Chapter 3

Bioremediation of Olive Mill Wastewater by Yeasts – A Review of the Criteria for the Selection of Promising Strains

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1. Introduction

The cultivation of olive trees and the production and use of olive oil has been a well-known and established practice in the Mediterranean region for more than 7000 years [1].

Olive is the most extensively cultivated fruit crop in the world, counting 9,2 million hectares of area harvested in 2009 and its cultivation area has tripled in the past 50 years [2].

Over the last decade, olive oil production has increased about 40% worldwide and Europe obtained an increase of 45% in production [3], due to its high dietetic and nutritional value (the high smoke point-210 °C- and an excellent lipid profile as the proportion of saturated, mono-unsaturated and poly-unsaturated fatty acids is 14:77:9) [1]. It is generally accepted that olive oil consumption brings benefits to human health, such as reduction of risk factors of coronary heart disease, prevention of several types of cancers, and modifications of immune and inflammatory responses [4].

Mediterranean Countries produce more than 98% of the world's olive oil, which is estimated at over 2.5 million metric tons *per* year. Three quarters of the annual production in the world comes from European Union, in particular Spain (36% of the worldwide production), Italy (24% of the world's total), and Greece (17% of the global production) [3].

These data reflect the importance of olive oil sector in the Mediterranean area and consequently the magnitude of the problems related with the disposal of large amounts of olive mill wastewaters (OMW). Many studies report that OMW is a major pollutant to surface and

ground water resources in the Mediterranean basin [5]. 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 OMW [6].

OMW (*acque reflue* in Italy; *alpechin* in Spain; *katsigaros* in Greece; *zebar* in Arab countries) is a dark red to black-coloured, mildly acidic liquid of high conductivity, obtained from mechanical olive processing during olive oil production [7]. Only in the Mediterranean area, OMW generation varies between 10×10^6 and 30×10^6 m^3 [3]. In general, the quality and quantity of OMW, and consequently the environmental impact, depends on many factors, such as the type of olives, the type of soil, the cultivation system and the production process [8]. The traditional cold press method typically generates about 50% of OMW relative to the initial weight of the olives, while the continuous centrifugation process generates 80–110% of OMW due to the continuous washing of the olive paste with warm water prior to oil separation from the paste [9].

The problems connected with OMW depend on their high chemical oxygen demand (COD) (up to 100 g/L), biological oxygen demand (BOD) (13-46 g/L), low pH (4–5), and other recalcitrant organic compounds, such as water-soluble phenols (hydroxytyrosol, tyrosol, catechol, methylcatechol, caffeic acid, vanillic acid, *p*-coumaric acid, etc.) and polyphenols originating from the olives [1]; the conductivity of OMW is around 18.0 mmhos/cm, while the average value of TSS (total suspended solids) and VSS (volatile suspended solids) are respectively 40-60 g/L and 30-50 g/L, with a TOC (total organic matter) of 10-30 g/L and TN (total nitrogen) of 0.6-1.4 g/L [1]. OMW contains also other mineral elements (P_2O_5, K_2O, Na, Mg, Fe, Cu etc.), but the amount of these compounds is greatly variable.

OMW is one of the most complex agro-industrial effluent [10]. Most of the problems associated with OMW pollution can be attributed to the phenolic fraction [11]. Monomeric phenols of OMW have been associated with the phytotoxic and antimicrobial properties of these wastewaters, while the dark brownish color of OMW, which is particularly recalcitrant to decolorization, has been attributed to the polymerization of tannins and low molecular weight phenolic compounds [12].

