

# Microbial Biotechnology in Olive Oil Industry

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## 1. Introduction

Microbial biotechnology is defined as any technological application that uses microbiological systems, microbial organisms, or derivatives thereof, to make or modify products or processes for specific use (Okafor 2007). Current agricultural and industrial practices have led to the generation of large amounts of various low-value or negative cost crude wastes, which are difficult to treat and valorize. Production of agro-industrial waste pollutants has become a major problem for many industries. The olive oil industry generates large amounts of olive mill wastes (OMWs) as by-products that are harmful to the environment (Roig et al. 2006).

However, OMWs have simple and complex carbohydrates that represent a possible carbon resource for fermentation processes. In addition, OMWs generally contain variable quantities of residual oil, the amount of which mainly depends on the extraction process (D'Annibale et al. 2006). Therefore, OMWs could be used as substrate for the synthesis of biotechnological high-value metabolites that their utilization in this manner may help solve pollution problems (Mafakher et al. 2010).

The fermentation of fatty low-value renewable carbon sources like OMWs to production of various added-value metabolites such as lipases, organic acids, microbial biopolymers and lipids, single cell oil, single cell proteins and biosurfactants is very interesting in the sector of industrial microbiology and microbial biotechnology (Darvishi et al. 2009). Thus, more research is needed on the development of new bioremediation technologies and strategies of OMWs, as well as the valorisation by microbial biotechnology (Morillo et al. 2009).

Few investigations dealing with the development of value-added products from these low cost materials, especially OMWs have been conducted. This chapter discusses olive oil microbiology, the most significant recent advances in the various types of biological treatment of OMWs and derived added-value microbial products.

## 2. Olive oil microbiology

In applied microbiology, specific microorganisms employed to remove environmental pollutants or industrial productions have often been isolated from specific sites. For example, when attempting to isolate an organism that can degrade or detoxify a specific target compound like OMW, sites may be sampled that are known to be contaminated by

this material. These environments provide suitable conditions to metabolize this compound by microorganisms.

Recent microbiological research has demonstrated the presence of a rich microflora in the suspended fraction of freshly produced olive oil. The microorganisms found in the oil derive from the olives' carposphere which, during the crushing of the olives, migrate into the oil together with the solid particles of the fruit and micro-drops of vegetation water. Having made their way to the new habitat, some microbic forms succumb in a brief period of time whereas others, depending on the chemical composition of the oil, reproduce in a selective way and the typical microflora of each oil (Zullo et al. 2010).

Newly produced olive oil contains numerous solid particles and micro-drops of olive vegetation water containing, trapped within, a high number of microorganisms that remain during the entire period of olive oil preservation. The microbiological analyses highlighted the presence of yeasts, but not of bacteria and moulds (Ciafardini and Zullo 2002). Several isolated genus of yeasts were identified as *Saccharomyces*, *Candida* and *Williopsis* (Ciafardini et al. 2006).

Some types of newly produced oil are very bitter since they are rich in the bitter-tasting secoiridoid compound known as oleuropein, whereas after a few months preservation, the bitter taste completely disappears following the hydrolysis of the oleuropein. In fact, the taste and the antioxidant capacity of the oil can be improved by the  $\beta$ -glucosidase-producing yeasts, capable of hydrolysing the oleuropein into simpler and less bitter compounds characterized by a high antioxidant activity. Oleuropein present in olive oil can be hydrolysed by  $\beta$ -glucosidase from the yeasts *Saccharomyces cerevisiae* and *Candida wickerhamii*. The absence of lipases in the isolated *S. cerevisiae* and *C. wickerhamii* examined that the yeasts contribute in a positive way to the improvement of the organoleptic characteristics of the oil without altering the composition of the triglycerides (Ciafardini and Zullo 2002).

On the other hand, the presence of some lipase-producing yeast can worsen oil quality through triglycerides hydrolysis. Two lipase-producing yeast strains *Saccharomyces cerevisiae* 1525 and *Williopsis californica* 1639 were found to be able to hydrolyse olive oil triglycerides. The lipase activity in *S. cerevisiae* 1525 was confined to the whole cells as cell-bound lipase, whereas in *W. californica* 1639, it was detected as extracellular lipase. Furthermore, the free fatty acids of olive oil proved to be good inducers of lipase activity in both yeasts. The microbiological analysis carried out on commercial extra virgin olive oil demonstrated that the presence of lipase-producing yeast varied from zero to 56% of the total yeasts detected (Ciafardini et al. 2006).

Some dimorphic species can also be found among the unwanted yeasts present in the olive oil, considered to be opportunistic pathogens to man as they have often been isolated from immunocompromised hospital patients. Recent studies demonstrate that the presence of dimorphic yeast forms in 26% of the commercial extra virgin olive oil originating from different geographical areas, where the dimorphic yeasts are represented by 3-99.5% of the total yeasts. The classified isolates belonged to the opportunistic pathogen species *Candida parapsilosis* and *Candida guilliermondii*, while among the dimorphic yeasts considered not pathogenic to man, the *Candida diddensiae* species (Koidis et al. 2008; Zullo and Ciafardini 2008; Zullo et al. 2010).

Overall, these findings show that yeasts are able to contribute in a positive or negative way to the organoleptic characteristics of the olive oil. Necessary microbiological research carried out so far on olive oil is still needed. From the available scientific data up to now, it is not possible to establish that other species of microorganisms are useful and harmful in stabilizing the oil quality. In particular, it is not known if the yeasts in the freshly produced olive oil can modify some parameters responsible for the quality of virgin olive oil. Further microbiological studies on olive oil proffer to isolation of new microorganisms with biotechnological potential. The OMWs due to their particular characteristics, in addition to fat and triglycerides, sugars, phosphate, polyphenols, polyalcohols, pectins and metals, could provide microorganisms with biotechnological potential and low-cost fermentation substrates. For example, the exopolysaccharideproducing bacterium *Paenibacillus jamilae* (Aguilera et al. 2001) and the obligate alkaliphilic *Alkalibacterium olivoapovlenticus* (Ntougias and Russell 2001) were isolated from olive mill wastes.

