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Suitability of Yeasts for the Treatment of Olive Mill Wastewater

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ABSTRACT

Olive mill wastewater (OMW) is strongly hindered by high concentration of phenolics, which is considered the main cause of its toxicity. Despite this high level of phenolics, yeasts are the dominant microorganisms with levels as high as 10^6 colony forming units/ml. The identification of yeast isolates from freshly produced OMW from olive oil plants, stored OMW in ponds and larvae of the olive fly *Bactrocera oleae* revealed that many species are common in these environments along with olives. Some strains of *Candida holstii*, *C. diddensiae*, *C. boidinii*, *Pichia guillermondii* and *Geotrichum candidum* were able to grow and reduce phenolics concentration in the OMW. However, phenolics reduction was lower than 50% raising the question if yeasts degrade or simply adsorb the phenolics to their cell walls. A review of the literature reveals that a few species of *Trichosposron cutaneum* and more recently *Candida tropicalis*, *C. matosa*, and *C. aquatextoris* can grow solely on phenol and other aromatics. Some investigations have also identified phenol degradation products while others revealed the ability of yeasts to produce aromatic-degrading enzymes such as phenol hydroxylase. These findings, along with the possibility of producing economically valuable products from yeasts, can contribute to design a successful treatment process of OMW using yeasts. The evaluation of ethanol production revealed that OMW-isolated strains, *C. holstii* and *C. ernobii*, produced ethanol with efficiencies higher than 90% theoretical yield. In addition, these two yeast strains can use xylose and galactose readily available in wood and whey wastes that could potentially be codigested with OMW.

Keywords: *Bactrocera oleae*, OMW, phenol degradation

INTRODUCTION

The phytotoxicity of olive mill wastewater (OMW) can be attributed to the phenolic compounds (Lanciotti *et al*. 2005). Some of the phenolics in OMW such as oleuropein and phenol originate from olives although their level might be different depending on the variety of olives with Picholine and Manzanillo varieties presenting a higher polyphenol level than Arbequina and Leccino (Vossen 1997). Other phenolics are generated from reactions of condensation and oxidation during the oil extraction process. Hence, many groups of phenolics were identified in OMW such as cinnamic acid derivatives with caffeic acid and 1-caffeil-glucose, benzoic acid derivatives such as cathechin and β-3,4-dihydroxyphenyl ethanol derivatives such as tyrosol and 4-hydroxytyrosol-*m*-glucoside (Knupp *et al*. 1996). There are also flavonoids such as apeginin, flavanones, luteoline and quercetine (Ramos-Cormenzana *et al*. 1996). OMW also contains other aromatic compounds such as *p*-hydroxybenzaldehyde, syringaldehyde, 2-*p*-hydroxyphenylethanol, 4-hydroxyphényl alcohol and 3,4-dihydroxyphenylethanol (Juárez *et al*. 2005).

The degradation of the phenolic fraction of OMW can be carried out by many chemical or physical treatments such as electrolysis (Adhoum and Monser 2004), photolysis (Miranda *et al*. 2002) or an advanced oxidation process (Mantzavinos *et al*. 2005). Biological treatment of phenolics relied mainly on the use of rot fungi such as the lignin-degrading *Phanaerochete chrysosporium* (Sayadi and Ellouz 1995). Trials using enzymes such as laccase of another white rot fungus *Lentinula edodes* were also evaluated for OMW treatment by D'Annibale *et al.* (2000).

In addition to the moulds, yeasts are increasingly being investigated for the treatment of OMW and especially the phenolic fraction. We report here a synthesis of research work carried out in the last 5 years in three Moroccan laboratories on yeast utilisation for OMW treatment. We compared yeast strains isolated from fresh OMW taken at the olive oil process plant, from OMW stored in ponds and from larvae of an olive insect parasite *Bactrocera oleae*. We also review recent literature data on phenolics degradation by yeasts.

