Residues of Olive Oil Extraction Process: Possible Biotechnological Approaches

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The disposal of olive mill wastewater (OMW) is a peculiar problem of the Mediterranean area. OMW is characterized by a highly variable chemical composition and a high organic load. This effluent can be regarded as a potentially interesting growth medium in microbial processes. Our research team has focused the attention on the technical feasibilities of polysaccharides and enzymes production and the agronomic use of OMW previously enriched in phosphorus by acidogenic fungi.

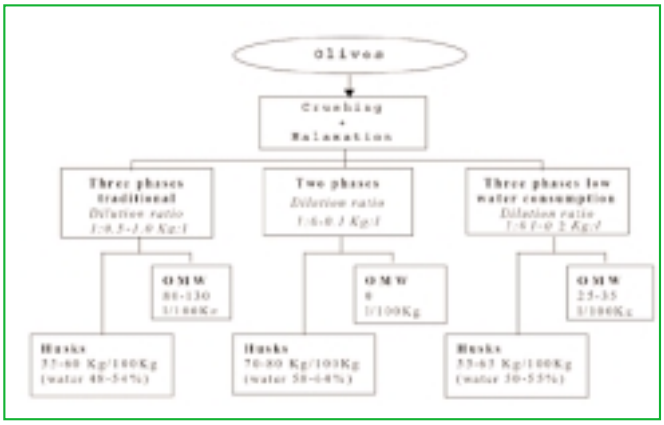

Figure 1 - Flow chart of continuous olive oil extraction process

Main residues of the olive-oil extraction process are vegetation water and husks. Extraction system, pressure and centrifugation play an important role in the amount and characteristics of both olive oil and residues [1].
In the traditional centrifugation system, 50-100 l of water are added to 100 kg of olive pastes to reduce viscosity and to improve oil separation (Figure 1). As a consequence, however, large amounts of vegetation water are produced. During the last ten years, new centrifugation systems have been developed that require either less (10-20 l) or no water at all added during the oil separation. In either case, however, residues of olive-oil extraction may represent a serious environmental problem. After recovery of residual oil by solvent extraction, husks from traditional processes can find valid utilization as animal feed or as alternative fuel and in compost preparation.
Vegetation water, that with washing waters make the oil-mill wastewaters (OMW) are generally characterized by large volumes and high polluting load [1]. Moreover, compounds with biostatic activity (*e.g.*, polyphenols) are largely present [2, 3]. Beside the traditional decantation, several disposal methods have been proposed for this waste, such as, e.g., physico-chemical treatments (decantation with lime and/or chemical oxidation, concentration, drying and incineration; ultrafiltration and reverse osmosis), agronomic (aquaculture, land-spreading) and animal-breeding methods (direct utilization as animal feed or after protein enrichment by, *e.g*, yeast or fungal fermentation) and of the "biotechnological" type (fermentation, biological treatments).
Each of the above approaches to the problem has its own essential validity for the reduction of the polluting load and, consequently, for the waste eventual disposal. With probably the only exception of field spreading, however, none appears to be a suitable and definitive solution to the problem on its complex. In our opinion, either a biotechnological approach or the combination of biotechnological approaches with other treatments (either physico-chemical or agronomic) [4] or a chemical use of the olive oil wastewaters to recover compound of high added value (*i.e*. antioxidants) might represent a new and possibly successful way of dealing with the problem. Besides being a serious environmental problem, OMW can represent a possible resource for the presence of simple and complex sugars and of other substances (Table 1) potentially useful as such (i.e. after chemical extraction) or to be used as a basis for fermentation processes.
Therefore, we report main results of few different biotechnological approaches tested by us for the industrial possible utilization of OMW.

