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Biological treatment of olive mill wastewater by non-conventional yeasts

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ABSTRACT

The ability of lipolytic yeasts to grow on olive mill wastewater (OMW)-based medium and to produce high-value compounds while degrading this waste, was tested. OMW collected from three-phase olive mills from the North region of Portugal were characterized and used. OMW with COD ranging from 100 g L^{-1} to 200 g L^{-1} were supplemented with yeast extract and ammonium chloride. Studies of OMW consumption were carried out in batch cultures of *Candida rugosa*, *Candida cylindracea* and *Yarrowia lipolytica*. All strains were able to grow in the OMW-based media, without dilution, to consume reducing sugars and to reduce COD. *C. cylindracea* was the best strain concerning the lipase production and the reduction of phenolic compounds and COD. For all strains, the phenols degradation was quite difficult, mostly when more easily degradable carbon source is still present in the medium. Among the phenolic compounds tested catechol is the most inhibitory to the cells.

1. Introduction

The olive oil consumed in the world is mainly produced in the Mediterranean basin countries. With an estimated olive oil production of 40 thousand tones per year, Portugal is one of the 10 major producers.

Pressing (traditional system) and continuous (three- or two-phase) are the most important extraction processes, applied for olive oil production. Three- and two-phase extraction technologies differ in the water supplies. Large amounts of water are required to an extraction process with a three-phase decanter and large amount of a liquid by-product is generated. This effluent is known as olive mill wastewater (OMW) and it is a stable emulsion composed of water, olive pulp and oil (Lanciotti et al., 2005). The OMW constituents, quality and quantity, depend on many factors, such as, type of olives and its maturity, climacteric conditions, region of origin, cultivation methods and specially the technology used for oil extraction (Roig et al., 2005). The organic fraction of OMW includes sugar, tannins, polyphenols, polyalcohols, pectins and lipids, which results in high values of chemical oxygen demand (COD) (Papanikolaou et al., 2008), with values up to 220 g L^{-1}.

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 but only 2% of the total phenolic content of the olive fruit remains 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). Thereby, uncontrolled OMW disposal can create several risks to the environment and it is urgent to develop a suitable treatment. Due to the seasonality of olive oil production the OMW treatment process should be flexible enough to operate in a non-continuous mode. Moreover, the olive mills are small enterprises, scattered around the olive production areas, making individual on-site treatment options unaffordable (Paraskeva and Diamadopoulos, 2006).

Several methods have been proposed, which include, mainly, physical–chemical treatments. The most common method applied has been the storage of OMW in lagoons, followed by evaporation during summer season (Azbar et al., 2004). This method is not satisfactory since it only reduces the volume of waste, without treating the pollutants, and a black foul-smelling sludge, difficult to remove, is produced. The anaerobic biological degradation of OMW can lead to methane production even if large periods of biomass adaptation have been reported as a disadvantage of the process. Biological treatment by aerobic microorganisms (fungi and yeasts) has also been proposed (Eusébio et al., 2002).

Instead of disposal solutions an approach of using this waste as a resource to be valorized is of greater interest. Some lipolytic yeast species can grow well in OMW media, consume the organic material and, at the same time, produce biomass and other valuable products (Scioli and Vollaro, 1997; D'Annibale et al., 2006), like enzymes and organic acids. 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

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reactive oxygen species are involved in the onset of several human diseases and in the oxidative degradation of food (De Marco et al., 2007).

The aim of the present investigation was the valorization of different OMW by producing high-value compounds from OMW while degrading this waste. Thus, the objective of this study was the OMW aerobic treatment with lipase and/or biomass production, with process conditions optimization and strains selection.

2. Methods

2.1. Microrganisms and OMW used

Strains of *Candida rugosa* (PYCC 3238 and CBS 2275), *Candida cylindracea* CBS 7869 and *Yarrowia lipolytica* (CBS 2073, W29 ATCC 20460 and IMUFRJ 50682) were maintained in YPD Agar at 4 °C. Cells were pre-grown in YPD medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone and 20 g L^{-1} glucose) and then harvested (12225 g, 5 min) from the pre-culture and re-suspended in the OMW-based media.

The OMW samples were collected from different three phases' olive oil mills, from the north of Portugal, and were stored at −20 °C, immediately after arrival to the laboratory. These samples were obtained during two consecutive campaigns (OMW1–OMW4) and were characterized for pH, COD, total solids and total volatile solids, nitrogen (Kjeldhal), phenols, reducing sugars and total protein (Table 1).