MATERIALS AND METHODS

OMW

Fresh olive oil mill wastewater was drawn during the oil extraction from a plant using the continuous process. Samples of stored OMW were obtained from a pond in the area of Fes in Morocco that receives OMW from different plants in the city. The pond was created to allow natural evaporation of the OMW. Larvae of *Bactrocera oleae* were obtained from infected olives. The characteristics of these effluents were determined using standard methods as described elsewhere (BenSassi *et al*. 2006; Chakri *et al*. 2007).

Isolation of yeast strains

Yeast were isolated from the OMW samples on YM agar (3 g/l malt extract (Difco), 3 g/l yeast extract (Biokar Diagnostics), 5 g/l peptone (Biokar Diagnostics), 10 g/l glucose (Labosi), 12 g/l agar (Biokar Diagnostics) at pH 6) containing 20 ppm tetracycline (Sigma). Strains were purified from isolation plates by subsequent streaking onto YM agar medium. The cultures were checked by microscope observations then maintained on slants of YM agar at 4°C.

The larvae of *Bactrocera oleae* collected from local infected olives were washed in ethanol, hypochlorite and thiosulfate as described by Cavados *et al*. (2001) to eliminate external microorganisms. The washed larvae were then crushed in 400 ml of YPG

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Research Note

medium (1% yeast extract, 2% peptone and 2% glucose, pH 4.5). A volume of 0.1 ml of this suspension was spread on agar plates containing the OMW. Yeast strains grown on this medium at 30°C were inoculated on agar plates containing non-diluted OMW. The grown strains were then purified on YPG medium containing three antibiotics (ampicillin, 100 µg/ml; kanamycin, 20 µg/ml and tetracycline, 20 µg/ml) to eliminate bacterial growth. The purified cultures were stored in slants of YPG medium at 4°C.

Sugar assimilation and ethanol fermentation

Sugar assimilation was assayed according to Middelhoven (2002): 0.5% of the sugars: xylose, galactose, lactose, sucrose and starch were added to a solution of 0.67% of Yeast Nitrogen Base (Difco). After sterilization, a fresh culture of yeast was inoculated, and the growth was estimated after 4 to 5 days of incubation at 30°C.

Ethanol production was assayed using a similar medium as the one used for sugar assimilation with a glucose concentration of 10 g/l. The ethanol concentration was measured in the vials' headspace using an Agilent Technologies 6800 gas chromatograph. We used a 30 m × 0.32 mm I.D., 0.25 µm film thickness Innowax column (J&W Scientific) with the following temperature program: 40°C for 2 min and then 40 to 160 at a rate of 2°C/min. Temperatures of the Flame Ionisation Detector (FID) and the injector were set to 250°C.

Total phenolic compound assay

To estimate the total phenolic compounds, 1 ml of filtrated OMW was added to 2.5 ml of Folin-Dennis reagent and 35 ml of distilled water. After homogenisation, 10 ml of a solution saturated with 20% sodium carbonate was added. Distilled water was then added to complete a volume of 50 ml. Colour was measured spectrophotometrically at 725 nm. The calibration curve was prepared by using different concentrations of tannic acid (0.05, 0.03, 0.025, 0.015 and 0.01) (Maestro-Duran *et al*. 1991).

Identification of yeast strains

Yeast strains were identified using molecular techniques based on PCR amplification and DNA sequencing of a fragment of the ribosomal RNA 5.8S gene as already described in detail in Ben Sassi *et al*. (2008).

RESULTS AND DISCUSSION

Abundance and diversity of yeasts in the different OMW

The level of phenolics was significantly higher in the OMW from evaporation ponds compared to the freshly OMW produced from continuous olive oil extraction systems (**Table 1**). This is certainly related to the evaporation in the ponds but also to the processes of oil extraction used to produce these OMW, as already shown in Ben Sassi *et al*. (2006). The higher level of phenolics along with the scarcity of degradable sources of carbon is probably the main cause of the lower microbial population observed for the OMW from the ponds. Nevertheless, the population of yeasts was still the dominant flora in both OMW. Hence, it seems that yeasts are well adapted in fresh as well as relatively concentrated OMW from evaporation ponds, while the bacteria are inhibited by the presence of phenolics (Paredes *et al*. 1986) suggesting an anti-bacterial effects of OMW. Furthermore, the development of moulds is at least partially limited because of low oxygen levels. This allows the yeast to dominate in high phenolics acidic effluent.