Table 1 - Average composition of olive vegetation waters [3]

Density	1.023-1.054
pH	4.6-6.7
Turbidity	11,000-65,000
Water (%)	82.4-96.0
Dry extract (%)	3.0-18.0
Suspended solids (%)	0.04-1.04
Mineral compounds (%)	0.4-7.2
Organic compounds (%)	3.9-16.5
Total sugars (%)	1.0-8.0
Total pectins (%)	0.05-0.15
Total polyphenols (%)	0.15-1.75
Total nitrogen (%)	0.1-7.2
BOD (mg l^{-1})	9,600-110,000
COD (mg l^{-1})	30,000-195,000

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Table 2 - Fungal growth and lipase production by various fungal strains cultivated in shaken culture on OMW*

Strain	*Growth (g l^{-1})*	*Lipase (U ml^{-1})*
Penicillium citrinum		
NRRL 1841	5.88±0.02	0.34±0.01
NRRL 3754	5.34±0.01	0.28±0.02
ISRIM 118	5.71±0.18	0.32±0.01
Aspergillus niger NRRL 334	7.07±0.63	0.33±0.01
Aspergillus oryzae		
NRRL 485	5.18±0.40	0.34±0.03
NRRL 1988	5.73±0.18	0.34±0.03
Geotrichum candidum		
NRRL Y-552	4.06±0.23	0.27±0.03
NRRL Y-553	3.16±0.05	0.52±0.01
Rhizopus sp. ISRIM 383	4.22±0.16	0.30±0.01
R. arrhizus NRRL 2286	3.07±0.15	0.30±0.02
R. oryzae NRRL 6431	3.27±0.15	0.32±0.01

* Data (means of three independent experiments ± standard deviations) are given at 168 h of fermentation

Enzyme production

Lipase

Microbial lipases catalyse the hydrolysis of ester linkages in lipids and, therefore, they are employed in food technology (mainly, in the dairy industry), detergent, pharmaceutical, cosmetic and leather industries. Their use can be also extended to biotechnological applications due to their ability to synthesize ester bonds in nonaqueous media [5]. The use of cheap growth substrates, such as agro-industrial wastes can lead to the reduction of production costs of microbial lipases.
OMW contains, among others, residual lipids as a consequence of incomplete olive oil extraction, that can stimulate and, sometimes, induce microbial production of lipases.
To this end, a screening was conducted in shaken culture on a series of microbial isolates (listed in Table 2) kindly provided from the ARS Culture Collection (NRRL) of Peoria (Usa) and from the Istituto Superiore di Ricerca e Formazione sui Materiali Speciali per le Tecnologie Avanzate (ISRIM), Terni, Italy. The OMW used was characterized by COD and total sugar and phenol contents of 43.0, 17.4 and 2.5 g l^{-1}, respectively. Before fermentation, the OMW was added with yeast extract, 0.5 g l^{-1} and $(NH_4)_2SO_4$, 1.0 g l^{-1}. Cells from 6-day-old PDA-slant cultures were suspended in 5 ml of sterile deionised water and used as inoculum for precultures (180 rpm at 28 °C for 72 h) in 500-ml Erlenmeyer flasks containing 95 ml of OMW integrated as above. These precultures were used as inoculum (5 ml per flask) for 95 ml of integrated OMW in 500-ml Erlenmeyer flasks; incubation was at 28 °C and 180 rpm for 168 h. Lipase activity was determined spectrophotometrically at 540 nm using β-naphtylmiristate as substrate [6]. Table 2 summarizes for each fungal strain the levels of growth and lipase production reached after 168 h of cultivation. All the strains were able to grow on the wastewater, producing lipase activities always higher than 0.30 U ml^{-1}. The highest enzyme activity was obtained with *G. candidum* NRRL 553 (0.52 U ml^{-1}), strain already known for its ability to produce lipases on defined media [7] but, so far, never tested on wastewaters. COD and polyphenol content were reduced reaching, at the end of fermentation, 32.6 and 30.5% of removal for COD and total phenol, respectively. Optimisation of culture medium, in terms of possible integrations (salts, inducers, etc.) to OMW, and fermentation conditions might significantly improve the enzyme production.

Laccase and Mn-dependent peroxidase

One of the most remarkable technical constraints in olive-mill wastewater upgrading by the use of microbial bioconversions is the presence of compounds such as polyphenols, that can exert significant toxicity and biostatic effects [8]. For this reason, the most appropriate microbial candidates to perform this task are fungi belonging to the ecological group of white-rot basidiomycetes, whose degradative capacity on aromatics has long been known [9]. The aromatic degrading ability is due to the release of extracellular oxidases, such as laccase and Mn-dependent peroxidase, characterized by a low substrate specificity and good intrinsic stability towards several potentially denaturing agents. As a consequence, the use of these biocatalysts in several commercial applications including textile and lignocellulosic fibers processing [10, 11], wine stabilization [12] and wastewater treatment [13], has been suggested. The production of laccase and Mn-dependent peroxidase by the white-rot basidiomycete *Panus tigrinus* CBS 577.79 was investigated using OMW as a low-cost growth medium both in liquid submerged (LSF) and solid state (SSF), where straw was added, fermentation [14]. To this aim, flasks and three dif-

