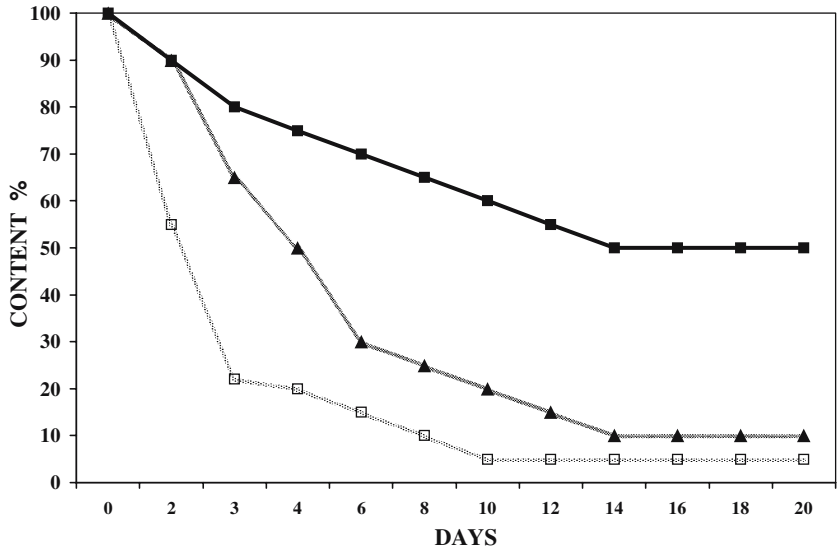

Fig. 2 Detection of proteins (■), sugars (□) and polyphenols (▲) during the fermentation process. The values are expressed as % of the content at time zero of fermentation

Table 2 Characteristics of the dehydrated OMWW biomass after microbial fermentation

Parameters considered	Sample A[a] (%)	Sample B[a] (%)
Humidity	53.8	63.2
Dry material	46.2	36.8
Organic substances	78.7	79.0
Ash	21.3	21.0
Total protein	13.6	13.4
Insoluble protein	12.7	12.7
Fiber	5.7	6.4
Sugars	nd	nd
Polyphenols	1.7	1.9
IVDMD[b]	54.9	51.2

nd not detectable

[a] Sample A—stabilized by heating; sample B—untreated

[b] IVDMD—biomass digestibility measured by means of 'in vitro' test

Table 3 Aminoacid content of dehydrated microbial biomass from OMWW

Aminoacids	Sample A (g%)	Sample B (g%)
Lysin	0.19	0.22
Histidin	0.28	0.33
Arginin	0.74	0.66
Aspartic acid	1.35	1.44
Threonin	0.64	0.66
Serin	0.76	0.79
Glutamic acid	1.58	1.60
Prolin	0.64	0.56
Glycin	0.63	0.63
Alanin	0.71	0.72
Valin	0.79	0.80
Methionin	0.21	0.19
Isoleucin	0.70	0.70
Leucin	1.04	1.06
Thyrosin	0.60	0.58
Phenylalanin	0.66	0.66

aminoacids. The lipid content (Table 4) revealed a residual oleic acid of 26–48 mg g^{-1} of biomass, as well as palmitic (6–10 mg g^{-1}) and linoleic acids (4–8 mg g^{-1}).

The content of four vitamins was considered (Table 5); of these only vitamin C was always absent. The stabilized biomass was shown to contain a considerable amount of vitamins A, E and niacin (78,000 UI kg^{-1}, 78 mg kg^{-1} and about 27 mg kg^{-1}, respectively) as compared to the untreated biomass. Among the minerals, copper was found in an unusual amount of 30 mg kg^{-1} (Table 5). High contents of iron (about 700 mg kg^{-1}), zinc (800–900 mg), manganese (22 mg), calcium (about 5%) and potassium (about 2%) were present.

