Research Article

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The use of olive mill wastewater by wild type *Yarrowia lipolytica* strains: medium supplementation and surfactant presence effect

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Abstract

BACKGROUND: The aim of this work was to study the ability of two different wild type strains of the yeast *Yarrowia lipolytica* to grow on olive mill wastewater (OMW) and their potential to produce high-value products such as lipases. Factors that affect cellular growth and OMW degradation were studied, such as nitrogen supplementation, cells concentration and surfactant addition.

RESULTS: Both strains, W29 and IMUFRJ 50682, were able to grow in OMW with 19 g L^{-1} of COD and approximately 800 mg L^{-1} of total phenols concentration. The strain W29 presented the highest potential for extracellular lipase production in OMW medium. Lipase productivity was improved by the medium supplementation with ammonium sulphate up to 6 g L^{-1}, leading to 80% of COD degradation and 70% of total phenols reduction. The surfactant Tween 80 enhanced cell growth and COD degradation, but had a negative effect on lipase activity.

CONCLUSIONS: *Y. lipolytica* has a great potential for OMW valorisation by its use as culture medium for biomass and enzymes production. The operating conditions that favoured lipase production differ from the conditions that improve COD reduction.

Keywords: olive mill wastewater; *Yarrowia lipolytica*; lipase; fermentation

INTRODUCTION

Mediterranean countries produce more than 98% of the world's olive oil, which is estimated at over 2.5 million metric tons per year and about 75% is produced in the European Union (EU). The largest olive oil producers are Spain, with 36%, Italy, with 24%, and Greece, with 17%, of the world's total production. The next largest producer is Portugal, with a production of one order of magnitude lower than the three leading countries, followed by France, Cyprus and Croatia[1]. The olive oil extraction results in a large amount of liquid waste. The quality and quantity of the constituents of olive mill wastewater (OMW) are dependent on many factors: type of olives, type of soil, cultivation system and production process. Batch (press) is the traditional method applied for olive oil production but has been replaced by the continuous operation. This method can be performed by a two- or three-phase process, the last one being the most popular and the major source of OMW.[2] The total quantity of OMW produced in Mediterranean countries has reached 30 million m^3 $year^{-1}$ and is produced in a short period of time (early November to late February).[3] OMW are characterised by an intensive dark brown colour, a strong acidic smell and a high organic content (COD values up to 220 g L^{-1}). The large diversity of components found in OMW (carbohydrates, polysaccharides, sugars, lipids and phenolic compounds) makes their treatment difficult, and their disposal becomes a critical environmental problem.[4] Thus, these wastewaters have great importance from an environmental and economical point of view and can be considered not only as a waste to be treated but also a resource to be recovered.

Although many methods have been proposed for the treatment of OMW, including physicochemical,[5] chemical, biological (aerobic and anaerobic),[3] evaporation (natural or forced) and land application,[6] the most commonly used has been the storage of OMW in lagoons, followed by liquid evaporation during summer season.[2]

The OMW treatment in traditional biological plants is limited by the inhibitory effects of phenolic and lipidic compounds on biomass activity. Some proposals have been reported to reduce this problem, including the use of aerobic microorganisms isolated in OMW but no valorisation of the OMW was attempted.[7] The use of fungi can lead to OMW valorisation through the enzymes

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production.[8] The anaerobic treatment of OMW can also represent an effluent recovery due to methane production, however large periods of biomass adaptation and low phenolic compounds degradation have been reported as a disadvantage of the process.[9] OMW detoxification by polyphenols reduction has been tested by chemical (Fenton's reagent) and enzymatic (laccase) methods. Yeasts can also be used to degrade phenolic compounds in OMW.[10]

Specifically, *Yarrowia lipolytica* strains are good candidates for the OMW treatment and recovery since they can grow well on OMW; degrade lipids and polyphenols; possibly consume the organic material; and produce, at the same time, biomass and other valuable products.[11,12] Nevertheless, the efficiency of organic and polyphenolic content reduction, as well as, the organic acids or lipases production from OMW are strongly dependent on the yeast strain.[13]

MATERIALS AND METHODS

Microorganisms and media

The strains of *Yarrowia lipolytica* W29 (ATCC20460; CLIB89) and IMUFRJ 50 682[14] were used. Cells were pre-grown in glucose medium as previously described.[15]

The OMW was obtained from a three-phases extraction olive oil mill of Vila Flor, Portugal. Table 1 shows the composition of OMW.

