

Olive mill wastewater valorization: Use by non-conventional yeasts

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Abstract

The ability of the strains *Yarrowia lipolytica* and *Candida rugosa* to grow on Olive Mill Wastewater (OMW) based medium and to produce high-value compounds from OMW (such as enzymes), while degrading this waste, was tested. Factors affecting cellular growth and OMW degradation were also studied, such as OMW composition, dilution and supplementation.

OMW collected from 3-phase olive mill of the north region of Portugal were used and characterized chemical and biochemically. OMW with COD ranging from 100 g·L⁻¹ to 200 g·L⁻¹ were supplemented with yeast extract and ammonium chloride proportionally to its organic composition. Preliminary studies of OMW consumption were carried out in batch cultures of *Y. lipolytica* W29 and *C. rugosa* PYCC 3238.

The strains were able to grow in the OMW used without dilution, to consume almost all of the sugars present in the media and to significantly reduce COD.

Y. lipolytica W29 was less affected by operating conditions changes such as, stirring rate variation and OMW medium supplementation.

Keywords: *Candida rugosa*; lipase; Olive Mill Wastewater; OMW; *Yarrowia lipolytica*

1. Introduction

Olive mill industry is a traditional agricultural industry in Mediterranean countries. These countries accounts for about 95% of the world olive production (Al-Malah et al, 2000) and produce almost all the olive oil sold worldwide. Olive oil production results on a large amount of liquid waste, known as Olive Mill Wastewater, which represents a major environmental problem.

The olive mill wastewater is a stable emulsion composed of “vegetation waters” of the olives, water from the processing, olive pulp and oil (Lanciotti et al, 2005). This important residue of the olive oil industry is characterized by an intensive brown to dark colour (due to recalcitrant compounds), a strong acidic smell and a high organic content.

The quality and quantity of olive mill wastewater (OMW) constituents depends on many factors, such as, type of olives and its maturity, climacteric conditions, region of origin, cultivation methods and especially the technology used for oil extraction (Roig et al, 2005). The organic fraction of OMW includes sugar, tannins, polyphenols, polyalcohols, pectins and lipids, that results in high values of chemical oxygen demand (COD) (Papanikolaou et al, 2008). This effluent has COD values up to 220 g·L⁻¹ and corresponding biochemical oxygen demand (BOD) values up to 100 g·L⁻¹ (Paraskeva and Diamadopoulos, 2006).

The main problems associated with OMW pollution can be attributed to the phenolic fraction. In fact, phenolic compounds are responsible for several biological effects, including antibiosis and phytotoxicity. (Lanciotti et al, 2005).

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 disposal methods have been proposed and mainly include physical-chemical treatments but the most common method applied has been the storage of OMW in lagoons, followed by evaporation during summer

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season (Azbar et al, 2004). Biological treatment by aerobic microorganisms (fungi and yeasts) has also been proposed (Eusébio et al, 2002). The anaerobic biological degradation of OMW can lead to methane production although large periods of biomass adaptation have been reported as a disadvantage of the process. Yeast species such as *Yarrowia lipolytica* and *Candida cylindracea* can grow well in OMW media, consume the organic material and, at the same time, produce biomass and other valuable products (Scioli et al, 1997; D'Annibale et al, 2006), like enzymes and organic acids.

The aim of the present investigation was the valorization of OMW by producing high-value compounds from OMW while degrading this waste. Thus, in a first stage, the objective of this work is the comparison of different non-conventional yeasts, for potential use of OMW to grow and to produce high-value compounds, such as enzymes (e.g.: lipases), while degrading and detoxifying this waste. This first stage will be followed by an anaerobic treatment to produce methane and to totally degrade the effluent. The tradability of the effluent and the efficiency of the biological treatment will be based on studies of toxicity and biodegradability of the effluent, before and after treatments, using anaerobic and aerobic microorganisms.

Firstly, the optimization of yeast growth on OMW based media was attempted, screening the effects of some operating parameters on cellular behavior during OMW utilization.

