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Characterization of phenolic compounds of OMW: toxicity and degradability by yeasts.

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Abstract

The characterization of Olive Mill Wastewaters (OMW), focusing the phenolic compounds, is one of the aims of the present work. As a first approach to characterize the phenolic compounds of OMW, the extraction methods used were: a liquid-liquid extraction by acidified ethyl acetate and a solid-liquid extraction with acidified methanol. The analysis of these extracts by reversed phase liquid chromatography confirmed that hydroxytyrosol was the most abundant phenolic compound in OMW, and that this compound was more efficiently recovered by the solid-liquid extraction technique. It was also a goal of this work to study the phenolic compounds toxicity to some yeast strains. Among the phenolic compounds tested catechol is the most inhibitory one to the cells. The phenols degradation was quite difficult, particularly when more easily degradable carbon source is still present in the medium.

1 Introduction

The olive oil consumed in the world is mainly produced in the Mediterranean area. The three -phase extraction system is the most common technology used in these countries, with exception of Spain, the most important producer, that mostly uses the two-phases process. These technologies differ in the water requirements. The three-phase extraction process requires large amounts of water, resulting in a large amount of effluent, known as Olive Mill Wastewater (OMW). OMW represents a major environmental problem due to its high organic content.

OMW is often characterized by a strong acidic smell and an intensive brown to dark color due to the presence of biodegradable and recalcitrant compounds (such as polyphenolic compounds). The phytotoxicity of the olive mill wastewaters 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 passes in the oil phase, while the remaining amount is lost in the OMW (approximately 53%) and in the pomace (approximately 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 is currently concentrated by evaporation in open pools, but this method is not satisfactory because it only reduces the volume of waste without treating the pollutants and a black foul-smelling sludge, difficult to remove, is produced. The research on OMW valorization is focused on the recovery or on the degradation of the phenolic compounds since its presence

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is considered to be the limiting step in the biotreatment of OMW (Tsioulpas et al, 2002).

Instead of disposal solutions an approach of using this waste as a resource to be valorized is of greater interest. 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. Thus, the extraction and purification of biologically active compounds (namely biophenols) turns OMW into a source of natural antioxidants. These compounds are object of growing interest in pharmaceutical and food industries since reactive oxygen species are involved in the onset of several human diseases and in the oxidative degradation of food (De Marco et al, 2007).

One of the aims of the present investigation was the characterization of different OMW, from north of Portugal, focusing the phenolic compounds identification. Subsequently to this identification 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.

2. Materials and Methods

Characterization of phenolic compounds in OMW

Extraction of phenolic compounds

The olive oil mill wastewater was collected in a continuous three phases olive oil factory in the north of Portugal and stored at -80 ºC right after arrival to the lab. The phenolic compounds were extracted from the OWM by two distinct extraction methods: (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. For the solid-liquid procedure, an OWM sample was lyophilized and 2 g of the resulting freeze-dried material was defatted 3 times with 20 mL of *n*-hexane, and the residue was extracted 5 times with 20 mL of methanol at pH 2. The methanolic extracts were filtered, combined, concentrated under vacuum and the dry residue was dissolved in 10 mL of methanol.

Colorimetric and HPLC analysis of the phenolic extracts

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.

HPLC analysis was performed on a HPLC apparatus from Knauer equipped with a Smartline 1000 bomb, an automatic Smartline Manager 5000 degassing unit, a Smartline 2500 UV/Vis detector and a JASCO automatic sampler, model AS-2057. The column (Nucleosil 100-5 C18 Macherey-Nagel, 250×4.6 mm) was kept at 30ºC using a Gecko 2000 oven. The results were recorded and processed using a Knauer Claritychrom software, version 2.4.4.80. 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 by the comparison of the retention time of the peak and the correlation of its area to the retention time and to the calibration curve of the standard, respectively.

Phenolic compounds toxicity to lipolytic yeast strains

Two OMW's samples (A and B) with the composition shown in Table 1 where used. Chemical Oxygen Demand (COD), Solids (total, volatile and dissolved), Nitrogen (Kjeldahl) were determined according to Standard Methods (APHA et al., 1989). Reducing sugars were measured by DNS method. Samples collected through fermentations were analyzed for COD, sugars, total phenols and by the methods above described.