Culture conditions

Cells were harvested (6000 *g*, 5 min) from the glucose pre-culture and resuspended in the OMW medium. Batch cultures were carried out with both strains in 500 mL Erlenmeyer flasks with 200 mL of initial medium. The pH of the medium was adjusted to 5.6 prior to sterilisation. OMW were supplemented with 6 g L^{-1} and 12 g L^{-1} of ammonium sulphate and 1 g L^{-1} of yeast extract, in order to counteract the lack of nitrogen and vitamins.[11] To investigate the possible nitrogen limitation on the OMW use by the yeast strains, a two-fold increase of ammonium concentration was performed. The cultures, with an initial concentration of 2×10^6 cells mL^{-1}, were incubated at 27 °C and 240 rpm of stirring rate. According to the results of previous experiments and to further improve OMW utilization by *Y. lipolytica* W29, trials with this strain were conducted in baffled conical flasks, with an increased initial cell concentration (10^8 cells mL^{-1}). OMW medium was supplemented with 6 g L^{-1} of ammonium sulphate and 1 g L^{-1} of yeast extract, with and without the addition of 1 g L^{-1} of Tween 80. An experiment with no OMW medium supplementation of yeast extract and ammonium sulphate was also performed.

Table 1. OMW characterization

Parameter	Value	Parameter	Value
pH	4.84		(mg L^{-1})
Colour units	8.2	K	700.33
COD	19 584 mg L^{-1}	Ca	39.53
Total solids	10 500 mg L^{-1}	Na	12.34
Total volatile solids	7 280 mg L^{-1}	Mg	19.52
Nitrogen (Kjeldhal)	50 mg L^{-1}	Cu	0.20
Phenols (caffeic acid)	796 mg L^{-1}	Fe	1.98
Reducing sugars	3 370 mg L^{-1}	Zn	0.75
Total protein	516 mg L^{-1}	Mn	0.30

All essays were repeated at least twice. Cultures were incubated for approximately 100 h and samples were taken along time to monitor and correct pH values. The final culture volume was around half the value of the initial one. Cell density was immediately determined by cell counting and samples were stored at −20 °C for further analysis.

Analytical methods

The OMW were characterised for the following parameters: chemical oxygen demand (COD), solids (total and volatile), colour and nitrogen (Kjeldahl) according to Standard Methods[16]. Total phenols were assessed by the Folin–Ciocalteau Method (Commission Regulation (EEC) N°2676/90) using as standard caffeic acid. Reducing sugars were measured by DNS method. Protein concentration was estimated according to Bradford's method using bovine serum albumin as a standard.

Metals concentrations were determined in a chemically digested sample by atomic absorption spectrometry (method EP 3051).

The samples COD, sugars and total phenols were analysed by the methods used for OMW characterization. Lipase activity in samples was estimated by a spectrophotometric assay method with *p*-nitrophenyl-butyrate (*p*NPB) as substrate.[17] One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of *p*-nitrophenol per minute at 25 °C and pH 7.3.

RESULTS AND DISCUSSION

Effect of ammonium concentration

The two *Y. lipolytica* strains studied were able to grow in OMW supplemented with yeast extract and ammonium sulphate (Fig. 1(A)). The growth curves of both strains were identical for the same culture conditions and a 15-fold cell number increase was obtained after 110 h of cultivation for the lowest value of ammonium sulphate concentration used. Increasing ammonium supplementation did not improve cell growth: on the contrary, a reduction of 30% in final cell density was obtained with 12 g L^{-1} of ammonium added. The increase in ammonium concentration also had a negative effect on extracellular lipase production for both strains (Fig. 1(B)).