### 3. Olive mill waste as renewable low-cost substrates

According to the last report of Food and Agriculture Organisation of the United Nations (FAOSTAT 2009), 2.9 million tons of olive oil are produced annually worldwide, 75.2% of which are produced in Europe, with Spain (41.2%), Italy (20.1%) and Greece (11.4%) being the highest olive oil producers. Other olive oil producers are Asia (12.4%), Africa (11.2%), America (1.0%) and Oceania (0.2%). Olive oil production is a very important economic activity, particularly for Spain, Italy and Greece; worldwide, there has been an increase in production of about 30% in the last 10 years (FAOSTAT 2009).

Multiple methods are used in the production of olive oil, resulting in different waste products. The environmental impact of olive oil production is considerable, due to the large amounts of wastewater (OMWW) mainly from the three-phase systems and solid waste. The three-phase system, introduced in the 1970s to improve extraction yield, produces three streams: pure olive oil, OMWW and a solid cake-like by-product called olive cake or *orujo*. The olive cake, which is composed of a mixture of olive pulp and olive stones, is transferred to central seed oil extraction plants where the residual olive oil can be extracted. The two-phase centrifugation system was introduced in the 1990s in Spain as an ecological approach for olive oil production since it drastically reduces the water consumption during the process. This system generates olive oil plus a semi-solid waste, known as the two-phase olive-mill waste (TPOMW) or *alpeorujo* (Albuquerque et al. 2004; McNamara et al. 2008; Morillo et al. 2009).

The olive oil industry is characterized by its great environmental impact due to the production of a highly polluted wastewater and/or a solid residue, olive skin and stone (olive husk), depending on the olive oil extraction process (Table 1) (Azbar et al. 2004).

Pressure and three-phase centrifugation systems produce substantially more OMWW than two-phase centrifugation, which significantly reduces liquid waste yet produces large amounts of semi-solid or slurry waste commonly referred to as TPOMW. The resulting solid waste is about 800 kg per ton of processed olives. This “*alpeorujo*” still contains 2.5–3.5% residual oil and about 60% water in the two-phase decanter system (Giannoutsou et al. 2004).

Production process	Inputs	Outputs
Traditional process (pressing)	Olives (1 ton)	Oil (~200 kg)
	Wash water (0.1-0.12 m <sup>3</sup> )	Solid waste (~400 kg)
		Wastewater (~600 kg)
	Energy (40-63 kWh)	-
Three-phase process	Olives (1 ton)	Oil (200 kg)
	Wash water (0.1-0.12 m <sup>3</sup> )	Solid waste (500-600 kg)
	Fresh water for decanter (0.5-1.0 m <sup>3</sup> )	Wastewater (800-950 kg)
	Water to polish the impure oil (10 kg)	-
	Energy (90-117 kWh)	-
Two-phase process	Olives (1 ton)	Oil (200 kg)
	Wash water (0.1-0.12 m <sup>3</sup> )	Solid waste (800 kg)
		Wastewater (250 kg)
	Energy (90-117 kWh)	-

Table 1. Inputs and outputs from olive oil industry (Adapted from Azbar et al. 2004)

The average amount of OMWs produced during the milling process is approximately 1000 kg per ton of olives (Azbar et al. 2004). 19.3 million tons of olive are produced annually worldwide, 15% of them used to produce olive oil (FAOSTAT 2009). As an example of the scale of the environmental impact of OMWW, it should be noted that 10 million m<sup>3</sup> per year of liquid effluent from three-phase systems corresponds to an equivalent load of the wastewater generated from about 20 million people. Furthermore, the fact that most olive oil is produced in countries that are deficient in water and energy resources makes the need for effective treatment and reuse of OMWW (McNamara et al. 2008). Overall, about 30 million tons of OMWs per year are produced in the world that could be used as renewable negative or low-cost substrates.

#### 4. Microbial biotechnology applications in olive oil industry

Microbial biotechnology applications in olive oil industry, mainly attempts to obtain added-value products from OMWs are summarised in Fig. 1. OMWs could be used as renewable low-cost substrate for industrial and agricultural microbial biotechnology as well as for the production of energy.

The chemical oxygen demand (COD) and biological oxygen demand (BOD) reduction of OMWs with a concomitant production of biotechnologically valuable products such as enzymes (lipases, ligninolytic enzymes), organic acids, biopolymers and biodegradable plastics, biofuels (bioethanol, biodiesel, biogas and biohydrogen), biofertilizers and amendments will be review.

##### 4.1 Olive mill wastes biological treatment

Ironically, while olive oil itself provides health during its consumption, its by-products represent a serious environmental threat, especially in the Mediterranean, region that accounts for approximately 95% of worldwide olive oil production.