OMW are often poured into the soil (up to 50 m^3 *per* hectar in Italy) or disposed of in sewage, causing soil and water pollution. In fact untreated OMW are able to change the microbial composition of the soil through their antibacterial activity and produce phytopathogenic effects due to their high toxicity [13] (i.e. 1 m^3 of OMW is equivalent to 100-200 m^3 of domestic sewage) [1]. Due to the high organic load of OMW, it may contribute significantly to eutrophication of recipients in which fluid exchange rates are low (closed gulfs, estuaries, lakes, etc.). An additional adverse impact of OMW on the environment is the aesthetic degradation caused by its strong odour and dark coloration [14]. Furthermore, environmental regulations and enforcements have become more and more strict [15], thus there is the need of new guidelines to manage these wastes; in fact the most olive oil is produced in Countries that are deficient in water and energy [3].

For these reasons, in recent years, several disposal methods have been proposed such as thermal processes (combustion and pyrolysis), physico-chemical treatments (e.g. precipita-

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tion/flocculation, ultrafiltration and reverse osmosis, adsorption, chemical oxidation processes and ion exchange), extraction of valuable compounds (e.g. antioxidants, residual oil, sugars), agronomic applications (e.g. land spreading), animal-breeding methods (e.g. direct utilisation as animal feed or following protein enrichment) and biological treatments [8]. Among the different options, biological treatments are considered the most environmentally compatible and the least expensive methods [9].

Two different approaches have been developed for OMW biological treatment: aerobic and anaerobic processes [16]. Some drawbacks of OMW bioremediation under anaerobic conditions are the difficulties to remove high-molecular weight phenols [16], the need for a long period for the adaptation of microorganisms, the high costs for the storage [17]; on the other hand, the aerobic protocols do not show these limits.

Early studies focused on the use of specific bacterial species, including *Bacillus pumilus* [18], *Arthrobacter* sp. [19], *Azotobacter vinelandii* [20], *Azotobacter chroococcum* [21], *Pseudomonas putida* and *Ralstonia* sp. [22] and various bacterial consortia [23-25]. 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 OMW [3].

Several strains of filamentous fungi have revealed interesting capacities for the removal of problematic OMW compounds [26]. A variety of white-rot fungi have been used including *Phanerochaete chrysosporium* [27], *Trametes versicolor* [28], *Pleurotus* spp. [29], *Funalia trogii* [30-31], *Lentinus edodes* [3]. Although Garcia et al. [32] studied the ability of *Aspergillus niger* and *Aspergillus terreus* to remove phenol compounds from OMW, the use of *Aspergillus* spp. is not so common as the application of white rot fungi.

According to a recent review, fungi - including white rot fungi - are more effective than bacteria for the degradation of the phenols of OMW [6]. The high efficiency of fungi relies upon the structure of the aromatic compounds present in OMW; they are analogous to those of many lignin monomers, and only a few microorganisms, mainly white rot fungi, are able to efficiently degrade lignin by producing ligninolytic enzymes such as lignin peroxidases, manganese peroxidases and laccases [6]. However, there is usually a need to employ a heat pre-treatment to facilitate establishment of introduced fungi [26, 33]. Starter cultures for bioremediation usually requires aeration, and the duration of treatment is ca. 8-24 days, depending on some process variables such as degree of dilution, aeration and supplementation [6]. In addition, only some white-rot fungi were reported as able to perform decolorization and COD reduction in OMW when the active COD is >50 g/L [34]. Finally, the application of fungi for OMW treatment on a large scale was limited by the difficulty of achieving continuous culture because of the formation of filamentous pellets and mycelia [16].

To overcome this limitation, the use of yeasts could be a promising way. In fact, among the mentioned microbiota, yeasts are the more adapted and resistant to high concentrations of phenols and low pH values of mill wastes, allowing them to dominate this environment [35].

Some genera have already been tested successfully to detoxify and/or decolourise OMW, including *Candida*, *Geotrichum*, *Pichia*, *Saccharomyces*, *Trichosporon* and *Yarrowia* (table 1). Little

information is now available on the indigenous yeasts present in the OMW and their possible use for performing biodegradation of the waste.