The strain IMUFRJ 50 682 has been reported to be an efficient lipase producer,[18] but the strain W29 showed a higher potential for lipase production in OMW based medium, considering the substrate used for lipase activity assessment. In fact, W29 strain has been recently reported to be a good candidate for the OMW use in lipase production.[13] The work herein presented is the first report on the behaviour of IMUFRJ 50 682 strain in OMW based medium. The low lipase activity level found for this strain in OMW medium can be explained by the use of the medium nutrients to produce other biomolecules, such as biosurfactants.[19] Both strains were able to consume the reducing sugars present in OMW (Table 2), but highest consumption was obtained for the lowest amount of ammonium supplied, which is in accordance with the cell growth profile (Fig. 1(A)). This was also observed for phenolic compounds degradation. However, the increase in ammonium supply slightly improved the COD degradation. The maximum values of COD degradation obtained (up to 80%) were similar to the maximum values previously reported for OMW aerobic treatment with fungi.[20]

Effect of cell concentration and surfactant

The main goal of this study was OMW use as a substrate for cell growth and lipase production. Therefore, the 6 g L^{-1} ammonium

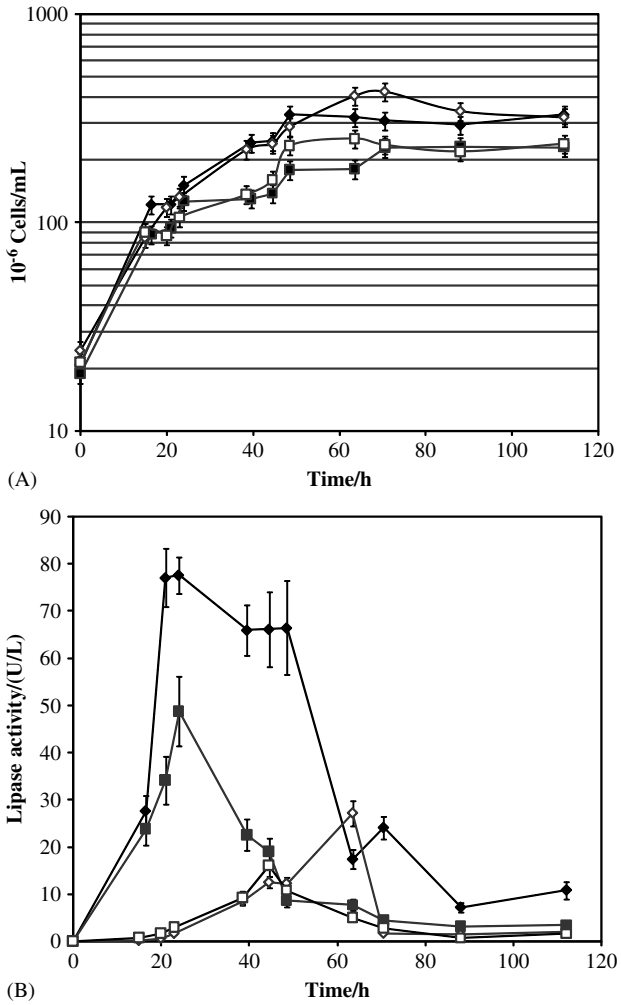

Figure 1. (A) *Y. lipolytica* strain W29 (closed symbols) and strain IMUFRJ 50 682 (open symbols) growth in OMW media with 6 g L^{-1} (◊) and 12 g L^{-1} (□) of ammonium sulphate at 240 rpm and 27 °C. (B) Time course of extracellular lipase activity. The values are the replicates mean ± standard deviation.