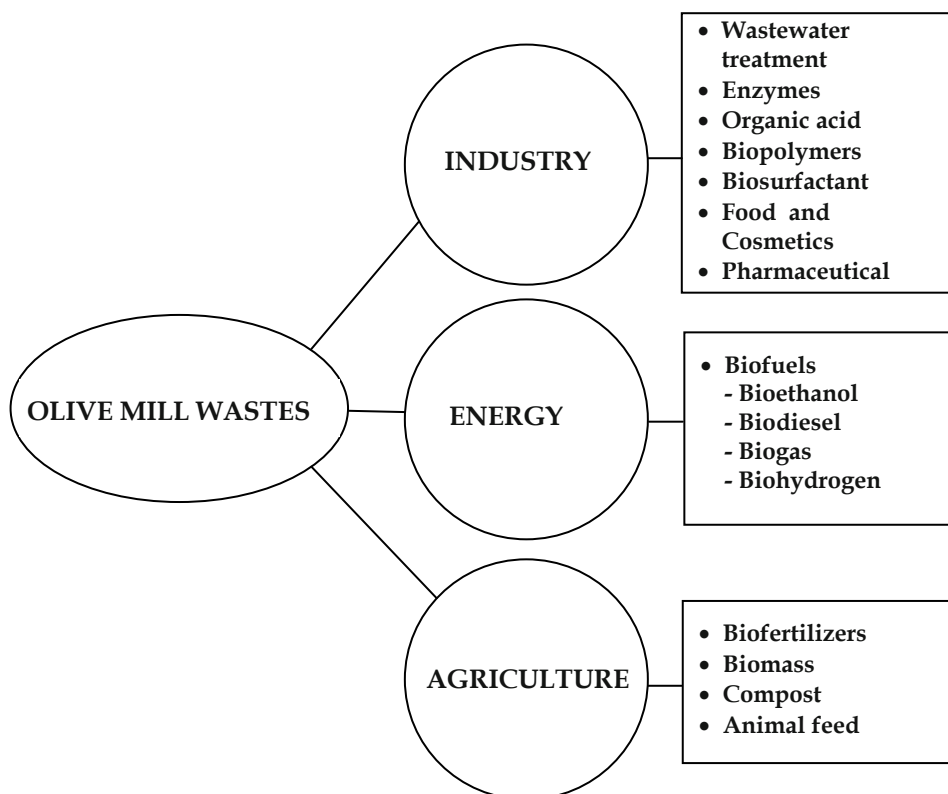


Fig. 1. Potential uses of olive mill wastes in microbial biotechnology.

Moreover, olive oil production is no longer restricted to the Mediterranean basin, and new producers such as Australia, USA and South America will also have to face the environmental problems posed by OMWs. The management of wastes from olive oil extraction is an industrial activity submitted to three main problems: the generation of waste is seasonal, the amount of waste is enormous and there are various types of olive oil waste (Giannoutsou et al. 2004).

OMWs have the following properties: dark brown to black colour, acidic smell, a high organic load and high C/N ratio (chemical oxygen demand or COD) values up to 200 g per litre, a chemical oxygen demand/biological oxygen demand (COD/BOD5) ratio ranging from 2.5 to 5.0, indicating low biodegradability, an acidic pH of between 4 and 6, high concentration of phenolic substances 0.5–25 g per litre with more than 30 different phenolic compounds and high content of solid matter. The organic fraction contains large amounts of proteins (6.7–7.2%), lipids (3.76–18%) and polysaccharides (9.6–19.3%), and also phytotoxic components that inhibit microbial growth as well as the germination and vegetative growth of plants (Roig et al. 2006; McNamara et al. 2008).

OMWs treatment processes tested employ physical, chemical, biological and combined technologies. Several disposal methods have been proposed to treat OMWs, such as traditional decantation, thermal processes (combustion and pyrolysis), agronomic applications (e.g. land spreading), animal-breeding methods (e.g. direct utilisation as animal feed or following protein enrichment), physico-chemical treatments (e.g. precipitation/flocculation, ultrafiltration and reverse osmosis, adsorption, chemical oxidation processes and ion exchange), extraction of valuable compounds (e.g. antioxidants, residual oil, sugars), and biological treatments (Morillo et al. 2009).

Among the different options, biological treatments or bioremediation are considered the most environmentally compatible and the least expensive (Mantzavinos and Kalogerakis 2005). Bioremediation is a treatment process employing naturally microorganisms (bacteria and fungi like yeasts, molds and mushrooms) to break down, or degrade, hazardous substances into less toxic or non-toxic substances. Bioremediation technologies can be classified as *in-situ* (bioaugmentation, bioventing, biosparging) or *ex-situ* (bioreactors, landfarming, composting and biopiles). *In-situ* bioremediation treats the contaminated water or soil where it was found, whereas *ex-situ* bioremediation processes involve removal of the contaminated soil or water to another location prior to treatment (Arvanitoyannis et al. 2008).

Bioremediation occurs either under aerobic or anaerobic conditions. Many aerobic biological processes, technologies and microorganisms have been tested for the treatment of OMWs, aimed at reducing organic load, dark colour and toxicity of the effluents (Table 2). In general, aerobic bacteria appeared to be very effective against some low molecular mass phenolic compounds but are relatively ineffective against the more complex polyphenolics responsible for the dark colouration of OMWs (McNamara et al. 2008). A number of different species of bacteria, yeasts, molds and mushrooms have been tested in aerobic processes to treat OMWs that are listed (Table 2).

A number of studies have utilized bacterial consortia for bioremediation of OMWW. Bioremediation of OMWW using aerobic consortia has been quite successful in these studies, achieving significant reductions in COD (up to 80%) and the concentration of phytotoxic compounds, and complete removal of some simple phenolics (Zouari and Ellouz 1996; Benitez et al. 1997). A combined bacterial-yeast system of *Pseudomonas putida* and *Yarrowia lipolytica* were used to degrade OMWW (De Felice et al. 1997).

Anaerobic bioremediation of OMWs has employed, almost exclusively, uncharacterized microbial consortia derived from municipal and other waste facilities. This technique presents a number of advantages in comparison to the classical aerobic processes: (a) a high degree of purification with high-organic-load feeds can be achieved; (b) low nutrient requirements are necessary; (c) small quantities of excess sludge are usually produced; and (d) a combustible biogas is generated (Dalis et al. 1996; Zouari and Ellouz 1996; Borja et al. 2006). Combined aerobic-anaerobic systems have also been used effectively in the bioremediation of OMWs (Hamdi and Garcia 1991; Borja et al. 1995). Aerobic processes are applied waste streams of OMWs with low organic loads, whereas anaerobic processes are applied waste streams with high organic loads.