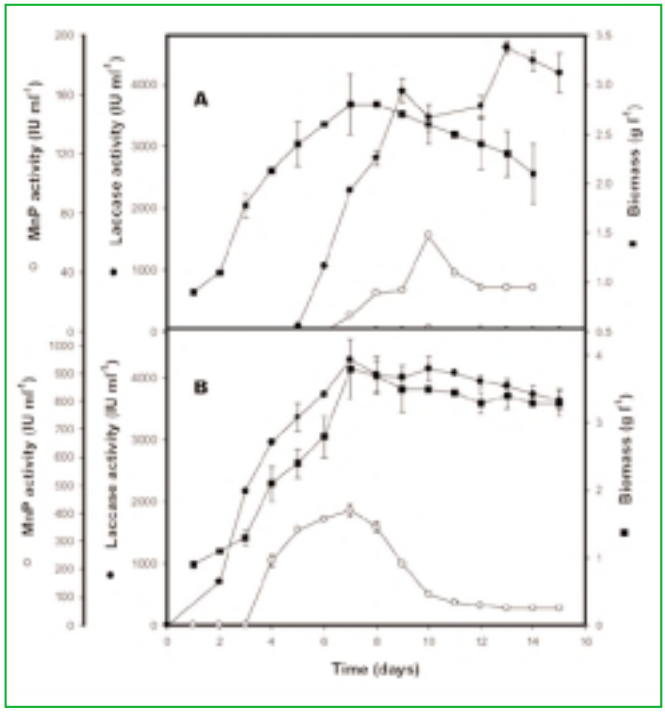

Figure 2 - Time course of biomass production (■) and laccase (●) and Mn-dependent peroxidase activity (○) in stirred-tank (A) and air-lift (B) reactor. Data are the mean ± SD of triplicate experiments [14]

ferent bioreactors, a stirred tank (STR), an air-lift (ALR) and a rotary drum (RDR) reactor [15, 16], were used and productivity and efficiency compared in view of a possible scale-up. Typical fermentations in STR and ALR are shown in Figure 2, while Table 3 reports the overall process performances for the reactor systems on OMW-based medium. Fermentation in the rotary drum reactor (RDR) gave the highest values for total enzyme activity (EA_{tot}) and in shorten time than in STR. This behavior would suggest the use of RDR particularly in presence of solid state materials such as olive husks to produce metabolites or enzymes useful in composting. Nevertheless, both submerged fermentation systems (air-lift in particular) showed unitary volumetric activity (EA_{vol}) and average volumetric productivity significantly higher than those of RDR [14]. It is worthwhile to note that the volume of processed effluent per unit of reactor volume (OMW_{tr}) with both LSF systems was at least ten fold higher than that of RDR.

Table 3 - Process performances of the three different bioreactors (RDR, STR and Air lift) with OMW-based medium [14]

	Laccase			*MnP*		
	RDR	*STR*	*Air lift*	*RDR*	*STR*	*Air lift*
Biomass (g l^{-1})	69±3.5^{§a}	2.4±0.26^{b}	3.8±0.45^{c}	65±5.7^{§a}	3.75±0.41^{b}	3.8±0.45^{b}
EA_{tot} (U)	22514±208^{a}	8990±280^{b}	10468±104^{c}	5022±156^{a}	701±35^{b}	993±35^{c}
V_{bio} (l)	20	3	3	20	3	3
EA_{max} (U l^{-1})	1309±20^{a}	4603±98^{b}	4300±23^{c}	292±12^{a}	360±20^{b}	410±22^{a}
T (h)	216	312	168	312	216	168
EA_{Vol} (U l^{-1})	1125±46^{a}	2997±94^{b}	3489±34^{c}	251±14^{a}	233±11^{a}	331±11^{b}
OMW_{tr} (l)	0.0325	0.33	0.42	0.0325	0.33	0.42
$Y_{a/s}$ (U g^{-1})	32.2±0.2	n.d.	n.d	7.2±0.04	n.d.	n.d
$Y_{p/s}$ (U l^{-1})	34615±1060^{a}	9206±196^{b}	8604±46^{c}	7723±140^{a}	719±41^{b}	820±35^{c}
EA_{sp} (U g^{-1})	466±28^{a}	1917±40^{b}	1131±6^{c}	110±4^{a}	96.0±5^{b}	108±6^{a}