Table 1 Characteristics of OMWW from oil extraction plant (fresh OMWW) and from storage ponds (stored OMWW).

Parameter	Fresh OMWW	Stored OMWW
pH	4.85	4.5
Dry matter ($g.l^{-1}$)	76	98
Chemical oxygen demand ($g\ O_2.l^{-1}$)	108	154
Total phenolics ($g.l^{-1}$)	7.2	9.7
Reducing sugrs ($g.l^{-1}$)	8	0.28
Total flora (cfu/ml)	$1.8\ 10^7$	$8.4.10^3$
Yeast (cfu/ml)	$8{,}4.10^6$	$7.6.10^3$
Moulds (cfu/ml)	-	$4.0.10^2$

The list of yeast species identified from fresh OMW, OMW from evaporation ponds and from the olive insect parasite *B. oleae* (**Table 2**) showed the dominance of the representatives of the two genera *Candida* and *Pichia*. Although the limited number of samples is not adequate to analyse the diversity of yeasts in OMW, it is noteworthy that the species *C. diddensiae* was found in all three environments and was shown to grow in the different types of OMW used in this study. Furthermore, this species was also found in black and green olive products (Coton *et al*. 2006). Many other yeast species were also found in olive products such as *C. boidinii*, *Debaryomyces hansenii* and *Geotrichum candidum* (Arroyo-Lopez *et al*. 2006). Hence, it seems that some yeast species encountered in the OMW originate from olives. A better approach of the diversity using molecular techniques might be required to confirm this hypothesis. In addition, only few species belonging to the species *C. diddensiae*, *C. holstii*, *C. boidinii* and *G. candidum* were able to grow in the OMW (**Table 2**) and reduce the concentration of phenolics. Except for *G. candidum* strains isolated from OMW and evaluated for its treatment (Assas *et al*. 2000), the other species were not previously found in OMW. Only few other yeast species originated from OMW with *C. tropicalis* (Fadil *et al*. 2003) and *Candida wickerhamii*, *C. molischian*a and *Saccharomyces cerevisiae* (Bambalov *et al*. 1989) and *Geotrichum candidum* (Assas *et al*. 2000).

Treatment of OMW and phenol degradation using yeasts

As stated above, the selection of yeast strains for OMW treatment was mainly based on their ability to grow in the liquid effluents with the idea of choosing strains that at least can resist the pollution load present in these wastewaters. The dry biomass obtained from the fresh OMW is greater than that obtained from the stored OMW (**Table 3**). This is obviously related to the different strains but it could also be a consequence of the lower reducing sugar fraction in the stabilised OMW from storage ponds along with the higher concentration of phenolics. The OMW stored in ponds for some time showed a concentration of organic compounds

Table 2 Yeast species identified in fresh and stored OMWW and in *Bactrocera* larvae.

Fresh OMWW	Stored OMWW	Yeasts in *Bacterocera*
*Candida holstii**	*Candida boidinii**	*Candida diddensiae**
*Candida diddensiae**	*Candida diddensiae**	*Debaryomyces hansenii*
*Candida ernobii**	*Candida wickerhamii**	*Pichia burtonii*
*Pichia guilliermondii**	*Geotrichum candidum**	*Pichia guilliermondii*
Pichia sp.*	*Hansenula kluyveri**	
	*Pichia membranaefaciens**	
	*Saccharomyces capensis**	
	*Zygosaccharomyces fermentati**	
Total number of yeast isolates: 105	Total number of yeast isolates: 71	Total number of yeast isolates: 39

* strains able to grow on OMWW

Table 3 Growth and phenolic removal from yeast strains isolated from OMWW and *Bactrocera oleae* larvae.