Yeasts	Method	Results		Reference
		Phenol Reduction	COD Reduction	
Candida boidinii	Fed-batch microcosm	42.2%	-	[36]
	Culture in OMW	40%	45%	[37]
C. cylindracea	Culture in OMW	27%	45.8-70.2%	[38]
	Bioreactor batch culture with OMW	12.8-31.3%	27.4-55.9%	[39]
	Culture in OMW	36.2%	48.4%	[40]
C. diddensiae	Culture in OMW	32.14-43.56%	55.40-64.84%	[41]
	Culture in OMW	10-72%	-	[42]
C. ernobii	Culture in OMW	34.09-35.23%	51.85-62.65	[41]
C. holstii	Culture in OMW	39%	57.93%	[41]
C. oleophila	Bioreactor batch culture with OMW	20.3% (tannins)	-	[43]
	Culture in OMW	83%	55%	[12]
C. rugosa	Culture in OMW	12.2-20.4%	20.4-62.2%	[38]
	Culture in OMW	15.3%	31.1-62.2%	[38]
C. tropicalis	Culture in OMW	51.7%	62.8%	[44]
	OMW from industrial mills	25%	18%	[45]
	Culture in OMW	12-36.5%	39.4-69.7%	[46]
	Culture in bioreactors with a mixture of OMW (75%) and pig slurry (25% v/v)	51%	62%	[47]
Geotrichum sp.	Culture in OMW	46.6%	55%	[44]
G. candidum	Culture in bioreactors with OMW	-	12.4-62%	[11]
	Fed-batch microcosm	42.9%	-	[36]
	OMW from industrial mills	25-31%	20-23%	[45]
	Culture in OMW	47%	77%	[48]
	Culture in bioreactors with OMW	-	25-65%	[16]
	Culture in OMW	20-41%	25-56%	[49]
	Culture in OMW	46%	51%	[37]

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Yeasts	Method	Results		Reference
		Phenol Reduction	COD Reduction	
Pichia guilliermondii	Culture in OMW	25.09-33.52%	34.47-53.21%	[41]
Pichia sp.	Culture in OMW	40%	41.04%	[41]
P. fermentans	OMW from industrial mills	26%	18%	[45]
P. holstii	OMW from industrial mills	17%	15%	[45]
Saccharomyces sp.	Fed-batch microcosm	38.8%	-	[36]
Trichosporon cutaneum	Culture in OMW	> 80%	>80%	[50]
	Culture in OMW	64%	88%	[48]
Yarrowia lipolytica	Culture in OMW	≤78.2%	1.47-41.22%	[51]
	Culture in OMW	-	67-82%	[52]
	Culture in bioreactors with OMW	-	80%	[53]
	Culture in OMW	19.2-31.3%	21.6-52.6%	[38]
	Culture in OMW	25.3%	23.5-51.3%	[38]
	Culture in OMW	20%	23.1-50.9%	[38]
	Culture in OMW	43-72%	54-79%	[54]
	Culture in OMW	39-68%	75-80%	[54]

Table 1. Phenol removal and COD decrease in OMW by yeasts. A review of the literature. -, data not available.

2. Yeast selection: A step-by step protocol

The selection of yeasts intended as functional starter for the bioremediation of OMW is a quite complex process, involving different steps; figure 1 proposes a possible scheme.

Namely, after strain isolation from OMW, yeasts should be characterized (step 1) and identified (2); then, some promising isolates could be studied in relation to their functional properties (phenol removal and COD/BOD decrease). Finally, a multivariate approach could be used to choose the best strains for the final validation under laboratory and factory-scale conditions.

In the following sections, there are some details on the most important assays for the selection of promising yeasts.

2.1. Isolation

This is a critical step as it important to recover yeasts and many times they are not able to grow under laboratory conditions.