2. Materials and Methods

2.1. Microorganisms, media and culture conditions

Strains of *Yarrowia lipolytica* W29 (ATCC 20460) and *Candida rugosa* PYCC 3238 were used. The OMW used was obtained from three phases olive oil mills, from the north of Portugal.

Cells, of both strains, were pregrown in YPD medium (composed of 10 g·L⁻¹ Yeast Extract, 20 g·L⁻¹ Glucose and 20 g·L⁻¹ Peptone), by 24 h. After that, cells were harvested (12225 g, 5 min) from the pre-culture and re-suspended in the OMW medium. Batch cultures were carried out, with the two strains, in 1000 mL and 500 mL Erlenmeyer baffled flasks filled with 400 mL and 200 mL, respectively, of OMW sterilized medium. The pH of the medium was adjusted to 7.2, prior to sterilization. OMW media were supplemented with ammonium chloride and yeast extract, proportionally to its organic composition in order to counteract the lack of nitrogen. The NH₄Cl concentration added was about 10% to 15% of the COD and reducing sugars of OMW, respectively. Yeast extract concentration used was approximately 40% of the NH₄Cl added.

The cultures, with an initial concentration of approximately 10⁶ cells·mL⁻¹, were incubated at 27 °C and 240 rpm of stirring rate, for approximately 300 h. Through the fermentation time, culture samples were collected for several analyses and pH was adjusted. Cell density was immediately determined by cell counting and samples were stored at -20 °C for further analysis.

Experiments were performed with OMW supplemented with the basal concentrations mentioned above and with the double of those concentrations, and compared with experiments in YPD medium. The influence of stirring rate on the yeast growth in OMW was also assessed.

2.2. Analytical methods

OMW from different olive mills were collected and characterized chemically and biochemically. After characterization, two OMW (OMW1 from *Beira Interior*, OMW2 and OMW3 from *Minho* region) were selected by their high organic load. The samples were previously centrifuged and analyzed. The characterization of OMW samples is shown on Table 1.

Chemical Oxygen Demand (COD), Solids (total, volatile and dissolved), Nitrogen (Kjeldahl) were determined according to Standard Methods (APHA et al., 1989). Total phenols were assessed by the Folin-Ciocalteu Method (Commission Regulation - EEC - N° 2676/90) using caffeic acid as a standard. Reducing sugars were measured by DNS method.

Extracellular lipase activity of the samples was measured using p-nitrophenyl-butirate (pNPB) as substrate. The enzymatic reaction was followed by the absorbance measurement at 400 nm during 3 min. Activities were calculated by linear regression of the absorbance vs time, using the molar extinction coefficient of pNP of 8 mmol⁻¹ l. One unit of activity was defined as the amount of enzyme that produces 1 μmol of p-nitrophenol per minute under assay conditions.

Cellular observations and cell images were performed and acquired, respectively, in an Olympus BX51 microscope coupled with a colour video camera linked to a personal computer by a frame grabber. Samples collected through fermentations were analyzed for COD, sugars, total phenols and lipases by the methods above described.

Table 1. Characterization of OMW's used

Parameter ^a	Olive Mill Wastewater		
	OMW1	OMW2	OMW3
pH	4.84	4.93	4.71
COD/(g·L ⁻¹)	184 ± 2	191 ± 2	99 ± 1
Total Solids/(g·L ⁻¹)	115 ± 3	119.6 ± 0.2	148 ± 3
Total Volatile Solids/(g·L ⁻¹)	84 ± 12	84 ± 42	117 ± 6
Nitrogen (Kjeldhal)/(g·L ⁻¹)	9 ± 7	6 ± 2	5 ± 4
Phenols (Caffeic acid)/(g·L ⁻¹)	9.7 ± 0.2	12.1 ± 0.2	5.5 ± 0.1
Reducing Sugars/(g·L ⁻¹)	45.5 ± 0.5	34.4 ± 0.9	12.9 ± 0.7
Total Lipids/(g·L ⁻¹)	-	-	39 ± 6
Total protein/(g·L ⁻¹)	0.8 ± 0.3	1.3 ± 0.0	1.2 ± 0.2

