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Characterization of phenolic compounds of OMW: toxicity and degrability by yeasts

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The olive oil extraction, performed by the 3-phases process, results on a large amount of an effluent, usually known as Olive Mill Wastewater (OMW). It is mainly produced in the Mediterranean area, with the exception of Spain that is one of the major producers but mostly use the 2-phases process. OMW represents a major environmental problem due to its high organic content, being characterized by a strong acidic smell and an intensive brown to dark color due to the presence of biodegradable, recalcitrant and biostatic compounds. OMW phytotoxicity can be attributed to the phenolic compounds (Lanciotti et al, 2005).
Owing to their instability, OMW phenols tend to polymerise during storage into condensed high-molecular-weight polymers that are difficult to degrade (Crognale et al, 2006). Thus, uncontrolled OMW disposal can create severe risks to water and soil quality. OMW is currently concentrated by evaporation in open pools, but this method is not satisfactory because a black foul-smelling sludge, difficult to remove, is produced. Instead of disposal solutions an approach of using this waste as a resource to be valorized is of greater interest. In fact, OMW contains sugars, lipids, mineral elements and phenolic compounds (10 % of the organic matter) that could be either directly recovered by chemical extraction and subsequent purification, or utilized as a basis for fermentative processes.
The research on OMW valorization is focused on the recovery or on the degradation of the phenolic compounds since its presence is considered to be the limiting step in the biotreatment of OMW (Tsioulpas et al, 2002).
One of the aims of the present investigation was the characterization of OMW, focusing the phenolic compounds.
As a first approach to characterize the phenolic compounds of OMW, two distinct extraction methods were used: (1) a liquid-liquid extraction by acidified ethyl acetate, according to the procedure of De Marco et al. (2007) and (2) a solid-liquid extraction with acidified methanol. The analysis of these extracts by reversed phase liquid chromatography showed that hydroxytyrosol was the most abundant phenolic compound in OMW, and that, this compound was more efficiently recovered by the solid-liquid extraction technique.
Hydroxytyrosol recovery from OMW is of obvious industrial interest due its well recognized nutraceutical properties as antioxidant (Leonardis et al, 2007).
Subsequently to the identification of the major components of the phenolic compounds on OMW, it was also a goal of this work to study its toxicity to yeast strains that are under investigation for potential use of OMW as culture media (Araujo et al, 2005).
For this purpose, strains of *Yarrowia lipolytica*, *Candida rugosa* and *Candida cilyndracea* where cultivated in YPD medium in the presence of different phenolic compounds commonly found in OMW. For the range of phenolic concentrations used (up to 1 g/L), no cell growth inhibition was observed. However, the phenols degradation was quite difficult, particularly when more easily degradable organic matter is still present in the medium.

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Characterization of phenolic compounds of OMW

Toxicity and degradability

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INTRODUCTION

The olive oil consumed in the world is mainly produced in the Mediterranean area. **OLIVE MILL WASTEWATERS (OMW)**, the effluent of olive oil production, represents a major **environmental problem** due to its high organic content. The OMW **phytotoxicity can be attributed to the phenolic compounds** (Lanciotti et al, 2005). In fact, the olive pulp is very rich in phenolic compounds (Cardoso et al, 2005), but only 2 % of the total phenolic content of the olive fruit goes to the oil phase, while the remaining amount is lost in the OMW (≈ 53 %) and in the pomace (≈ 45 %) (Rodis et al, 2002).

Due to their instability, **OMW phenols** tend to polymerize, during storage, into condensed high-molecular-weight polymers that are **difficult to degrade** (Crognale et al, 2006). Thus, uncontrolled OMW disposal can create **severe risks to water and soil quality**. OMW are currently concentrated by evaporation in open pools, which constitutes an unsatisfactory method because it only reduces the volume of waste with no pollutants treatment, and produces a black foul-smelling sludge, difficult to remove.