2.2. Culture conditions

2.2.1. Degradation trials

The OMW used to the degradation trials were enriched with ammonium chloride and yeast extract and, after sterilization, its pH was adjusted to 5.6. The supplementation was made proportionally to its organic composition, in order to counteract the lack of nitrogen. The NH_4Cl concentration added was about 10% (p/p) to 15% (p/p) of the OMW COD and reducing sugars, respectively. Yeast extract concentration used was approximately 40% (p/p) of the NH_4Cl added.

The batch cultures, with OMW base media, were carried out in Erlenmeyer baffled flasks, with 1000 mL of total volume, or in a 2000 mL bioreactor (Biolab, B. BRAUN).

The cultures, with an initial concentration of approximately 10^6 cells mL^{-1}, were incubated at 27 °C. The stirring rate in the Erlenmeyer flasks and in the lab-scale bioreactor was 240 rpm and 400 rpm, respectively.

Throughout the process time, culture samples were collected for several analyses. Cell density, assessed by cell counting in the microscope, and pH adjustment were made at each sampling time. The pH was automatically adjusted on the bioreactor. The samples were stored at −20 °C for further analyses.

Table 1
OMW characterization.

Parameter[a]	OMW 1	OMW 2	OMW 3	OMW 4
	Campaign 2006/2007		Campaign 2007/2008	
pH	4.8 ± 0.1	4.9 ± 0.2	4.7 ± 0.3	5.5 ± 0.1
COD (g L^{-1})	184 ± 2	191 ± 2	115 ± 1	179 ± 2
Total solids (g L^{-1})	115 ± 3	119.6 ± 0.2	148± 3	142.7 ± 0.2
Total volatile solids (g L^{-1})	84 ± 12	84 ± 42	117 ± 6	113.9 ± 2.3
Nitrogen (Kjeldhal) (mg L^{-1})	95 ± 17	60 ± 12	192 ± 17	–
Phenols (tyrosol) (g L^{-1})	9.7 ± 0.2	12.1 ± 0.2	5.5 ± 0.1	5.7 ± 0.1
Reducing sugars (g L^{-1})	45.5 ± 0.5	34.4 ± 0.9	12.9 ± 0.7	52.4 ± 0.4
Total protein (g L^{-1})	0.8 ± 0.3	1.3 ± 0.0	1.2 ± 0.2	1.3 ± 0.0

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

2.2.2. OMW phenolic compounds toxicity

Phenolic compounds toxicity to yeast strains was studied, in batch cultures, in 96-wells microplates during 100 h, approximately. Cells were pre-grown in YPD medium, for 24 h. Then, 20 μL of cells suspension were transferred to each microplate well with 300 μL of 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, caffeic acid, catechol, oleuropein, syringic acid and vanillic acid.

Respiratory activity essays 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 re-suspended in sodium phosphate buffer 50 mM pH 7.0 in order to obtain a suspension with a final cellular concentration of 9×10^6 cell mL^{-1}. 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 °C. Each vessel contains an oxygen probe connected to an oxygen meter. The vessels were closed and the decrease in dissolved oxygen tension (DOT) 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 (OUR), due to substrate oxidation, solutions of glucose (0.48 g L^{-1}), catechol (0.32 g L^{-1}) or OMW (3.8 g COD L^{-1}) were injected into the vessel. An essay without carbon source addition was performed simultaneously and used as control for endogenous OUR determination.

2.3. Analytical methods

The OMW were characterized by the following parameters: chemical oxygen demand (COD), Solids (total, volatile and dissolved) and nitrogen (Kjeldahl) according to standard methods (APHA et al., 1989). Total phenols were assessed by the Folin-Ciocalteau method (Commission Regulation (EEC) No. 2676/90) using tyrosol as standard. Reducing sugars were measured by the DNS method (Miller, 1959) and were expressed as glucose. Extracellular lipase was measured in the samples supernatant using *p*-nitrophenyl-butyrate (pNPB) in sodium acetate buffer 50 mM at pH 5.6 as a substrate, at 37 °C for 15 min. One unit of activity was defined as the amount of enzyme that produces 1 μmol of *p*-nitrophenol per minute under essay conditions.

Cells were observed in an Olympus BX51 microscope immediately after sampling (48 h of growth) for the morphology changes detection.