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

3. Results and discussion

3.1. Use of diluted OMW by *Candida rugosa* for lipase production

Lipase production by *Y. lipolytica* W29 grown on OMW based medium was already demonstrated in previous work (Araujo et al, 2005). In the herein presented work, lipase activity in *C. rugosa* PYCC 3238 cultures with OMW based medium was detected (Figure 1). It was shown the ability of *C. rugosa* strain to grow in OMW medium and to produce up to 200 U·L⁻¹ of lipase. In spite of the high organic charge of the OMW used, dilution did not reveal to be useful. Cells grew better and produced the highest amount of enzyme in the more concentrated medium.

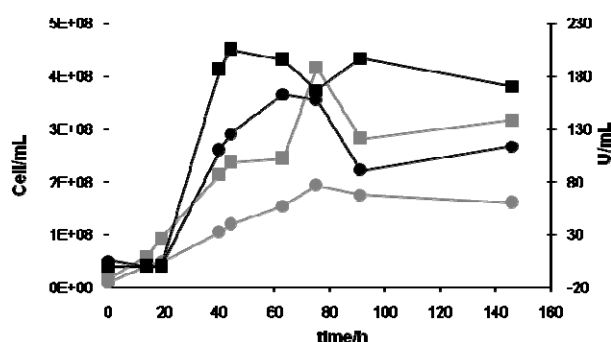


Figure 1. Cell Growth (■,●) and lipase activity (□,○) for cultures of *C. rugosa* strain PYCC 3238 in OMW1 with 1:5 dilution (■,●) and 1:10 dilution (□,○).

3.2. Comparison of two different strains degrading two different OMW

Assays with *Yarrowia lipolytica* W29 and *Candida rugosa* PYCC 3238, in OMW1 and OMW2, using 1000 mL Erlenmeyer baffled flasks were made at 240 rpm of stirring rate. Both strains were able to grow on OMW1 and OMW2 media, without dilution (Figure 2), 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).

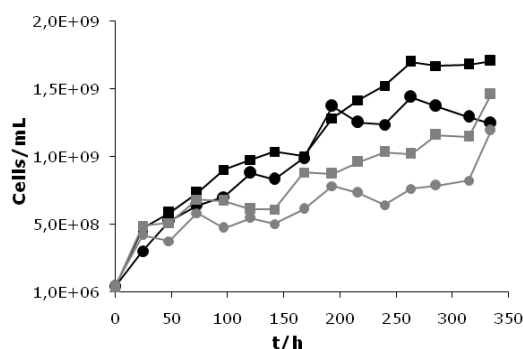


Figure 2. Growth of both strains in OMW1 [*Y. lipolytica* (●); *C. rugosa* (■)] and OMW2 [*Y. lipolytica* (●); *C. rugosa* (■)], through time of fermentation.

In spite of the low degradation of phenolic compounds, no cell growth inhibition was noticed. The reduction of COD, reducing sugars and phenols was greater in OMW1. The strain of *Candida rugosa* seemed to be more efficient than *Yarrowia lipolytica* degrading the COD in OMW, but *Y. lipolytica* degraded better the phenolic compounds (Table 2). Lipase production was detected for both cultures at identical levels mentioned above.

Table 2. Overall results of organic matter degradation

Conditions	COD reduction (%)	Sugars reduction (%)	Phenols reduction (%)
OMW1 with <i>Y. lipolytica</i>	52.6	90.5	19.2
OMW1 with <i>C. rugosa</i>	62.2	80.2	12.2
OMW2 with <i>Y. lipolytica</i>	29.5	71.8	20.6
OMW2 with <i>C. rugosa</i>	35.8	64.2	ND ¹

3.3. Comparison of two different strains using different media supplementation

Assays with *Yarrowia lipolytica* W29 ATCC20460 and *Candida rugosa* PYCC 3238, in OMW3, have been made in 500 mL Erlenmeyer flasks with baffles with two concentration values of the media supplements, yeast extract and NH₄Cl. Cells growth in OMW of both strains was compared with cultures in YPD medium, used as control. In order to assess the effect of stirring rate a lower value of agitation rate was used (140 rpm).