Microorganism	Waste type	Method	Results	Reference
<b>Bacteria</b>				
<i>Azotobacter vinelandii</i>	OMWW	Culture in OMWW	70% COD reduction	(Piperidou et al. 2000)
<i>Bacillus pumilus</i>	OMWW	Culture in OMWW	50% phenol reduction	(Ramos-Cormenzana et al. 1996)
<i>Lactobacillus paracasei</i>	OMWW	Culture in OMWW with cheese whey's	47% colour removal 22.7% phenol reduction	(Aouidi et al. 2009)
<i>Lactobacillus plantarum</i>	OMWW	Culture in OMWW	Increase of simple polyphenols content	(Kachouri and Hamdi 2004)
<i>Pseudomonas putida</i> and <i>Ralstonia</i> spp.	OMWW	Culture two strains in OMWW	Biodegradation of aromatic compounds	(Di Gioia et al. 2001)
<b>Yeasts</b>				
<i>Candida boidinii</i>	TPOMW	Fed-batch microcosm	57.7% phenol reduction	(Giannoutsou et al. 2004)
<i>Candida cylindracea</i>	OMWW	Culture in OMWW	reduction of phenolic compounds and COD	(Gonçalves et al. 2009)
<i>Candida holstii</i>	OMWW	Culture in OMWW	39% phenol reduction	(Ben Sassi et al. 2008)
<i>Candida oleophila</i>	OMWW	Bioreactor batch culture with OMWW	Tannins content reduction	(Peixoto et al. 2008)
<i>Candida rugosa</i>	OMWW	Culture in OMWW	reduction of phenolic compounds and COD	(Gonçalves et al. 2009)
<i>Candida tropicalis</i>	OMWW	Culture in OMWW	62.8% COD reduction 51.7% phenol reduction	(Fadil et al. 2003)
<i>Geotrichum candidum</i>	OMWW	Culture in bioreactors with OMWW	60% COD reduction	(Asses et al. 2009)
<i>Geotrichum candidum</i>	TPOMW	Fed-batch microcosm	57% phenol reduction	(Giannoutsou et al. 2004)
<i>Saccharomyces</i> spp.	TPOMW	Fed-batch microcosm	61% phenol reduction	(Giannoutsou et al. 2004)
<i>Trichosporon cutaneum</i>	OMWW	Culture in OMWW	removal of mono- and polyphenols	(Chtourou et al. 2004)
<i>Yarrowia lipolytica</i>	OMWW	Culture in OMWW	20-40% COD reduction < 30% phenol reduction	(Lanciotti et al. 2005)
<i>Yarrowia lipolytica</i> W29	OMWW	Culture in OMWW	67-82% COD reduction	(Wu et al. 2009)
<b>Molds</b>				
<i>Aspergillus niger</i>	OMWW	Culture in OMWW	73% COD reduction 76% phenol reduction	(García García et al. 2000)
<i>Aspergillus</i> spp.	OMWW	Culture in OMWW	52.5% COD reduction 44.3% phenol reduction	(Fadil et al. 2003)
<i>Aspergillus terreus</i>	OMWW	Culture in OMWW	63% COD reduction 64% phenol reduction	(García García et al. 2000)
<i>Fusarium oxysporum</i>	DOR	Culture in DOR	16-71% phytotoxicity reduction	(Sampedro et al. 2007a)
<i>Penicillium</i> spp.	OMWW	Culture in OMWW	38% COD reduction 45% phenol reduction	(Robles et al. 2000)

Microorganism	Waste type	Method	Results	Reference
<i>Phanerochaete chrysosporium</i>	OMWW	Culture in bioreactors with OMWW	75% COD reduction 92% phenol reduction	(García García et al. 2000)
<i>Phanerochaete chrysosporium</i>	TPOMW	Culture in TPOMW	9.2% TOC reduction 14.5% phenol reduction	(Sampedro et al. 2007b)
<i>Phanerochaete flavido-alba</i>	OMWW	Culture in bioreactors with OMWW	52% phenol reduction	(Blánquez et al. 2002)
<i>Phanerochaete flavido-alba</i> Mushrooms	TPOMW	Solid-state culture	70% phenol reduction	(Linares et al. 2003)
<i>Coriolopsis rigida</i>	TPOMW	Culture in OMWW	9% TOC reduction 89% phenol reduction	(Sampedro et al. 2007b)
<i>Coriolopsis polyzona</i>	OMWW	Culture in OMWW	75% colour removal	(Jaouani et al. 2003)
<i>Coriolus versicolor</i>	OMWW	Culture in OMWW	65% COD reduction 90% phenol reduction	(Yesilada et al. 1997)
<i>Funalia trogii</i>	OMWW	Culture in OMWW	70% COD reduction 93% phenol reduction	(Yesilada et al. 1997)
<i>Lentinula edodes</i>	OMWW	Culture in OMWW	65% COD reduction 88% phenol reduction	(D'Annibale et al. 2004)
<i>Lentinus tigrinus</i>	OMWW	Culture in OMWW	Effective in decolorization	(Jaouani et al. 2003)
<i>Pleurotus eryngii</i>	OMWW	Culture in OMWW	> 90% phenol reduction	(Sanjust et al. 1991)
<i>Pleurotus floridae</i>	OMWW	Culture in OMWW	> 90% phenol reduction	(Sanjust et al. 1991)
<i>Pleurotus ostreatus</i>	OMWW	Culture in OMWW	100% phenol reduction	(Tomati et al. 1991)
<i>Pleurotus ostreatus</i>	OMWW	Culture in bioreactors with OMWW	Phenol reduction nearly complete	(Aggelis et al. 2003)
<i>Pleurotus ostreatus</i>	OMWW	Solid-state culture	67% phenol reduction	(Fountoulakis et al. 2002)
<i>Pleurotus ostreatus</i>	TPOMW	Plastic bag	22% TOC reduction 90% phenol reduction	(Saavedra et al. 2006)
<i>Pleurotus pulmonarius</i>	TPOMW	Culture in TPOMW	9.7% TOC reduction 66.2% phenol reduction	(Sampedro et al. 2007b)
<i>Pleurotus sajor-caju</i>	OMWW	Culture in OMWW	> 90% phenol reduction	(Sanjust et al. 1991)
<i>Pleurotus spp.</i>	OMWW	Culture in OMWW	76% phenol reduction	(Tsioulpas et al. 2002)
<i>Phlebia radiata</i>	TPOMW	Culture in TPOMW	13% TOC reduction 95.7% phenol reduction	(Sampedro et al. 2007b)
<i>Poria subvermispora</i>	TPOMW	Culture in TPOMW	13.2% TOC reduction 72.3% phenol reduction	(Sampedro et al. 2007b)
<i>Pycnoporus cinnabarinus</i>	TPOMW	Culture in TPOMW	7.6% TOC reduction 88.7% phenol reduction	(Sampedro et al. 2007b)
<i>Pycnoporus coccineus</i>	OMWW	Culture in OMWW	Effective in decolorization	(Jaouani et al. 2003)

OMWW: olive oil wastewater, TPOMW: two-phase olive-mill waste, COD: chemical oxygen demand, TOC: Total organic carbon, DOR: olive-mill dry residue.