3. Results and discussion

3.1. Comparison of different strains degrading different OMW

Experiments with *Y. lipolytica* W29 and *C. rugosa* PYCC 3238, in OMW were performed using 1000 mL Erlenmeyer baffled flasks, with 400 mL of OMW-based media. The OMW used were OMW1 and OMW2. Both strains were able to grow on both OMW, without dilution (Fig. 1), increasing about 1.7 log the cell number. Cell mass production was higher for OMW1 than for OMW2, for both strains, probably due to the higher content of sugars and lower content of phenolic compounds in this medium (Table 1). Both strains were able to consume almost all of the sugars present in the media and to significantly reduce COD (Table 2). In spite of the low degradation of phenolic compounds, no cell growth inhibition was noticed. The COD, reducing sugars and phenolic compounds reductions were greater in OMW1. The strain of *C. rugosa* seemed to be more efficient than *Y. lipolytica* reducing the OMW COD, but *Y. lipolytica* degraded better the phenolic compounds (Table 2).

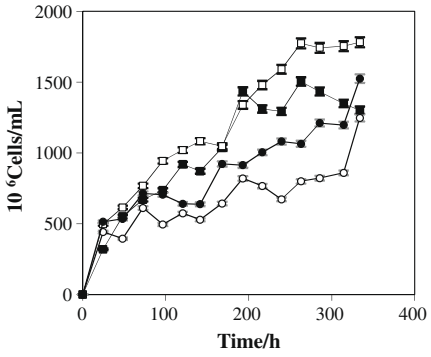

Fig. 1. Growth of *Y. lipolytica* W29, in OMW 1 (■) and OMW 2 (○); and *C. rugosa* PYCC 3238, in OMW 1 (□) and OMW 2 (●).

Table 2
OMW organic matter degradation, for different conditions of OMW and strains.

Yeast strain	Reducing sugars reduction (%)		Phenolic compounds reduction (%)		COD reduction (%)	
	OMW 1	OMW 2	OMW 1	OMW 2	OMW 1	OMW 2
Y. lipolytica W29	90.5	71.8	19.2	20.6	52.6	29.5
C. rugosa PYCC 3238	80.2	64.2	12.2	N[a]	62.2	35.8

[a] Negligible.

Yeast cells were observed by optical microscopy. Cells displayed a typical oval form in all the essays, demonstrating that cell growth in OMW medium did not induce hyphae formation for both strains but the cells seems to be more aggregated than in YPD medium. Yeasts are capable of forming aggregates, as a survival strategy in adverse conditions (Calleja, 1987). Moreover, the presence of lipids in the OMW can induce the cell aggregation around oil droplets, particularly for strains with hydrophobic cell surfaces, as *Y. lipolytica* (Aguedo et al., 2005). A greater cell aggregation occurred for *C. rugosa* strains, which could probably cause substrates availability limitations, to the cells. These limitations could explain the weak cell growth observed, for this strain.

Further trials, with the six strains of *C. rugosa*, *C. cylindracea* and *Y. lipolytica*, using the OMW samples OMW3 and OMW4 (Table 1), were conducted, using 1000 mL Erlenmeyer baffled flasks, with 400 mL of working volume. All strains were able to grow on both OMW, without dilution and with similar growing profiles previously presented. OMW4 seems to be more easily degradable, since higher reduction values of sugars, phenolic compounds and COD (Table 3) were obtained for this sample than for OMW3. The phenolic compounds degradation in OMW3 was negligible for all strains. The strains of *Y. lipolytica* W29 and *C. cylindracea* CBS 7869, presented the better performance degrading phenolic compounds of OMW4. Moreover, *C. cylindracea* was the most efficient strain on COD degradation in both OMW samples. Concerning the reducing sugars consumption, no significant differences were found among the cultures but the strains of *Y. lipolytica* and *C. cylindracea* presented a slight better performance.

Table 3
OMW organic matter degradation, for different conditions of OMW and strains.

Yeast strain	Reducing sugars reduction (%)		Phenolic compounds reduction (%)		COD reduction (%)	
	OMW 3	OMW 4	OMW 3	OMW 4	OMW 3	OMW 4
Y. lipolytica W29	55.5	85.1	N[a]	31.3	21.6	36.9
Y. lipolytica CBS 2073	56.5	85.3	N[a]	25.3	23.5	51.3
Y. lipolytica IMUFRJ 50682	58.8	76.0	N[a]	20.0	23.1	50.9
C. rugosa PYCC 3238	56.5	82.6	N[a]	20.4	20.4	58.7
C. rugosa CBS 2275	55.6	68.7	N[a]	15.3	31.1	40.9
C. cylindracea CBS 7869	54.8	84.3	N[a]	27.0	45.8	70.2

[a] Negligible.