Legend: EA_{tot} = total enzyme activity from a single fermentation batch; V_{bio} = total bioreactor volume; EA_{max} = maximal enzyme activity; T = time to reach the maximal activity; EA_{vol} = unitary volumetric activity: activity per unit of reactor volume; OMW_{tr} = amount of OMW treated in a single fermentation batch per unit of reactor volume; $Y_{a/s}$= yield of activity per g of substrate; $Y_{p/s}$ = production yield: enzyme production per unit of OMW; EA_{sp}= specific activity: activity referred to weight unit of the mycelial biomass. Results are mean of three replicates ± standard deviation. Row means followed by the same superscript letter were not significantly different ($P < 0.05$) as determined by the Tukey test.
§ Data are expressed in g mycelium (Kg solid substrate)$^{-1}$.

Chemicals

Exopolysaccharides

Microbial exopolysaccharides (EPS) might be a valid alternative to plant and algal products [17]. Furthermore, they can have unusual molecular structures and peculiar conformations, thus conferring them unique and potentially interesting properties in view of eventual industrial uses [18, 19]. However, with the exceptions of xanthan, gellan and curdlan that come from bacteria, for economic reasons commercial polysaccharides are, at present, mainly derived from plants [17]. Commercial usage of fungal exopolysaccharides such as, *e.g.*, scleroglucan and schyzophillan might be encouraged by the development of fermentative processes possibly using cheap substrates such as agro-industrial residues and/or wastes.

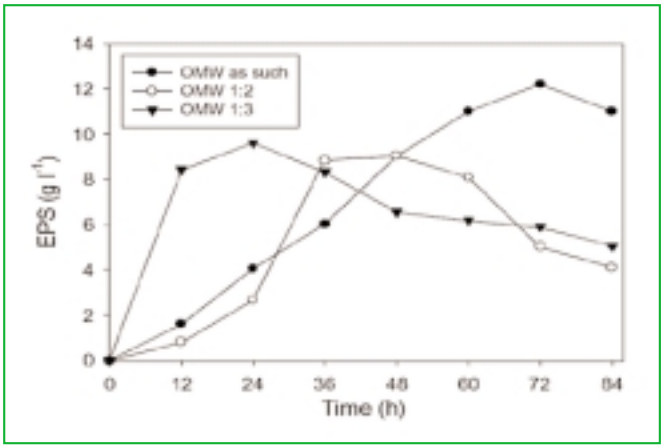

Figure 3 - Time course of esopolysaccharide (EPS production by Botryosphaeria rhodina DABAC-P82 grown on OMW as such and diluted

Three fungal strains were tested on OMW: *Sclerotium glucanicum* NRRL 3006 and *Sclerotium rolfsii* ATCC 15206, already well known as good producers of beta-glucans [20, 21], and *Botryosphaeria rhodina* DABAC-P82, previously selected for the high productions of an EPS that was characterized as a beta-glucan very similar to the scleroglucan [22].
An OMW with COD, total sugars and phenol content of 74.0, 39.2 and 7.2 g l^{-1}, respectively, was used. Fermentations were performed in shaken cultures (at 180 rpm and 28 °C) using OMW both as such and diluted 1:2 and 1:3. Surprisingly, the strains of *S. glucanicum* and *S. rolfsii* did not growth on OMW, even if diluted 1:3. On the contrary, *B. rhodina* DABAC-P82 grew well (approximately, 13.0 g/l of fungal biomass) also on undiluted OMW producing high levels of EPS (more than 10 g/l after only 96 h of fermentation). Figure 3 shows the time course of EPS production by this strain grown on OMW as such and diluted. Also, a significant reduction of COD and total phenol content of the effluent (around 50%) was observed at the end of the fermentative process when OMW were used as such. The potential of this fungal strain to use OMW as substrate for the production of EPS appears to be noticeable. However, further studies, aimed to assess the technical feasibility of the process using, for example, different OMW typologies in terms of origins, polluting loads and time and way of storage, are yet needed.