Strain	Growth (dry weight g/l)	Phenolics removal (%)
Candida holstii	2.6	39
Pichia guilliermondii	3.5	25
Candida diddensiae	6.0	44
Candida boidinii	1.0	7
Geotrichum candium	1.5	10

(**Table 1**) certainly related to the water evaporation and the reduction of reducing sugars used quickly by the existing microorganisms. These facts may explain the relatively slow decrease in phenolics observed in the stabilised and more concentrated OMW although there might also be differences due to the strains (**Table 3**). However, overall the reduction of phenolics by the different strains in this study remains very limited as it hardly reaches 50% (**Table 3**). Although some investigations (Aissam *et al*. 2007; Chakri *et al*. 2007) have reported better removal of phenolics after acclimation of the yeast strains to OMW, the level of removal was still relatively low barely reaching 50% removal (Aissam *et al*. 2007) or at best reaching around 70% but only after 60 days incubation (Chakri *et al*. 2007). These levels are comparable to published data using other yeasts such as *C. tropicalis* that removed around 51% of phenolics (Fadil *et al*. 2003). A maximum of 70% phenolics removal by *G. candidum* (Assas *et al*. 2000) was based on light absorbance at 280 nm and not on the extraction and measurement of phenolics as carried out in our work. In addition to the limited reduction of phenolics none of these investigations has evaluated phenolic adsorption on yeast biomass that account for some phenolic removal (Rizzo *et al*. 2006). This raises the question if yeasts are able to degrade phenolics.

The best answer to this question was first demonstrated by the yeast-like fungus *Trychosporon cutaneum.* Strains of *T. cutaneum* were among the first yeasts identified for their ability to degrade phenols by Neujah and Gaal (1973). Other authors have also shown the ability of other strains of this species to degrade phenols (Shivarova *et al*. 1999; Alexievaa *et al*. 2004). A strain of this species has also been evaluated on OMW by Chtourou *et al*. (2004). Many strains of *C. tropicalis* from China (Yan *et al*. 2006), Morocco (Ettayebi *et al*. 2003), Brazil (Rocha *et al*. 2007) and Mexico (Galíndez-Mayer *et al*. 2008) were shown to grow and use phenols as the sole carbon source. Varma and Gaikwad (2008) identified some degradation products of phenols by strains of *C. tropicalis* that were able to degrade up to 95% of 2 g/l phenols in 16 hours incubation. Similarly, Vallini *et al*. (2001) reported phenol degradation products in a culture of *C. aquaetextoris*. Furthermore, some yeast strains showed the production of phenol-degrading enzymes such as phenol hydroxylase or catechol di-oxygease (Fialova *et al*. 2004). The contribution of catalase activity from yeast peroxisomes to phenolic degradation claimed by some authors (Ettayebi *et al*. 2003) needs to be confirmed using purified enzymes.

However, the reduction of phenolics in OMW by yeasts currently reported is far less important that those obtained by white rot fungi such as *P. chrysosporium* (Sayadi and Ellouz 1995). Future developments should focus on improving the phenolic degradation ability of potential yeast strains such as those of *C. tropicalis* through physiological adaptation. Another alternative might be developing genetically engineered yeast strains with white rot fungi genes responsible for lignin degradation enzymes. This would make it easier and more economic to develop a process adapted to OMW treatment while producing high value products such as ethanol or yeast enzymes.