Table 4
Maximum of lipase activity produced for the different strains and OMW, in Erlenmeyer baffled flasks.

Yeast strain	Maximum of lipase activity/(U L^{-1})	
	OMW 3	OMW 4
Y. lipolytica W29	451.8 ± 253.8	320.4 ± 194.9
Y. lipolytica CBS 2073	1041.1 ± 182.8	828.1 ± 140.5
Y. lipolytica IMUFRJ 50682	533.4 ± 185.9	371.7 ± 128.4
C. rugosa PYCC 3238	433.7 ± 194.9	494.1 ± 207.0
C. rugosa CBS 2275	832.6 ± 87.6	373.2 ± 39.3
C. cylindracea CBS 7869	2200.1 ± 831.1	877.9 ± 329.4

Data are mean values ± standard deviation ($n = 2$).

3.2. OMW use for lipase production

Lipase production by *Y. lipolytica* W29 grown on OMW-based medium was already demonstrated in previous work (Lopes et al., 2008 II). In the herein presented work was also possible to detect lipase activity, in undiluted OMW3 and OMW4. The maximum lipase activity, obtained for the six strains, referred above, is shown in Table 4. The kinetic profile demonstrates that the activity increases up to a maximum, after what it decays, which is in accordance with the ones obtained by other authors (Lopes et al., 2008; 2008 II). In both OMW, *C. cylindracea* was the strain that expressed the highest value of lipolytic activity. This result agree with the one obtained by D'Annibale et al. (2006), in which the authors demonstrated the high potential of lipase production by one strain of *C. cylindracea*. An increase of the lipase activity in OMW3 with *C. cylindracea* was obtained, compared with the experiment with *Y. lipolytica* W29. Except for *C. rugosa* PYCC 3238, all strains reached the highest value of lipolytic activity in OMW3, probably due to lower content in sugars that can repress lipase production (Fickers et al., 2003; Dalmau et al., 2000). After these experiments, in Erlenmeyer flasks, and since *C. cylindracea* has presented higher lipase production values, batch cultures with this strain were performed in the bioreactor (Fig. 2). Lipase production up to 3511 U L^{-1}, approximately, was obtained, showing that this strain can be used for the scale-up of lipase production, from OMW.

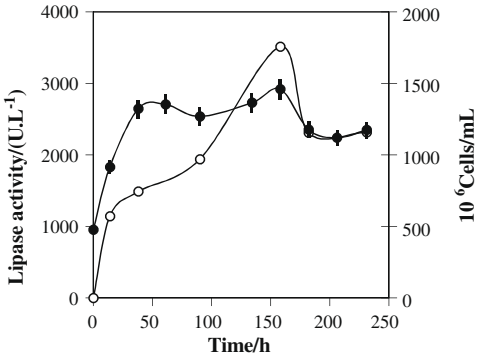

Fig. 2. Cell growth (●) and lipase activity (○) for *C. cylindracea* CBS culture in OMW 3, in the bioreactor.

3.3. OMW phenolic compounds toxicity

The effect of different phenolic compounds, commonly found in OMW, on each yeast strain growth was evaluated by essays in 96-wells microplates. Typical growth curves profile for the experiments with *Y. lipolytica* W29 and *C. rugosa* CBS 2275 strains are shown in Fig. 3. Similar curves were obtained to the other strains. For the phenolic compounds concentration used (1 g L^{-1}) a final biomass concentration decrease was observed, in phenolic media, comparatively to YPD medium, for all strains used, except for *Y. lipolytica* W29. Beside this difference, it was found 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 essays 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 confirm the results obtained in the microplate trials, in which the presence of catechol inhibits the cell growth, respirometric short-term essays were made with this phenolic compound, glucose and OMW, as carbon sources.

Oxygen uptake rate (OUR), due to carbon source oxidation, was assessed by the slope of linear decrease in the dissolved oxygen tension with time and compared with the endogenous one obtained without any carbon source present. Fig. 4 shows an example of the respiratory profiles of a cellular suspension, with and without injection of carbon source (OMW 3.8 g COD L^{-1} and catechol 0.32 g L^{-1}). The injection of OMW in cell suspension increase threefold the OUR comparatively to that found in the essay without this addition. A twofold OUR increase was found in the essay with addition of 0.5 g L^{-1} glucose solution (data not shown). The addition of catechol to *Y. lipolytica* suspension leads to an inhibitory effect in the respiratory activity of this strain, since the OUR value obtained with catechol in the medium was 93% of the endogenous value, that is in accordance with the results obtained in the essays on microplates and other studies (Obied et al., 2005).