Table 2. Aerobic treatment of OMWs by microorganisms



In general, available scientific information shows that fungi are more effective than bacteria at degrading both simple phenols and the more complex phenolic compounds present in olive-mill wastes. For example, several species of the genus *Pleurotus* were found to be very effective in the degradation of the phenolic substances present in OMWs (Hattaka 1994). For OMWs biotreatment in large-scale, the use of filamentous fungi have considerable problems because of the formation of fungal pellets and other aggregations. The use of yeast in bioreactors could be a way forward to overcome this limitation.

## 4.2 Enzymes

In recent years, many researchers have utilized OMWs as growth substrates for microorganisms, obtaining a reduction of the COD level, together with enzyme production. The addition of nutrients can modify the pattern of degrading enzymes production by specific microorganisms from OMWs. (De la Rubia et al. 2008).

Lipases (EC 3.1.1.3) are among the most important classes of industrial enzymes (Darvishi et al. 2009). Many microorganisms are known as potential producers of lipases including bacteria, yeast, and fungi. Several reviews have been published on microbial lipases (Arpigny and Jaeger 1999; Vakhlu and Kour 2006; Treichel et al. 2010).

Lipolytic fungal species, such as *Aspergillus oryzae*, *Aspergillus niger*, *Candida cylindracea*, *Geotrichum candidum*, *Penicillium citrinum*, *Rhizopus arrhizus* and *Rhizopus oryzae* were preliminarily screened for their ability to grow on undiluted OMWW and to produce extracellular lipase. A promising potential for lipase production was found by *C. cylindracea* NRRL Y-17506 on OMWW (D'Annibale et al. 2006).

Among the different yeasts tested, the *Y. lipolytica* most adapted to grow on OMW. the *Y. lipolytica* strains were produced 16-1041 U/L of lipase on OMWs and also reduced 1.5-97% COD, 80% BOD and 0-72% phenolic compounds of OMWs (Fickers et al. 2011). The yeasts *Saccharomyces cerevisiae* and *Candida wickerhamii* produce  $\beta$ -glucosidase enzyme to hydrolyse oleuropein present in olive oil (Ciafardini and Zullo 2002).

Olive oil cake (OOC) used as a substrate for phytase production in solid-state fermentation using three strains of fungus *Rhizopus* spp. OOC of initial moisture 50% was fermented at 30°C for 72 hours and inoculated with *Rhizopus oligosporus* NRRL 5905, *Rhizopus oryzae* NRRL 1891 and *R. oryzae* NRRL 3562. The results indicated that all three *Rhizopus* strains produced very low titers of enzyme on OOC (Ramachandran et al. 2005).

Tannase could be utilized as an inhibitor of foam in tea production, clarifying agent in beer and fruit juices production, in the pharmaceutical industry and for the treatment of tannery effluents. *Aspergillus niger* strain HA37, isolated from OMW, was incubated on a synthetic medium containing tannic acid and on diluted OMW on a rotary shaker at 30°C. On the medium containing tannic acid, tannase production was 0.6, 0.9 and 1.5 U/ml at 0.2%, 0.5% and 1% initial tannic acid concentration, respectively (Aissam et al. 2005).

Extracellular ligninolytic enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac) were produced by the white rot fungus *Phanerochaete flavid-alba* with a concomitant decoloration and decrease in phenolic content and toxicity of OMWW. Laccase was the sole ligninolytic enzyme detected in cultures containing monomeric aromatic compounds. Laccase and an acidic manganese-dependent peroxidase (MnPA, pI

62.8) were accumulated in cultures with OMWW or polymeric pigment. Furthermore, modified manganese-dependent peroxidases were observed mainly in OMWW-supplemented cultures. Laccase was more stable to the effect of OMWW toxic components and was accumulated in monomeric aromatic-supplemented cultures, suggesting a more important role than manganese-dependent peroxidases in OMWW detoxification. Alternatively, MnPA accumulated in cultures containing the polymeric pigment seemed to be more essential than laccase for degradation of this recalcitrant macromolecule by *P. flavido-alba*. (Ruiz et al. 2002).

Enzyme laccase, produced by fungus *Pycnoporus coccineus*, is responsible for OMWW decolorization and decrease COD and phenolic compounds. The highest laccase level was 100 000 U/l after 45 incubation-days. The enzyme was stable at pH 7, at room temperature and showed a half-life of 8 and 2 h at 50 and 60°C, respectively (Jaouani et al. 2005). In order to decolourise OMWW efficiently, production and differential induction of ligninolytic enzymes by the white rot *Corioloropsis polyzona*, were studied by varying growth media composition and/or inducer addition (Jaouani et al. 2006). The production of lignin peroxidase (LiP), manganese peroxidase (MnP) and lipases by *Geotrichum candidum* were performed in order to control the decolourisation and biodegradation of OMWW (Asses et al. 2009).

Sequential batch applications starting with adapted *Trametes versicolor* FPRL 28A INI and consecutive treatment with *Funalia trogii*, possible to remove significant amount of total phenolics content and higher decolorization as compared to co-culture applications. Also highest laccase and manganese peroxidase activities were obtained with *F. trogii* (Ergul et al. 2010).