Results obtained for the cellular growth are depicted in Figure 3. It was possible to conclude that *Candida rugosa* has a larger phase of adaptation under this condition of agitation. A significant difference of the cell growth kinetics was observed for cultures of *C. rugosa* in YPD medium and in OMW based media (Figure 3A). The increase in medium supplements concentration had a positive influence in the use of OMW by this strain. Contrarily, *Yarrowia lipolytica* cells grew identically on the three media tested, OMW based media and YPD (Figure 3B). No influence of supplementation increasing on cellular growth was observed.

¹ ND: No phenols degradation was observed.

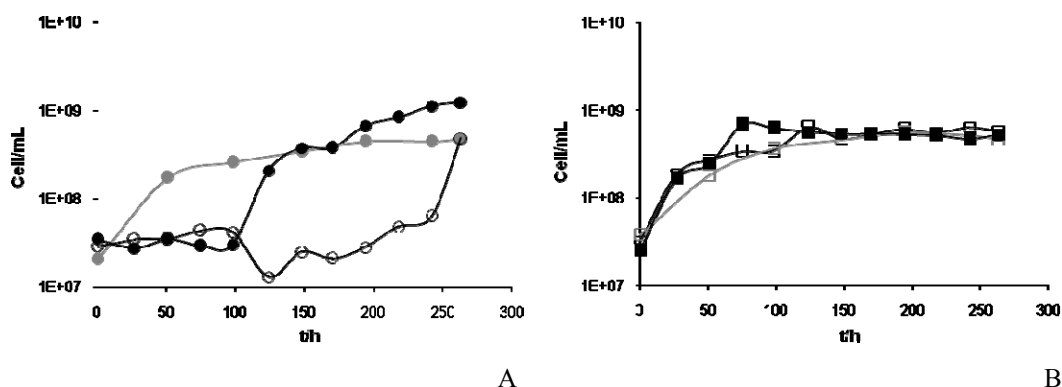


Figure 3. Growth of *Candida rugosa* (A) and *Yarrowia lipolytica* (B) in OMW3 supplemented (○, □), in synthetic medium (●, ■) and in OMW3 two fold supplemented (●, ■), through time.

The pH of these assays was controlled and its mean values were 6 and 7, approximately, for *Candida* and *Yarrowia* experiments, respectively. Organic matter degradation was affected by the reduction of stirring rate for both stains used (Table 3). In fact, a lower COD and reducing sugars consumption was notice compared to the previous results obtained at higher agitation rates (Table 2). Increase of medium supplements concentration favored the OMW degradation, for *C. rugosa* experiments. The higher ability to the phenolic compounds degradation of OMW by the *Y. lipolytica* strain was confirmed. Moreover, in experiences with *Y. lipolytica*, a progression of color (being more intensive brown in the last samples taken) was noticed. This can be explained by the presence of tyrosine in OMW3. Some strains of *Y. lipolytica* have a unique ability, among yeasts, to produce brown pigments when grown in presence of tyrosine (Carreira and Loureiro, 1998).

Table 3. Overall results of organic matter degradation

Conditions	COD reduction (%)	Sugars reduction (%)	Phenols reduction (%)
OMW3 with <i>C. rugosa</i>	17.9	24.6	11.0
OMW3 (two fold supplemented) with <i>C. rugosa</i>	32.6	41.8	ND ²
OMW3 with <i>Y. lipolytica</i>	32.9	29.3	20.7
OMW3 (two fold supplemented) with <i>Y. lipolytica</i>	33.3	45.1	10.8

3.4. Effect of OMW medium on cell morphology

Yeast cells were observed by optical microscopy. Several differences were found between the morphology of *Y. lipolytica* and *C. rugosa* in different medium conditions, as shown on the figures below (Figure 4 and Figure 5). Cells displayed a typical oval form in all the assays but seams to be more aggregated in OMW than in synthetic medium. Yeasts are capable of forming aggregates as a survival strategy in adverse conditions (Calleja, 1987). Also, the presence of lipids in the OMW can induce the cell aggregation around oil droplets, particularly for strains with hydrophobic cell surfaces as is the case of *Y. lipolytica* (Aguedo et al., 2005). However, a greater cell aggregation was observed for *C. rugosa* strains that can be responsible for the weak cell growth observed, probably caused by limitations of substrates availability to the cells.