The research on **OMW valorization** focuses on the **phenolic compounds recovery** or **degradation**, since its presence is considered to be the limiting step in the biotreatment of OMW (Tsioulpas et al, 2002). Instead of disposal solutions, an approach of waste utilization as a resource to be valorized, is of great interest. The extraction and purification of biologically active compounds turns OMW into a source of natural antioxidants.

The most important aims of the this investigation are:

» The characterization of different OMW, focusing in the phenolic compounds identification;

» The study of phenolic compounds toxicity to yeast strains, of potential interest to use OMW as culture media.

EXTRACTION AND ANALYSIS OF PHENOLIC COMPOUNDS

OMW were collected in a continuous 3-phases olive mills in the north of Portugal and stored at -80 ºC immediately after arrival to the lab. The phenolic compounds were extracted from the OWM by two distinct extraction methods (Figure 1).

Liquid-liquid extraction

❖ De Marco et al., 2007

Solid-liquid extraction

❖ An OMW sample was lyophilized;
❖ The resulting freeze-dried material was defatted with n-hexane;
❖ The residue was extracted with methanol at pH 2;
❖ The methanolic extracts were filtered, combined and concentrated;
❖ The dry residue was dissolved in methanol;

Figure 1. Extraction methods used.

The total concentration of phenolic compounds in the phenolic extracts was determined by an adaptation of the Folin-Ciocalteu method (Singleton and Rossi, 1965), using tyrosol as a reference. The column of the HPLC was kept at 30ºC. The mobile phase was (A) formic acid 0.1% and (B) acetonitrile containing 0.1% formic acid. The solvent gradient started with 97% A and 3% B reaching 91% A at 4 min, 85% A at 15 min, 84% A at 25 min, 60% A at 70 min, 10% A at 80 min followed by an isocratic plateau for 5 min and return to initial conditions. Hydroxytyrosol in the phenolic extracts was identified and quantified.

» The total amount of phenolic compounds recovered from the OMW by the **solid-liquid extraction** procedure was approximately **three times higher** than that obtained by the **liquid-liquid extraction** methodology;

» For both OMW extracts, the **main phenolic** chromatographic peak was eluted at 8.9 min (Figure 2), which corresponded to **hydroxytyrosol**;

» The recovery of hydroxytyrosol accounted for **0.81 g·L^{-1}** and **0.06 g·L^{-1}**, for the **solid-liquid** and the **liquid-liquid** extraction procedures, respectively.

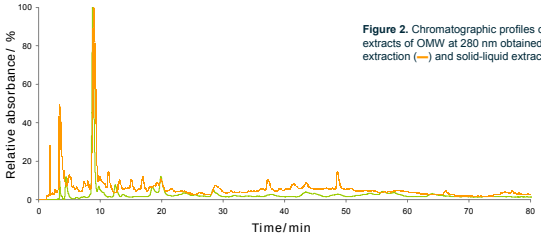


Figure 2. Chromatographic profiles of phenolic extracts of OMW at 280 nm obtained by liquid-liquid extraction (—) and solid-liquid extraction (—).

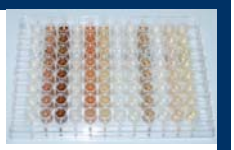
PHENOLIC COMPOUNDS TOXICITY

Two OMW's samples (A and B) with the composition shown in Table 1 were used.

Table 1. Characterization of OMW's used

Parameter *	Olive Mill Wastewater A	B
pH	4.71	4.93
COD/(g L^{-1})	99 ± 1	191 ± 2
Total Solids/(g L^{-1})	148± 3	119.6 ± 0.2
Total Volatile Solids/(g L^{-1})	117 ± 6	84 ± 42
Nitrogen (Kjeldhal) /(mg L^{-1})	192 ±17	-
Phenols (Tyrosol)/(g L^{-1})	5.5 ± 0.1	12.1 ± 0.2
Reducing Sugars/(g L^{-1})	12.9 ± 0.7	34.4 ± 0.9
Total protein/(g L^{-1})	1.2 ± 0.2	1.3 ± 0.0

* Data are mean values ± standard deviation (n=20)



The **phenolic compounds degradation and toxicity experiments** were carried out in 96-wells microplates for 100 hours, approximately. Six different **yeast strains** were used: *C. rugosa* PYCC 3238 and CBS 2275, *C. cylindracea* CBS 7869, *Y. lipolytica* CBS 2073, W29 (ATCC 20460) and IMUFR 50682. The sterilized phenolic medium was composed by YPD medium (10x diluted) with the different phenolic compounds.