### 4.3 Organic acids

Some *Y. lipolytica* strains are good candidates for the reduction of the pollution potential of OMWW and for the production of enzymes and metabolites such as lipase and citric acid (Lanciotti et al. 2005). *Y. lipolytica* strain ACA-DC 50109 demonstrated efficient growth on media containing mixtures of OMWs and commercial glucose. In nitrogen-limited diluted and enriched with high glucose quantity OMWW, a noticeable amount of total citric acid was produced. The ability of *Y. lipolytica* to grow on relatively high phenolic content OMWs based media and produce in notable quantities citric acid, make this non-conventional yeast worthy for further investigation (Papanikolaou et al. 2008).

The biochemical behavior and simultaneous production of valuable metabolites such as lipase, citric acid (CA), isocitric acid (ICA) and single-cell protein (SCP) were investigated by *Y. lipolytica* DSM 3286 grown on various plant oils as sole carbon source. Among tested plant oils, olive oil proved to be the best medium for lipase and CA production. The *Y. lipolytica* DSM 3286 produced  $34.6 \pm 0.1$  U/ml of lipase and also CA, ICA and SCP as by-product on olive oil medium supplemented with yeast extract. Urea, as organic nitrogen, was the best nitrogen source for CA production. The results of this study suggest that the two biotechnologically valuable products, lipase and CA, could be produced simultaneously by this strain using renewable low-cost substrates such as plant oils in one procedure (Darvishi et al. 2009).

In the other study, a total of 300 yeast isolates were obtained from samples of agro-industrial wastes, and M1 and M2 strains were investigated for their ability to produce lipase and

citric acid. Identification tests showed that these isolates belonged to the species *Y. lipolytica*. M1 and M2 strains produced maximum levels of lipase on olive oil, and high levels of citric acid on citric acid fermentation medium (Mafakher et al. 2010).

The highest oxalic acid quantity (5 g/l) was obtained by the strain *Aspergillus* sp. ATHUM 3482 on waste cooking olive oil medium. For strain *Penicillium expansum* NRRL 973 on this medium sole organic acid detected was citric acid with maximum concentration achieved 3.5 g/l (Papanikolaou et al. 2011).

#### 4.4 Biopolymers and biodegradable plastics

Exopolysaccharides (EPSs) often show clearly identified properties that form the basis for a wide range of applications in food, pharmaceuticals, petroleum, and other industries. The production of these microbial polymers using OMWW as a low-cost fermentation substrate has been proposed (Ramos-Cormenzana et al. 1995). This approach could reduce the cost of polymer production because the substrate is often the first limiting factor. Moreover, OMWW contains free sugars, organic acids, proteins and other compounds such as phenolics that could serve as the carbon source for polymer production, if the chosen microorganism is able to metabolize these compounds (Fiorentino et al. 2004).

Xanthan gum, an extracellular heteropolysaccharide produced by the bacterium *Xanthomonas campestris* has been obtained from OMWW. Growth and xanthan production on dilute OMWW as a sole source of nutrients were obtained. Addition of nitrogen and/or salts led to significantly increased xanthan yields with a maximum of 7.7g/l (Lopez and Ramos-Cormenzana 1996).

The fungus *Botryosphaeria rhodina* has been used for the production of  $\beta$ -glucan from OMWW with yield of 17.2 g/l and a partial dephenolisation of the substrate (Crognale et al. 2003). A metal-binding EPS produced by *Paenibacillus jamilae* from OMWs. Maximum EPS production (5.1 g/l) was reached in batch culture experiments with a concentration of 80% of OMWW as fermentation substrate (Morillo et al. 2007).

Polyhydroxyalkanoates (PHAs) are reserve polyesters that are accumulated as intracellular granules in a variety of bacteria. Of these polymers, poly- $\beta$ -hydroxybutyrate (PHB) is the most common. Since the physical properties of PHAs are similar to those of some conventional plastics, the commercial production of PHAs is of interest. However, these biodegradable and biocompatible 'plastics' are not priced competitively at the present, mainly because the sugars (i.e. glucose) used as fermentation feed-stocks are expensive. Finding a less expensive substrate is, therefore, a major need for a wide commercialisation of these products. Large amounts of biopolymers containing  $\beta$ -hydroxybutyrate (PHB) and copolymers containing  $\beta$ -hydroxyvalerate (P[HB-co-HV]) are produced by *Azotobacter chroococcum* in culture media amended with alpechin (wastewater from olive oil mills) as the sole carbon source (Pozo et al. 2002).

#### 4.5 Biosurfactants

Rhamnolipids, typical biosurfactants produced by *Pseudomonas aeruginosa*, consist of either one or two rhamnose molecules, linked to one or two fatty acids of saturated or unsaturated alkyl chain between C8 and C12. The *P. aeruginosa* 47 T2 produced two main rhamnolipid

homologs, (Rha-C10-C10) and (Rha-Rha-C10-C10), when grown in olive oil waste water or in waste frying oils consisting from olive/sunflower (Pantazaki et al. 2010).

#### 4.6 Food and cosmetics

A few edible fungi, especially species of *Pleurotus*, can also be grown using OMWs as the source of nutrients by the application of different strategies. Recently the cultivation of the oyster mushroom *Pleurotus ostreatus* was suggested on OMWW (KalmIs et al. 2008).

Hydroxy fatty acids (HFAs) are known to have special properties such as higher viscosity and reactivity compared to other normal fatty acids. These special properties used in a wide range of applications including resins, waxes, nylons, plastics, lubricants, cosmetics, and additives in coatings and paintings. Some HFAs are also reported as antimicrobial agents against plant pathogenic fungi and some of food-borne bacteria. Bacterium *Pseudomonas aeruginosa* PR3 produce several hydroxy fatty acids from different unsaturated fatty acids. Of those hydroxy fatty acids, 7,10-dihydroxy-8(E)-octadecenoic acid (DOD) was efficiently produced from oleic acid by strain PR3. DOD production yield from olive oil was 53.5%. Several important environmental factors were also tested. Galactose and glutamine were optimal carbon and nitrogen sources, and magnesium ion was required for DOD production from olive oil (Suh et al. 2011).