Table 1. Characterization of OMW's used

Parameter [a]	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

[a] Data are mean values ± standard deviation (n=20)

Phenolic compounds toxicity and biodegradability

The phenolic compounds degradation and toxicity tests were carried out in microplates for 4 days and 6 yeast strains were used: *C. rugosa* PYCC 3238, *C. cylindracea* CBS 7869, *C. rugosa* CBS 2275, *Y. lipolytica* CBS 2073, *Y. lipolytica* W29 ATCC 20460 and *Y. lipolytica* IMUFR. Cells were pre-grown in YPD medium, for 24 h. After that, 20 µL of cells suspension were transferred to each microplate well with the sterilized phenolic medium. The phenolic mediums were composed of YPD medium (10 fold diluted) with different phenolic compounds (commonly found in OMW), such as tyrosol, hydroxytyrosol, cafeic acid, cathecol, oleuropein, syringic acid and vanillic acid.

In order to evaluate the induction of phenolic metabolism in yeast strains, assays with pre-adaptation to phenolic compounds were carried out. Cells were pre-grown in YPD medium by 24 h, harvested (12225 g, 5 min), re-suspended in the phenolic medium (YP and phenolic solution in phosphate buffer pH 7.2, 0.1 M) and grown for 24 h in this conditions. After this pre-adaptation phase, cells were transfered to OMW mediums (A and B). Batch cultures were carried out in 500 mL Erlenmeyer baffled flasks filled with 200 mL of OMW sterilized medium, supplemented with ammonium chloride at a concentration corresponding to 10% (w/w) of the COD value and yeast extract (40% of the NH_4CL added).

Respiratory activity assays were carried on a Biological Oxygen Monitor System (YSI 5300A) with a stirred thermostatic bath. *Y. lipolytica* W29 culture with, approximately, 22 h of growth was harvested, washed and ressuspended in sodium phosphate buffer in order to obtain a final suspension with an optical density of 0.3 (λ = 600 nm). This suspension was aerated for 30 min to ensure the oxygen saturation and after this time was placed in the temperature-controlled vessel at 27 ºC. The vessels were closed and the decrease in air saturation percentage was monitored over time. The linear decrease observed between time zero and carbon source addition corresponds to the endogenous respiration rate. To determine the oxygen uptake rate due to substrate oxidation, different volumes of a glucose solution (10 g·L^{-1}), phenolic solution (tyrosol, oleuropein, syringic acid and gallic acid 2.5 g·L^{-1} each and hydroxytyrosol 1.25 g·L^{-1}) or OMW (COD 115 g·L^{-1}) were injected into the vessel. The slope of the linear decrease in the air saturation percentage immediately after carbon source injection corresponds to the total respiration rate. In order to calculate the influence of exposition of the yeast suspension to catechol solution, the cell suspension was incubated with a catechol solution (0.3 g·L^{-1}) for 30 min with aeration. After the required contact time, the cell suspension was placed in the vessels in order to assess the metabolic state of the yeast culture by respiratory activity as described above.

3. Results and discussion

Characterization of phenolic compounds in OMW

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 (5.1 mg/mL and 1.7 mg/mL, respectively). For the two extracts, the main phenolic chromatographic peak was eluted at 8.9 min (Figure 1), and this corresponded to hydroxytyrosol. The recovery of this compound accounted for 0.81 mg/mL and 0.06 mg/mL of OMW, for the solid-liquid and the liquid-liquid extraction procedures, respectively.