Production of ethanol as a value-added product from yeasts

In this work, besides the treatment of OMW, we have evaluated the potential of ethanol production by some yeast strains (**Fig. 1**). The highest ethanol production was obtained for the strain *C. ernobii* with a 94% theoretical yield followed by the strains *C. holstii* with 93%, *C. diddensiae* with 72% and *P. guilliermondii* with only 49%. The evaluation of ethanol production was carried out in synthetic medium containing glucose because the concentration of sugars in the OMW was too low to produce ethanol. This has already been reported by Bambalov *et al*. (1989), who used a collection of yeasts such as *S. cerevisiae* as well as OMW-originating yeast strains. Only the OMW yeast isolates produced ethanol, the best producers were *Torulopsis* sp. MK-1 with 87% and *S. oleaginosus* MC-5 with 80% theoretical yield. In order to increase the concentration of ethanol the authors concentrated the OMW to 13-14% of dry matter but none of the strains was able to ferment this concentrated effluent. Hence, it seems that the more concentrated the OMW is, the more it becomes toxic for the yeasts. A better alternative method to increase the sugars in the OMW decreasing its toxicity is the co-digestion with another waste such as cheese whey (Azbar *et al*. 2008). The yeast strains evaluated in this work showed the ability to use some sugars that are available in other wastes such as wood waste for xylose, lactose and galactose from cheese whey and starch (**Table 4**). In addition to ethanol, some authors have showed the possibility of producing other viable economical products. Thus, Amat *et al*. (1986) evaluated the production of single cell proteins using *Saccharomyces cerevisiae* biomass on hydrogen peroxide-treated OMW. The yeast used mainly sugars and residual oil, while pectin, tannins and polyphenols were not metabolized. *Yarrowia lypolytica* is also one of the yeast species that was shown to grow well in OMW with an important biomass production of 22 g/l (Scioli and Valloro 1997). In addition, some strains are capable of producing citric acid (Lanciottti *et al*. 2005) and lipases (Scioloi and Valloro 1997; Lanciotti *et al*. 2005). However, most evaluated strains of *Y. lipolytica* in the two investigations produced little change in phenolic content with some seemingly increasing it. The production of other yeast enzymes was also evaluated for strains of *Cryptococcus albidus* by Petruccioli *et al*. (1988) for the production of pectinases.

CONCLUSION

There has been a relatively low number of published studies on the treatment of OMW using yeasts despite their dominance over bacteria and moulds in this acidic effluent with high phenolic content. A few of yeast strains isolated from fresh and stored OMW and from larvae of the olive fly *Bactrocera oleae* grew well in this wastewater and lowered its toxicity and also its phenolic content. Some of these identified strains such as *Candida diddensiae*, found in all three prospected environments in this work, were also reported in

Table 4 Sugar utilisation by *Pichia guilliermondii*, *Candida ernobii*, *Candida diddensiae*, *Candida holstii* yeast strains isolated from fresh OMWW.

Yeast strains	Sugars				
	Xylose	Sucrose	Galactose	Lactose	Starch
Pichia guilliermondii	+	+	+	+	-
Candida ernobii	+	+	+	+	-
Candida diddensiae	+	+	+	-	+
Candida holstii	+	+	+	-	-