#### 4.7 Pharmaceutical

The enhancing effect of various concentrations of 18 oils and a silicon antifoam agent on erythromycin antibiotic production by *Saccharopolyspora erythraea* was evaluated in a complex medium containing soybean flour and dextrin as the main substrates. The highest titer of erythromycin was produced in medium containing 55 g/l black cherry kernel oil (4.5 g/l). The titers of erythromycin in the other media were also recorded, with this result: black cherry kernel > water melon seed > melon seed > walnut > rapeseed > soybean > (corn = sesame) > (olive = pistachio = lard = sunflower) > (hazelnut = cotton seed) > grape seed > (shark = safflower = coconut). In medium supplement with olive oil, concentration of erythromycin was  $2.15 \pm 0.03$  and  $2.75 \pm 0.02$  g/l before and after optimization, respectively (Hamed et al. 2004).

#### 4.8 Biofuels

It is widely recognised that clean and sustainable technologies, e.g. biofuels, are only part of the solution to the impending energy crisis. Comparing the heating value of biohydrogen (121 MJ/kg), methane (50.2 MJ/kg) and bioethanol (23.4 MJ/kg), the production of hydrogen will be more attractive. Nevertheless, the use of biohydrogen is still not practical and thus there is a higher demand for methane and bioethanol because they can be used directly as biofuels with the existing technology (Duerr et al. 2007).

Ethanol production as a biofuel from OMWs with high content of organic matter is interesting (Li et al. 2007). The two main components of TPOMW (stones and olive pulp) as substrates were used to production of ethanol by a simultaneous saccharification and fermentation process (Ballesteros et al. 2001). In recent study, an enzymatic hydrolysis and subsequent glucose fermentation by baker's yeast were evaluated for ethanol production

using dry matter of TPOMW. The results showed that yeasts could effectively ferment TPOMW without nutrient addition, resulting in a maximum ethanol production of 11.2 g/l and revealing the tolerance of yeast to TPOMW toxicity (Georgieva and Ahring 2007).

Anaerobic digestion is a biological process in which organic material is broken down by microorganisms. Unlike composting, the process occurs in the absence of air. Anaerobic digestion is a practical alternative for the treatment of TPOMW, which produces biogas. The TPOMW is biodegradable by anaerobic digestion at mesophilic temperatures in stirred tank reactors, with COD removal efficiencies in the range of 72–89% and an average methane yield coefficient of 0.31 dm<sup>3</sup> CH<sub>4</sub> per gramme COD removed. Hydrogen production was coupled with a subsequent step for methane production, giving the potential for production of 1.6 mmol H<sub>2</sub> per gramme of TPOMW (Borja et al. 2006).

The OMW used as a sole substrate for the production of hydrogen gas with *Rhodobacter sphaeroides* O.U.001. The bacterium was grown in diluted OMW media, containing OMW concentrations between 20% and 1% in a glass column photobioreactor at 32°C. The released gas was nearly pure hydrogen, which can be utilized in electricity producing systems, such as fuel cells. The maximum hydrogen yield (145 ml) was obtained with 3% and 4% OMW concentrations. However, as well as hydrogen production, COD, BOD and phenol reduction from OMW were recorded (Eroglu et al. 2004).

Biodiesel, a fuel that can be made from renewable biological sources such as vegetable oils or animals fats, has been recognized recently as an environment friendly alternative fuel for diesel engines. Among liquid biofuels, biodiesel derived from vegetable oils is gaining ground and market share as diesel fuel in Europe and the USA. A mixture of frying olive oil and sunflower oil for the production of methyl esters that can be used as biodiesel (Encinar et al. 2005).

#### 4.9 Biofertilizers

As far as agronomic use of the waste is concerned, the idea of re-using microbially treated OMWW as fertiliser has been also proposed. An acidogenic fungus strain *Aspergillus niger* was grown in either free or immobilised form on OMWW with rock phosphate added in order to solubilise it. It was found that at optimized process conditions (moisture 70%; corn steep liquor as a nitrogen source; inoculum size of 3–4 ml; presence of slow release phosphate), the filamentous fungal culture was able to produce 58 U phytase/g dry substrate and 31 mg soluble phosphate per flask (Vassilev et al. 1997; Vassilev et al. 2007).

#### 4.10 Biomass

Already 50 years ago, the production of yeast biomass using OMWW in a chemostat for use in industrial applications was reported. The microbial biomass produced from OMW fermentations either as an additive to animal feed or to improve its agronomic use. For example, an intense degradation of most polluting substances of OMWW and the production of biomass could be used as an animal feed integrator using a chemical-biological method (Morillo et al. 2009).

Seven strains of *Penicillium* isolated from OMWW disposal ponds were tested for biomass production and biodegradation of undiluted OMWW. Best results were obtained by using

strain P4, which formed 21.50 g (dry weight) of biomass per litre of undiluted wastewater after 20 days of cultivation. This and other strains also carried out an outstanding reduction of the COD and the phenolic content of OMW, as well as a pH raise (Robles et al. 2000). The *Y. lipolytica* strain ATCC 20255 strain has been effective in the treatment of OMWW that yield of the biomass (single-cell protein) was 22.45 g/l (Scioli and Vollaro 1997).

Microalgal biomass is as a potential source of proteins, carbohydrates, pigments, lipids, and hydrocarbons. In addition, the biomass can be used as a low-release fertilizer. This chemical composition has great variation, depending on the species, culture medium, and the operating conditions. Microalga *Scenedesmus obliquus* was used to biomass production from rinse water (RW) from two-phase centrifugation in the olive-oil extraction industry. Maximum specific growth rate, 0.044 per hour was registered in the culture with 5% RW and reduces 67.4% BOD when operating with 25% RW. The greater specific rate of protein synthesis during the exponential phase was 3.7 mg/g h to 50% RW (Hodaifa et al. 2008).

Microbial lipid (single cell oil or SCO) production has been an object of research and industrial interest for more than 60 years. Microorganisms can store triacylglycerol (TAG) as intracellular oil droplets. *Gordonia* sp. DG accumulated more than 50% lipid with most tested wastes, while only 29, 36 and 41% was accumulated in presence of olive mill waste, hydrolyzed barely seeds and wheat bran, respectively (Gouda et al. 2008).