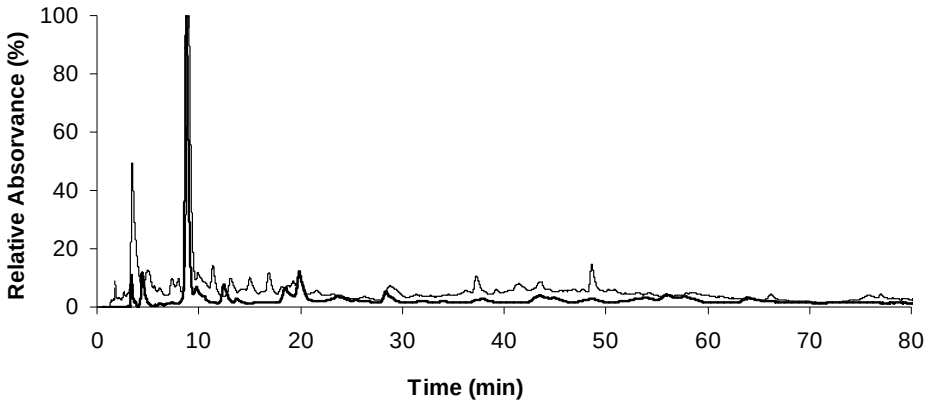

Figure 1. Chromatographic profiles of phenolic extracts of OMW at 280 nm obtained by liquid-liquid extraction (▬) and solid-liquid extraction (▬) procedures.

Phenolic compounds toxicity and biodegradability

The effect of different phenolic compounds on each yeast strain growth was evaluated by the assays in microplate. Typical batch growth curves profile for the experiments with *Y. lipolytica* W29 and C. *rugosa* CBS strains are shown in Figure 2.

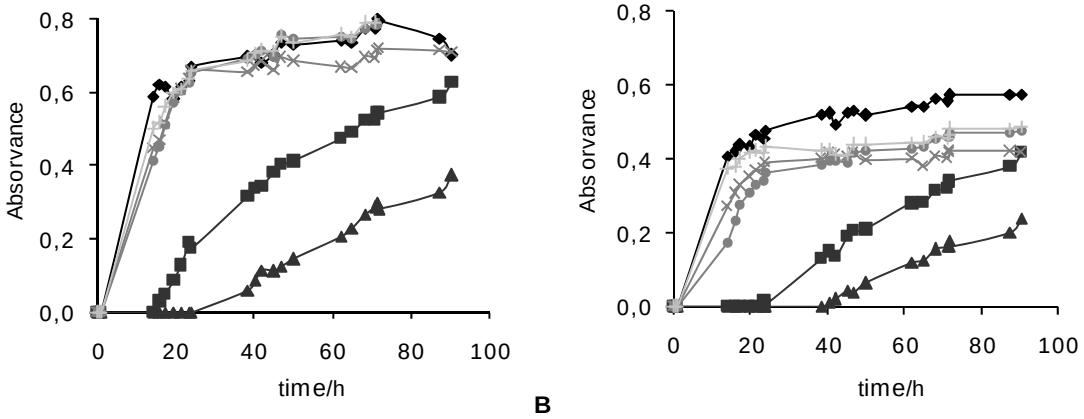

Figure 2. Batch growth profile of *Yarrowia lipolytica* W29 (A) and *Candida rugosa* CBS (B) in YPD medium (♦) and phenolic mediums: Catechol (▲), Hydroxityrosol (●), Caffeic Acid (■), Tyrosol (×) and Oleuropein (+).

For the phenolic concentrations used (1 $g \cdot L^{-1}$), it was observed that the final biomass concentration decrease in phenolic mediums comparatively to YPD medium for all strains used, except for *Yarrowia lipolytica*. Beside this difference, it was verified that the cells still grown in the presence of phenolics. However, in the presence of catechol the cell growth (for all strains) decreased substantially. An adaptation phase is noticed for catechol and caffeic

acid. In all the assays no phenolic compounds degradation was observed, particularly when more easily degradable carbon source, such as glucose, is still present in the medium.

In order to investigate the influence of a pre-adaptation phase of cells to a phenolic medium, assays were conducted in Erlenmeyer flasks, as described above. It was observed that the phenolic compounds degradation was not improved, suggesting that the pre-adaptation phase used was not efficient to induce the metabolism of *C. cylindracea* CBS, *Y. lipolytica* W29 and *C. rugosa* CBS strains.