In spite of some authors (Obied et al., 2005; El Hadrami et al., 2004) reported catechol as one of the most abundant compounds in traditional OMW, the results presented in herein work suggested that the OMW used has a lower concentration of this phenolic compound, once OMW was not inhibitory to growth and respiratory activity of *Y. lipolytica* W29.

4. Conclusions

The results of this study confirmed the potential application of the non-conventional lipolytic yeasts for OMW valorization, by its use as culture medium for biomass and enzymes production. The ability of all strains used, to produce lipase from undiluted OMW was shown. Moreover, *C. cylindracea* was the best strain concerning the lipase production and also for the COD reduction.

The OMW samples did not inhibit the yeasts cell growth, comparably to YPD medium. Catechol was found to be the most inhibitory phenolic compound for the yeast cells used. However, tyrosol, hydroxytyrosol, oleuropein, syringic acid and vanillic acid did not affect the cell growth. Therefore, catechol is supposed to have null or insignificant concentrations on the OMW used. This conclusion was confirmed by respirometry experiments where a strong respiratory activity inhibition was caused by catechol to *Y. lipolytica*, in contrast to the OMW samples effect.

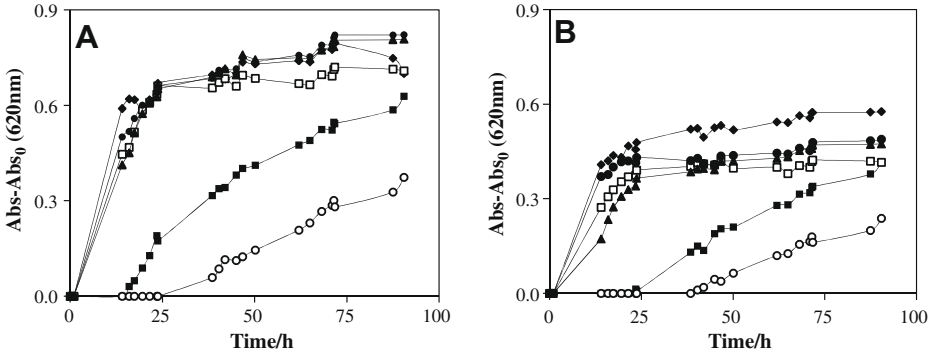

Fig. 3. Batch growth profile of *Y. lipolytica* W29 (A) and *C. rugosa* CBS (B) in YPD medium (♦) and phenolic mediums: catechol (○), hydroxytyrosol (▲), caffeic acid (■), tyrosol (□) and oleuropein (●).

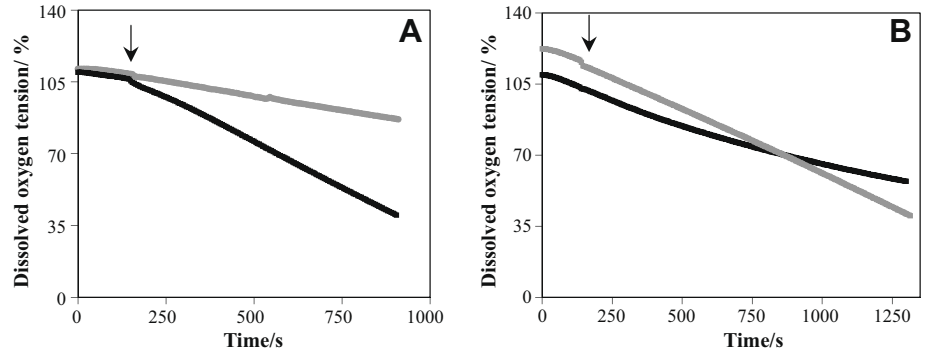

Fig. 4. Comparison of the oxygen consumption by *Y. lipolytica* suspension in phosphate buffer without carbon source (grey) and with the addition of OMW (A) or catechol (B) (black). The arrow represents the injection of carbon source.

Thus, the utilization of OMW as a resource to be valorized is of greater interest, since it could be used to produce high-value products while is being degraded.

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