² ND - No phenols degradation was observed.

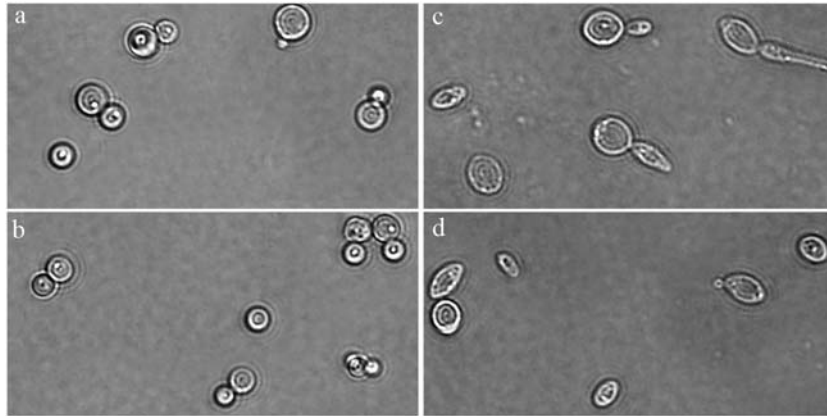


Figure 4. *Candida rugosa* (a, b) and *Yarrowia lipolytica* (c, d), in synthetic medium.

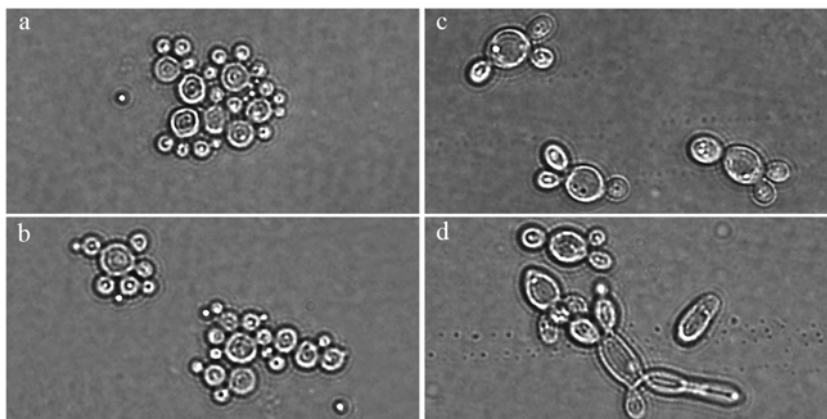


Figure 5. *Candida rugosa* (a, b) and *Yarrowia lipolytica* (c, d), in OMW medium two fold supplemented.

3. Conclusions

The results of this study confirmed the potential application of the non-conventional lipolytic yeasts *Yarrowia lipolytica* W29 and *Candida rugosa* PYCC 3238 for OMW valorisation by its use as culture medium for biomass and enzymes production. The ability of *C. rugosa* to produce lipase from OMW was shown. Both strains were able to use undiluted OMW and OMW dilution led to lower cellular growth and lipase production. However, the use of OMW based media by *Y. lipolytica* strain is less affected by operating conditions variation, such as stirring rate decrease and media supplements concentration. This can be particularly interesting, considering that the possibility of working with lower stirring rates and lower nutrients supplementation present important operating cost savings. Moreover, *Y. lipolytica* presented an additional advantage over *C. rugosa* which was the higher phenolic compounds degradation.

The utilization of olive mill wastewaters for biological production of high value products may have a positive impact on the environmental problem of OMW management, since it can act also as a first step of effluent treatment.

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