Generally, OMW are stored under controlled conditions (for example at 25 °C) and let to ferment; for example, authors of reference [55] analyzed OMW for 90 days. Periodically, the

samples are serially diluted and plated on opportune media, like acidified Malt Extract Agar [55], Potato Dextrose Agar and Yeast Malt Agar [56], YEPD agar (Yeast Extract Potato Dextrose) supplemented with 50 μg/mL of ampicillin [45]. Then, yeasts are selected on the basis of colony morphology.

An interesting approach was proposed by other authors [57]; they optimized the protocol for the isolation of bacteria able to remove phenols from wastewater and slurry, but their method, with some modifications, could be used successfully for yeasts. OMW should be added to a mineral salt medium (MSM) containing (g/L): Na_2HPO_4, 1.6; KH_2PO_4, 0.4; NH_4NO_3, 0.5; $MgSO_4*7H_2O$, 0.2; $CaCl_2$, 0.025; $FeCl_3$, 0.0025 with and without 1% glucose (w/v) as an additional carbon source.

Different concentrations of phenol (100, 200, 300, 500, 700, and 900 mg/L) should be added to the medium; after adjusting the pH to 7.0, the samples can be stored at 25°C for at least 5 days and then plated onto MSM agar plates, with and without glucose.

2.2. Technological characterization

The technological characterization of yeasts deals with both the taxonomic assays and the technological traits (growth requirements and enzymatic traits).

The most important trait is the effect of phenols on yeast growth/survival; this characteristic includes both the ability to use phenols as carbon sources and the growth/survival in OMW. Phenol assimilation can be assessed on Yeast Nitrogen Base (a laboratory medium without carbon source), added with either caffeic, vanillic or *p*-coumaric acid [55]. Another way to assess the suitability of yeasts for bioremediation is the evaluation of growth in OMW or in solid/liquid media containing OMW [58].

A modification of this last assay was proposed by Aissam et al. [37], who cultured yeasts into lab media containing increasing amounts of OMW (from 50 to 100%) to induce yeast adaptation to such a stressful environment.

Although the assimilation of phenols and the growth in OMW are the most important traits for the selection of promising yeasts for bioremediation, some other interesting characteristics are the thermal profile (*i.e.* the minimal and maximum temperatures of growth, the optimal temperature), the effect of pH, and nitrogen assimilation. The effects of temperature and pH can be evaluated through a spectrophotometric measurement, followed by the calculation of Growth Index, as proposed for yeasts intended as starters for table olives [59].

On the other hand, nitrogen assimilation should be assessed in a poor medium, containing a single nitrogen source (for example KNO_3 or ethylamine) [55]; this assay, as well as spore production, has also a taxonomic potential: for example *Saccharomyces cerevisiae* is not able to use nitrate as the only nitrogen source, whereas other yeasts do it.

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2.3. Enzymatic traits

Yeasts intended for bioremediation should be assessed for different enzymatic traits; some of them (pectinolytic, lipolytic and protease activities) rely on the ability to persist in a stressful environment, whereas other traits are strongly related with the ability to remove phenols.

For example, Taccari and Ciani [60] reported that ligninolytic enzymes lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase, characterized by a low substrate specificity, are involved in the degradation of polyphenols in OMW. Reference [61] reports the most common protocols to assess enzymatic traits.

2.4. Identification

For long time yeasts have been identified through the fermentation/assimilation profiles of sugars; a good profile should include the assays for the following sugars: D-glucose, D-galactose, maltose, α-methyl-D-glucoside, sucrose, trehalose, melibiose, lactose, cellobiose, melezitose, raffinose, inulin, starch and D-xylose. Nowadays, these assays are usually run through some commercial miniaturized kits [61].

It is well known that the phenotypic identification shows some limits and drawbacks, therefore yeast identification should be performed through genotypic method. One of the most used approach is PCR amplification of the region spanning ITS1 and ITS2 and the 5.8S rRNA gene (5.8S–ITS region) and subsequent restriction analysis, following the protocols by the references [62, 63]; the results of amplification and restriction are used as input data for an analysis through a specific database (for example Yeast-id database, CECT, University of Valencia, Spain).