+ sugar utilised, - sugar non utilised

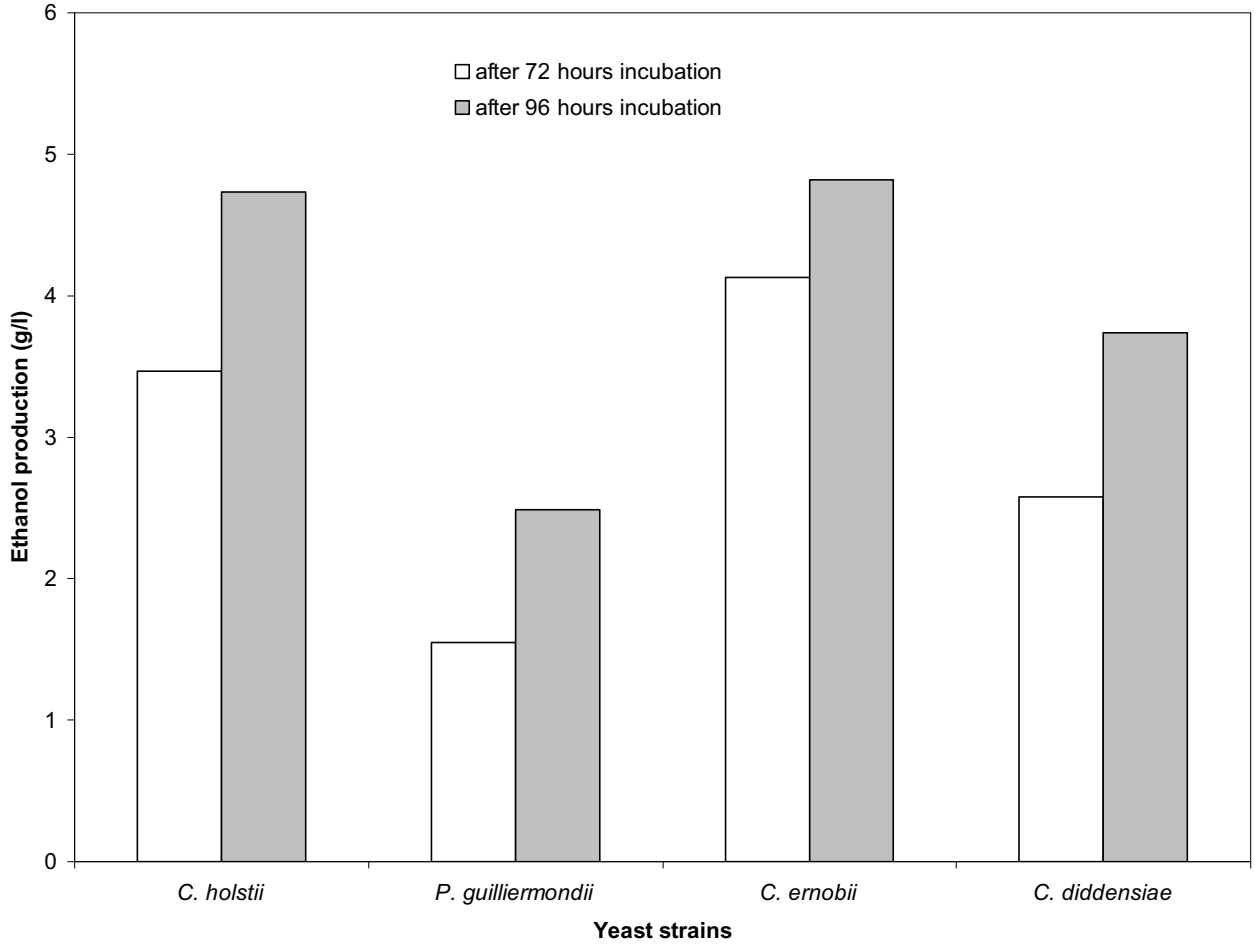

Fig. 1 Average (n=2) ethanol production (g/l) for different yeast strains isolated from fresh OMW after 72 hours (□) and 96 hours incubation (■).

olive products.

The reduction of the OMW phenolic content, albeit lower than what was reported for white rot fungi, raises the question if yeasts possess the enzymatic arsenal needed for OMW polyphenol degradation. Certain strains of the yeast-like fungus *Trichsporon cutaneum* along with *Candida maltosa* have been shown to produce phenol hydroxylase activity. Moreover, phenol degradation metabolites were also detected for certain strains of *C. tropicalis*. These results seem to indicate that at least some yeast strains are able to degrade some aromatics with the proven presence of phenol-degrading enzymes. Nevertheless, some improvement of the existing strains might be needed to improve the removal of phenolics from OMW using these strains. An alternative route could be the development of genetically engineered yeasts with lignin-like compounds degradation capabilities from white rot fungi. Developing a process using yeasts should be more suitable and more economically attractive, especially if the yeasts produce high value by products such as enzymes or ethanol.

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