Discussion

Alkaline-oxidative pretreatment of OMWW with $Ca(OH)_2$ and H_2O_2 considerably reduces the total phenolic compound concentration, which is the major cause of the biotoxicity of this industrial by-product. A further amount of polyphenols

Table 4 Analytical fat content of microbial biomass from OMWW

Fatty acids	Sample A (mg g^{-1})	Sample B (mg g^{-1})
C16	6.15	10.67
C16:1 *cis*	0.68	1.31
C17	0.04	0.06
C18	0.82	1.36
C18:1 *trans*	0.22	0.47
C18:1 *cis*	25.92	47.93
C18:2 *cis*	4.30	7.95
C18:3 *cis*	0.43	0.79
CLA[a]	0.22	0.35

[a] CLA conjugated linoleic acid

Table 5 Vitamin and mineral content of microbial biomass

Vitamins and minerals	Samples	
	A	B
Vitamin A (UI kg^{-1})	78,237	14,559
Vitamin E (mg kg^{-1})	78.00	38.00
Vitamin C	0.00	0.00
Nicotinic acid (mg kg^{-1})	26.80	0.00
Calcium (%)	4.76	4.74
Phosphorus (%)	0.13	0.13
Sodium (%)	0.19	0.19
Potassium (%)	1.59	1.73
Magnesium (%)	0.13	0.13
Iron (mg kg^{-1})	702.00	721.00
Manganese (mg kg^{-1})	22.00	23.00
Copper (mg kg^{-1})	30.00	29.00
Zinc (mg kg^{-1})	928.00	848.00

is eliminated via fungal metabolism. The cocktail strains chosen for the fermentation experiments can induce successful OMWW bioremediation and produce potentially useful biomasses. Strains were adapted for fast growth on OMWW at different concentrations, but were indistinguishable from those grown on conventional substrates. Since in our country olive milling takes place between December and February, the fermentation process was run at a quite low (13–18°C) temperature. In any case, fresh OMWW had to be used for this experiment, since undesired anaerobic fermentation can alter the stored OMWW composition. In our experience in laboratory conditions, OMWW was processed at even higher temperatures with a considerable reduction of the fermentation time. *Pleurotus* species were already found to be able to strongly reduce organic substances in OMWW absorbed into solid supports (Capasso et al. 1995; Palmieri et al. 2000). A B.O.D. decrease of about 90%, from 101.7 to 10.7 g l^{-1}, was also obtained; this finding is consistent with data previously reported on the growth of single Pleurotus fungi (Fountoulakis et al. 2002; Isadori et al. 2004; Kissi et al. 2001; Rigoni-Stern et al. 1988; Sayady and Ellouz 1993; Maullu et al. 1999; Pompei et al. 1994; Sanjust et al. 1991). The final B.O.D. value obtained is still higher than that required by legal requirements and needs further treatment in wastewater urban plants. Biomasses obtained from pressure-produced OMWW represented up to 160 g l^{-1} of the fluid treated, measured as a wet weight. The biomass obtained shows an interesting composition of organic matter (proteins and fibers), which resembles that of soy peal and may be considered as a potential animal food integrator, although biomass digestibility (IVDMD) was found to be a little over 50%, with a nutritional value that can be considered of average quality. The almost complete absence of sugars was expected, since it is probable that all the sugar content was metabolized by the fungi and other microorganisms present in the fermentation mixture. Polyphenols decreased by more than 90% in the OMWW, but they were still present at a final concentration of about 1.8 g% (w/w) in the dehydrated biomass; this probably means that some fungi are able to uptake and concentrate a small amount of unmetabolized polyphenols. Among the lipids found in the biomass, the prevalent amount of oleic acid was expected; furthermore, there was an interesting content of linoleic acid, an important unsaturated fatty acid which, together with conjugated linoleic acid (CLA), is considered as having extremely beneficial properties in both animal and human nutrition (Cartens et al. 1996; Rosa et al. 2005). In addition, an interesting content of vitamins A, E and nicotinic acid was found. Some minerals were present in unusual amounts; in particular copper was found in a concentration considered close to the highest values compatible with sheep feeding. It is probably a residue of the antiparasitic treatment of the olive trees.

Conclusion

The chemical–biological method presented in this work allows an intense degradation of most polluting substances contained in the OMWW and at the same time allows a possible transformation of most of the organic compounds in potentially useful microbial biomass, which can be used as an animal feed integrator. The system is easy to handle and has low-energy requirements. Further studies will be necessary for defining the best way to obtain high-nutritional value biomass from OMWW using different mixtures of fungi or probiotic microorganisms and to reach a final B.O.D. that will meet with legal requirements. Finally, it will be necessary to perform in vivo studies to ascertain the real possibility of using OMWW obtained biomasses for the nutrition of ruminant or monogastric animals, bred for the production of either meat or milk, as has already been done with untreated olive oil plant by-products (Cabiddu et al. 2004).

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