Carbon-limited cultures were performed on waste cooking olive oil, added in the growth medium at 15 g/l, and high biomass quantities were produced up to 18 g/l. Cellular lipids were accumulated in notable quantities in almost all cultures. *Aspergillus* sp. ATHUM 3482 accumulated lipid up to 64% (w/w) in dry fungal mass. In parallel, extracellular lipase activity was quantified, and it was revealed to be strain and fermentation time dependent, with a maximum quantity of 645 U/ml being obtained by *Aspergillus niger* NRRL 363. Storage lipid content significantly decreased at the stationary growth phase (Papanikolaou et al. 2011).

#### 4.11 Compost

Composting is the aerobic processing of biologically degradable organic waste to produce a reasonably stable, granular material and valuable plant nutrients. Composting removes the phytotoxicity of the residues within a few weeks and allows the subsequent enrichment of croplands with nutrients that were originally taken up by olive tree cultivation. Composting of OMWs requires the proper adjustment of pH, temperature, moisture, oxygenation and nutrients, thereby allowing the adequate development of the microbial populations (Arvanitoyannis and Kassaveti 2007).

Among the possible technologies for recycling the TPOMW, composting is gaining interest as a sustainable strategy to recycle this residue for agricultural purposes. Dry olive cake alone or mixed with municipal biosolids vermicomposted for 9 months in order to examine the behaviour of three specific humic substance-enzyme complexes. During the process,  $\beta$ -glucosidase synthesis and release was observed, whereas no significant change in urease and phosphatase activity was recorded. The vermicomposted olive cake, alone or in blends with biosolids, could be effectively used as amendment due to their ability to reactivate the C, P and N-cycles in degraded soils for regeneration purposes (Benitez et al. 2005).

Olive pomace, a wet solid waste from the three-phase decanters and presses, was composted by using a reactor for a period of 50 days in four bioreactors. Urea was added to

adjust C/N ration between 25-30. At the end of 50 days of composting using *Trichoderma harzianum* and *Phanerochaete chrysosporium*, cellulose and lignin were highly degraded. It was found that after 30 days, *P. chrysosporium* and *T. harzianum* degraded approximately 71.9% of the lignin and 59.25% of the cellulose, respectively (Haddadin et al. 2009).

#### 4.12 Animal feed

Treated OMW may find applications as a raw material in various biotechnological processes or as animal food. The appropriate utilization of by-products in animal nutrition can improve the economy and the efficiency of agricultural, industrial and animal production.

The olive pomace was alkali-treated, transferred to culture flasks and inoculated with the above fungi. After inoculation, the fermentation process was carried out at 25°C for 60 days. The results indicated that *Oxysporus* spp. degraded lignin up to 69%, whereas *Phanerochaete chrysosporium* and *Schizophyllum commune* delignified olive pomace 60% and 53%, respectively. However, the potential use of treated olive pomace as a feed for poultry is still under investigation. The fermented olive pomace can be used as a feed for the poultry industry (Haddadin et al. 2002).

### 5. Conclusion

The olive oil industry generates large amounts of olive mill wastes (OMWs) as by-products that are harmful to the environment. About 30 million tons of OMWs per year are produced in the world. Thus, more research is needed on the development of new bioremediation technologies and strategies of OMWs, as well as the valorisation by microbial biotechnology. The fermentation of fatty low-value renewable carbon sources like OMWs aiming at the production of various added-value metabolites is a noticeable interest in the sector of industrial microbiology and microbial biotechnology.

Microbiological studies show that presence of yeasts, but not of bacteria and moulds in the olive oil. Some of the yeasts are considered useful as they improve the organoleptic characteristics of the oil during preservation, whereas others are considered harmful as they can damage the quality of the oil through the hydrolysis of the triglycerides. Olive oil and its by-products could provide a source of low-cost fermentation substrate and isolation of new microorganisms with biotechnological potentials.

OMWs treatment processes that employ physical, chemical, biological and combined technologies have been tested. Among the different options, biological treatments or bioremediation are considered the most environmentally compatible and the least expensive. Bioremediation occurs either under aerobic or anaerobic conditions. Aerobic processes are applied waste streams of OMWs with low organic loads, whereas anaerobic processes are applied waste streams with high organic loads.

Microbial biotechnology strategies and methods in olive oil industry were used to reduce chemical oxygen demand (COD), biological oxygen demand (BOD) and phenolic compounds of OMWs with a concomitant production of biotechnologically valuable products such as enzymes (lipases,  $\beta$ -glucosidase, phytase, tannase, lignin peroxidase, manganese peroxidase, laccase and pectinases), organic acids (citric, isocitric and oxalic acids), biopolymers and biodegradable plastics (xanthan,  $\beta$ -glucan and polyhydroxyalkanoates), biosurfactants, food

and cosmetics, pharmaceutical, biofuels (bioethanol, biogas, biohydrogen and biodiesel), biofertilizers and amendments, biomass (single cell proteins, single cell oil), compost and animal feed.

What has been discussed in this review indicate that microbial biotechnology can be used for the production of value-added products from olive oil by-products and can facilitate a significant reduction in waste treatment costs.

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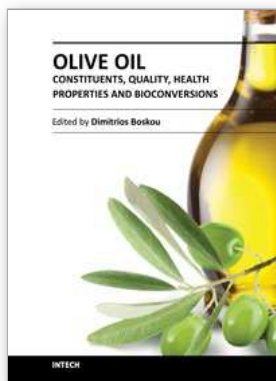
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The health-promoting effects attributed to olive oil, and the development of the olive oil industry have intensified the quest for new information, stimulating wide areas of research. This book is a source of recently accumulated information. It covers a broad range of topics from chemistry, technology, and quality assessment, to bioavailability and function of important molecules, recovery of bioactive compounds, preparation of olive oil-based functional products, and identification of novel pharmacological targets for the prevention and treatment of certain diseases.

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