2.5. Functional characterization

For yeasts intended for bioremediation, phenol removal, the decrease of BOD/COD and OMW decolorization could be referred to as the functional traits, as they are strictly related to the decrease of the pollutant impact of OMW.

Focusing on phenol removal, yeasts should be inoculated onto aliquots of OMW under laboratory conditions (static temperature and agitation) for some days [55]. Thereafter, the amount of residual phenols can be assessed through HPLC equipments or simply using the method by Folin-Ciocalteau [64]. Other authors [12,65] evaluated indirectly phenol removal through toxicity attenuation, thus they studied the phytoxicity of OMW towards seeds and the microbial toxicity towards *Bacillus cereus*.

Other traits are the reduction of COD and BOD [45, 66], as well as waste decolorization; for this last assay, OMW should be diluted with distilled water and then analyzed through absorbance measurement at 390 nm.

2.6. Selection of promising strains and validation

Choosing the most promising strains is the final step for a starter selection; as reported elsewhere [59], the management of a such large amount of data (many strains, each of them

studied for different parameters) is quite difficult and complex. A possible solution could be the use of multivariate statistical approaches, like the Principal Component Analysis, Cluster Analysis or Multiple Correspondence Analysis or all of them in a sequence.

The main result of the multivariate approach is the choice of the best strains (3-10) for an *in vivo* validation; however, yeasts require a preliminary optimization and/or validation in small volumes and under controlled conditions.

Some variables that should be assessed are:

1. **Use of coadjutants**. It has been reported that yeast metabolism could be enhanced by the addition of some ingredients; for example, Sinigaglia et al. [55] proposed the use of $(NH4)_2SO_4$ (1.5-6.0 g/L), while authors of the reference [46] used hexadecane and yeast extract.

2. **Temperature and shaking**. Some authors [46, 58] proposed a bioremediation with agitation (100-150 rpm) and at relatively high temperatures to increase the yield of the process.

3. **State of cells (free or immobilized in a bioreactor)**. OMW can be detoxified by free cells, as proposed by many authors or using the novel method proposed in the reference [46], who loaded a strain of *Geotrichum candidum* in Na-alginate beads and increased by 2-2.2 fold the yield of removal.

4. **Use of a single strain or a multiple strain starter**. The use of a multiple strain starter could be a promising way to enhance the yield and avoid a stop in the detoxification; thus, validation should focus on the composition of the starter (amounts of the different strains) and the way of inoculation (single inoculum or step-by-step inoculum).

5. **OMW dilution**. It was proposed a 10-fold dilution to increase fungal bioremediation by *Aspergillus wentii*, *A niger* and *Pleurotus ostreatus* [67] and these data were confirmed by a preliminary investigation performed on yeasts on our laboratory with a 3-fold dilution.

6. **Kind of process (aerobic or anaerobic)**.The use of an aerobic step could increase the yield [68]. The authors of the reference a preliminary and aerobic step with *G. candidum* to reduce COD and phenolic and fatty acid contents and increase substrate up-taking during the anaerobic treatment.

3. Conclusions

The use of yeasts for the bioremediation of OMW is a promising and open way; the starting question of this paper was: why yeasts?

We can try to point out some-key elements/benefit for the use of yeasts in OMW:

1. yeasts represent the dominant microflora of OMW and many strains are well adapted and able to grow in this stressful environment;

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2. yeasts can be used for the aerobic and anaerobic treatment of wastes;
3. the yield of moulds in phenol removal is high, many times higher than for yeasts; however, micelia could absorb phenols and release them again in the case of a prolonged storage;
4. some yeasts could be used to produce biomass and useful metabolites (for example lipases) using OMW as medium;
5. yeasts can be used in continuous or in batch cultures, while moulds do not;
6. some yeasts are able to remove both low and high molecular weight phenols, whereas bacteria do not.