Table 4 - Preliminary agricultural tests on wheat in greenhouse using treated and untreated OMW [23]

	Spikes/plant (N°)	*Kernel weight (mg)*	*Grain yield (g/pot)*	*Harvest Index*
Control (soil)	1.4±0.7	27.3±0.06	0.49±0.18	0.20±0.06
Soil + P fertilizer	2.0±0.6	30.2±0.03	0.98±0.34	0.25±0.01
Soil + Untreated OMW	1.0±0.0	30.1±0.25	0.26±0.03	0.22±0.04
Soil + Treated OMW	3.8±0.2	48.3±0.02	3.05±0.57	0.53±0.02

Improved agronomic characteristics

Microbial enrichment of OMW with soluble phosphate

This work was aimed to perform the partial removal of pollutants from OMW with the concomitant enrichment in soluble phosphorus in order to use the treated effluent for land spreading as a low cost P fertilizer.
OMW, obtained by a 3-phase continuous olive mill (located in Viterbo, Italy) and showing low levels of soluble phosphorus (ca 0.2 g l^{-1}) and COD (ca 30 g l^{-1}), were centrifuged, filtered and supplemented with 3 g l^{-1} of rock phosphate (RP) powder. This material was directly biotreated with *Aspergillus niger* immobilised in Ca-alginate and cultivated in shaken flasks by batch and repeated-batch processes. The fungus grew well and reduced the COD of the waste to ca 35% (batch fermentation) and 64% (repeated-batch fermentation, 4th batch) of its initial level (Figure 4). Total sugar content was reduced to ca. 60% in both processes (data not shown) while reduction of total phenols was minimal. RP was solubilised, maximum soluble P was 0.63 and 0.75 g l^{-1} in the batch and repeated-batch (3rd batch) processes, respectively [23].
Several types of OMW±RP, microbially-treated or not, were tested for their fertilising ability on a soil-wheat (*Triticum durum*) model system in green house in order to simulate the effects of possible land spread of the effluent. Beneficial effects were highest using the OMW treated by the repeated-batch process (Table 4): plants grown on such treated soil showed an increase seed biomass, spike number and kernel weight. Harvest Index was highest (0.53) after treatment with OMW from repeated-batch process [23].

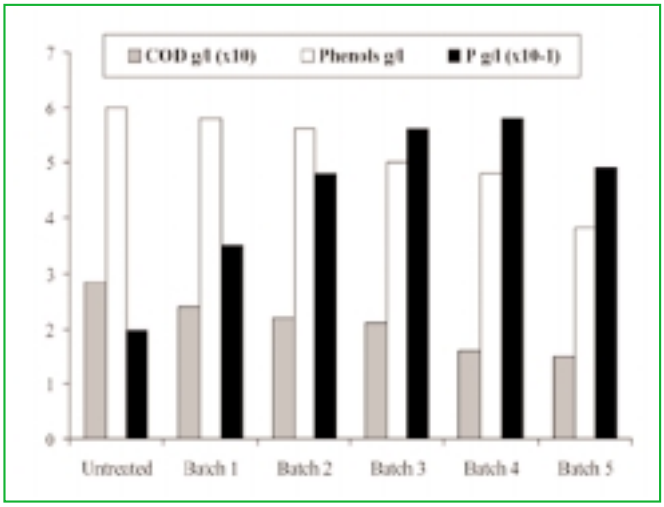

Figure 4 - Repeated batch fermentation using immobilised Aspergillus niger on OMW + RP, shaken flasks

Conclusions

The above reported results suggest that OMW can be regarded as an useful residue for different biotechnological applications such as fine chemicals and metabolites production, and biotreatment to improve its characteristics as fertilizers. Nevertheless, it should be taken into account that there are several obstacles and difficulties for OMW upgrading at an industrial scale. These technical constraints include the seasonality of the olive oil production, the highly variable chemical composition of OMW as well as the need for an accurate storage. Therefore it seems that fermentative biotechnologies can be applied successfully for limited amount of OMW, while in the case of huge amounts controlled compost production might be more indicated.

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Acknowledgments: The researches were supported by grants from the Italian Ministry of Research and University - COFIN2000, the Italian CNR/Murst - Project 95/95 "Reflui oleari", the INCA Project "Piano agro alimentare Nazionale" and the European Community - Project "Medusa Water" (ICA3-1999-10032).