Respirometric assays

The toxicity of phenolic compounds and OMW on the activity of *Y. lipolytica* cells was evaluated through respiratory activity. The measurement of the oxygen uptake rate due to carbon source oxidation gives the fraction of the cells that are active. Figure 3 shows an example of the respiratory profiles of a cellular suspension with and without an injection of carbon source. The slope of linear decrease in the air saturation percentage represents the OUR.

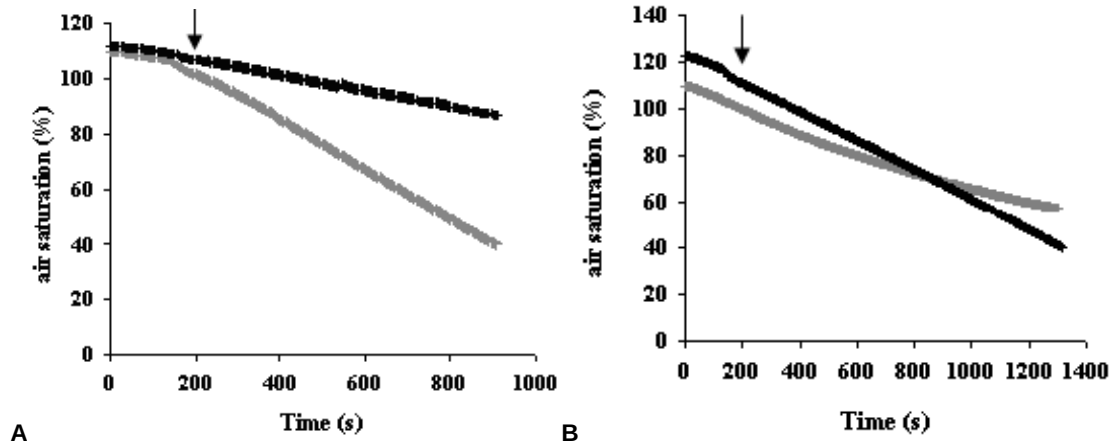

Figure 3. A - Air saturation percentage curves of a *Y. lipolytica* suspension (+) and cellular suspension with injection (+) of OMW (A) or catechol (B). The arrow represents the injection of carbon source.

The injection of a carbon source in cell suspension increase the oxygen uptake rate comparatively to that found in the assay without this addition, as proved with the injection of OMW. However, the addition of catechol to *Y. lipolytica* suspension leads to an inhibitory effect in the respiratory activity of this strain. The respiratory activity factor (RAF) for the different carbon sources was also determined and it was defined as a ratio between the total and the endogenous respiration rate (Table 2).

Table 2. *Y. lipolytica* respiratory activity factor (RAF) with different carbon sources

	Glucose	Phenolic solution	OMW (COD)	Catechol
Concentration/($g \cdot L^{-1}$)	0.48	0.83	3.8	0.63
RAF	2.01	2.24	3.09	0.82

It was observed that the injection of OMW and phenolic solution, without catechol, improved the total respiration rate even more than glucose. However, catechol injection leads to a respiratory activity inhibition.

The loss of activity factor was determined for assays with 30 min of catechol incubation and injection of glucose. The loss of activity obtained was, approximately, 99.6% for a concentration of catechol of 0.32 $g \cdot L^{-1}$.

Conclusions

The phenolic extracts obtained from the OMW have a large number of phenolic compounds, but these are mainly rich in hydroxytyrosol. The recovery of this important compound is improved in the solid-liquid extraction procedure, compared to that obtained by the liquid-liquid extraction methodology.

The less toxic compound, hydroxityrosol, is the most abundant in both OMW but there was no significant inhibition by the other phenolic compounds existing in the OMW. Thus the OMWs collected could be used by the lipolytic strains studied (*C. rugosa* PYCC 3238, *C. cylindracea* CBS 7869, *C. rugosa* CBS 2275, *Y. lipolytica* CBS 2073, *Y. lipolytica* W29 ATCC 20460 and *Y. lipolytica* IMUFR).

From the studied concentrations, no evidences of *Y. lipolytica* respiratory activity inhibition, by OMW and it's phenolic compounds, was observed. However, the results for catechol solution proved that exist considerable inhibition to yeast culture by this compound.

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