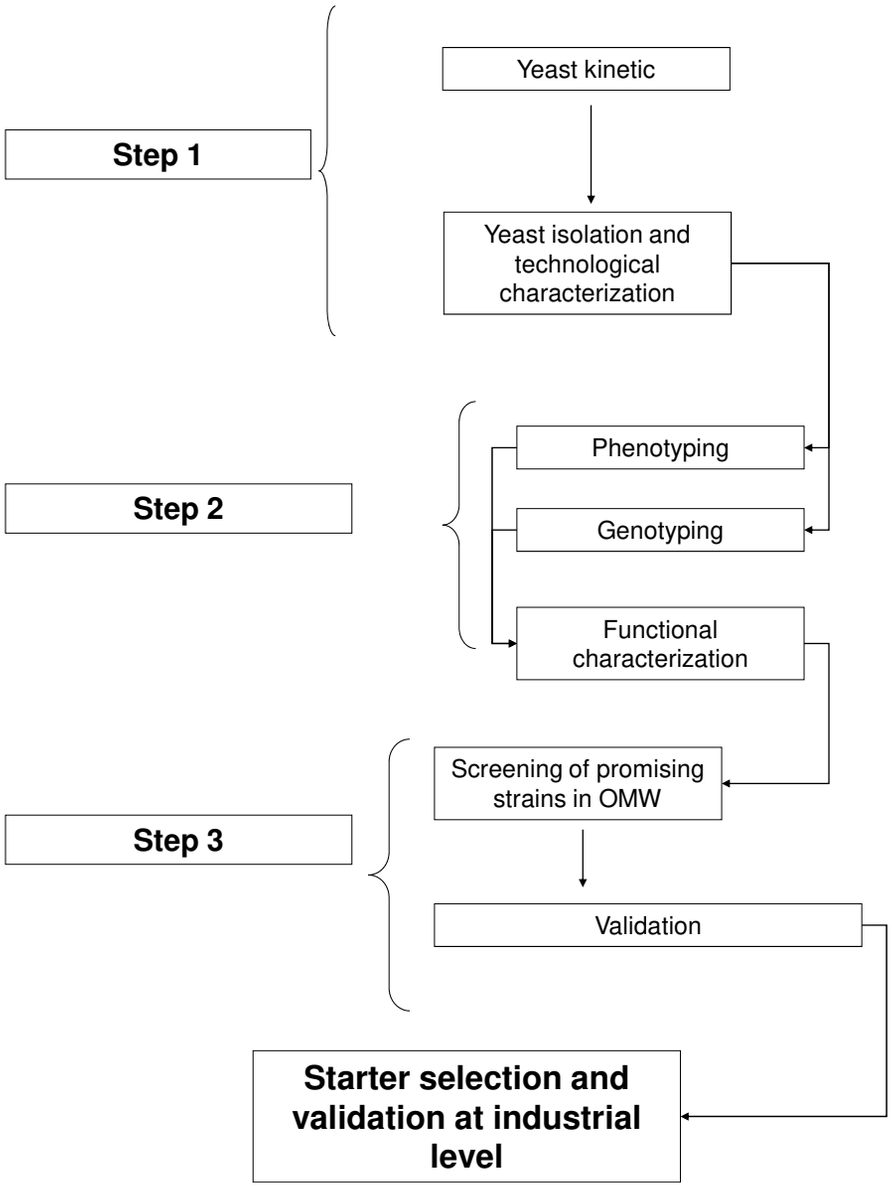

Figure 1. Selection of yeasts for phenol removal in OMW

Taxonomy
Spore production
Growth requirements
Nitrogen assimilation
Phenol assimilation
Growth in OMW
Effect of temperature and pH
Enzymatic traits
Catalase activity
Hydrolysis of pectins and xylans
Cellulolytic activity
Lipolytic activity
Protease activity
Polyphenoloxydase activity
Peroxidase activity

Table 2. Technological and taxonomic characterization

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