Typical batch growth curves profile, for these experiments are shown in Figure 3.

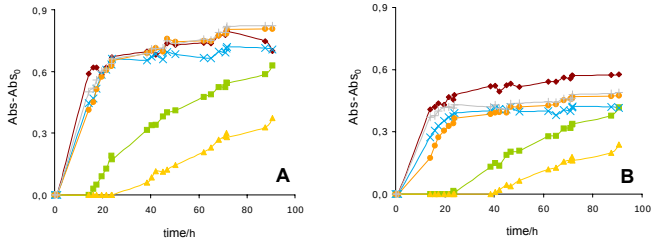


Figure 3. Batch growth profile of *Yarrowia lipolytica* W29 (A) and *Candida rugosa* CBS (B) in YPD medium (♦) and phenolic mediums: Catechol (▲), Hydroxityrosol (●), Caffeic Acid (■), Tyrosol (×) and Oleuropein (+).

» The **final biomass concentration decrease in phenolic mediums** comparatively to YPD medium for all strains used, except for *Y. lipolytica*;

» A cellular growth **inhibition by catechol and caffeic** acid was found, being **catechol the major inhibitor**. An **extended lag-phase** of cell growth was observed in the presence of these compounds;

» In all the assays **no phenolic compounds degradation** was observed;

» Essays with **pre-adaptation phase** to phenolic compounds did **not** improve the phenolic **compounds degradation.**

The **toxicity of phenolic compounds and OMW** on the activity of *Y. lipolytica* W29 cells was evaluated through respiratory activity. The decrease in dissolved oxygen tension (DOT), measured as percentage of saturation, was monitored on a ***Biological Oxygen Monitor System***.

Endogenous respiration rate was calculated from the slope of the decrease of DOT with time (green line in Figure 4), for a **cell suspension** (with 22 hours of growth) in phosphate buffer, pH 7.0.

This suspension was aerated by 30 minutes, to achieve a total oxygen saturation, before being monitored at 27 ºC. The respiration rate was also determined for **cell suspension with the addition of** glucose, **OMW** and **catechol** (red line in Figure 4) and compared with the endogenous one**.**

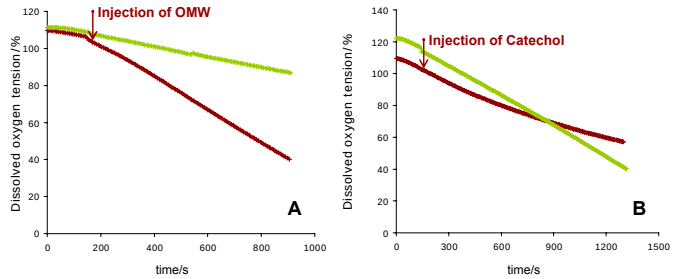


Figure 4. A – DOT/% curves of a *Y. lipolytica* suspension without (+) and with injection (+) of OMW (A) or catechol (B). The arrow represents the injection of carbon source.

» The injection of OMW (diluted 1:50) as a **carbon source** in cell suspension **increase the OUR** comparatively to that found in the assay without this addition (Figure 4.A),

» **Catechol** suspension leads to an **inhibitory effect** to *Y. lipolytica* (Figure 4.B). The loss of activity obtained for catechol was, approximately, 99.6% for a concentration of catechol of 0.32 g·L^{-1},

» The injection of **OMW** improved the total respiration rate, even **more (24,6 %) than glucose (0,5 g·L^{-1})**. Thus it is proved the non-inhibitory effect of OMW to the strain W29, possibly due to the low concentration of the most inhibitory phenolic compounds (eg.: catechol).

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