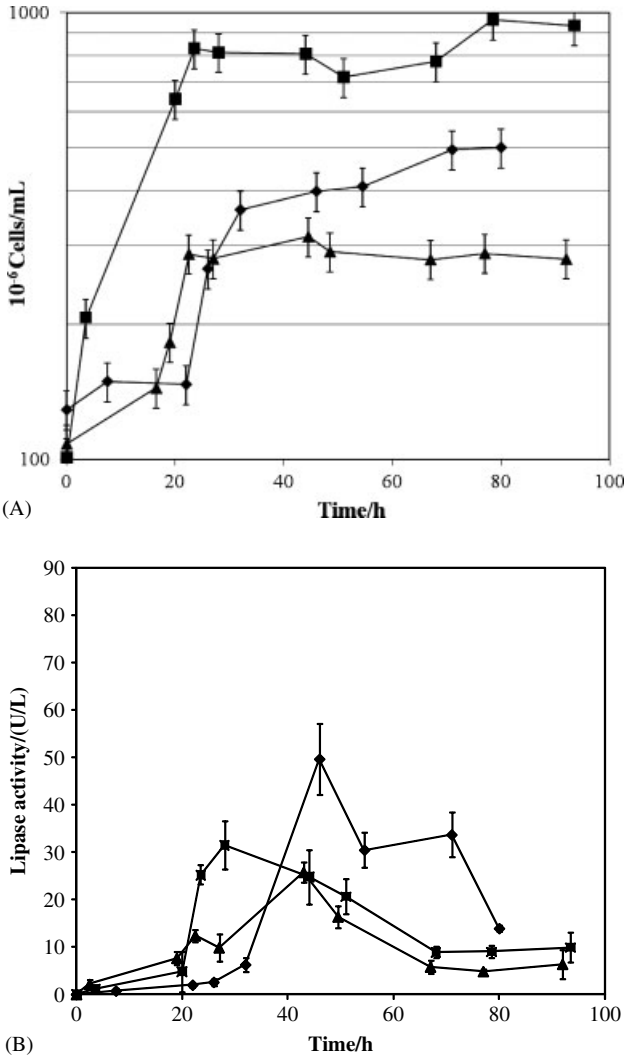

Figure 2. (A) *Y. lipolytica* W29 growth in OMW media in baffled conical flasks with an initial cell concentration of 10^8 cells mL^{-1} at 240 rpm and 27 °C. (B) Time course of extracellular lipase activity: (▲) OMW without supplementation, (♦) OMW supplemented with 1 g L^{-1} yeast extract and 6 g L^{-1} $(NH4)_2SO_4$; (■) OMW supplemented as previous and with the addition of 1 g L^{-1} of Tween 80. The values are the replicates mean ± standard deviation.

Table 2. Total degradation percentage of sugars, phenols and COD; maximum values of lipase activity and productivity for the cultures of strains W29 and IMUFRJ 50 682, supplemented with 6 g L^{-1} (N6) and 12 g L^{-1} (N12) of ammonium sulphate. The values are the replicates mean ± standard deviation

	Reducing sugars (%)	Phenols (%)	COD (%)	Lipase activity (U L^{-1})	Productivity (U L^{-1} h^{-1})
W29-N6	90 ± 8	72 ± 8	61 ± 5	78 ± 6	3.7 ± 0.4
W29-N12	63 ± 5	57 ± 7	79 ± 8	49 ± 3	2.3 ± 0.3
IMUFRJ-N6	90 ± 9	68 ± 8	75 ± 6	27 ± 3	0.43 ± 0.12
IMUFRJ-N12	76 ± 6	39 ± 5	80 ± 7	16 ± 2	0.36 ± 0.08

concentration and the strain with highest values of lipase activity (W29) were selected for further studies of the influence of culture conditions on the process. In these batch essays, the initial cell concentration was increased (10^8 cells mL^{-1}) and baffled flasks were used to ensure strong mixing in this increased cell density culture. For these culture conditions, in comparison with the experiments with a lower cell density inoculum, an increase in final cell density was reached (around 1.6-fold, for the trial with ammonium sulphate and no surfactant) (Fig. 2). The 1 g L^{-1} of Tween 80 addition to the medium strongly improved cell growth and a final cell concentration rise of 1.9-fold was observed, which indicates that the surfactant enhanced mass transfer between substrates in the liquid phases, thus improving cell growth.

On the other hand, the use of a cell culture with higher cell density in the OMW-based medium did not favour the kinetics of lipase production (Fig. 2(B)). In fact, a 36% reduction in the maximum value of extracellular lipase activity was found (Table 2) compared with values with lower cell density. The reduction in the lipase production by Tween 80 in synthetic media with *Y. lipolytica* cultures was also observed by other authors.[21]

Cell growth on OMW with no supplementation was also studied. The results obtained show the ability of *Y. lipolytica* W29 to grow in OMW, as well as the induction of lipase secretion in OMW

Table 3. Total degradation percentage of sugars, phenols and COD; maximum values of lipase activity and productivity for W29 cultures, supplemented with 6 g L^{-1} (N6) of ammonium sulphate, with 1 g L^{-1} of Tween 80 (N6 – T) and without supplementation (N0). The values are the replicates mean ± standard deviation

	Reducing sugars (%)	Phenols (%)	COD (%)	Lipase activity (U L^{-1})	Productivity (U L^{-1} h^{-1})
N6	86 ± 7	70 ± 6	54 ± 5	50 ± 4	1.1 ± 0.1
N6-T	88 ± 8	47 ± 4	74 ± 7	32 ± 3	1.1 ± 0.2
N0	75 ± 6	43 ± 4	63 ± 6	26 ± 3	0.60 ± 0.08

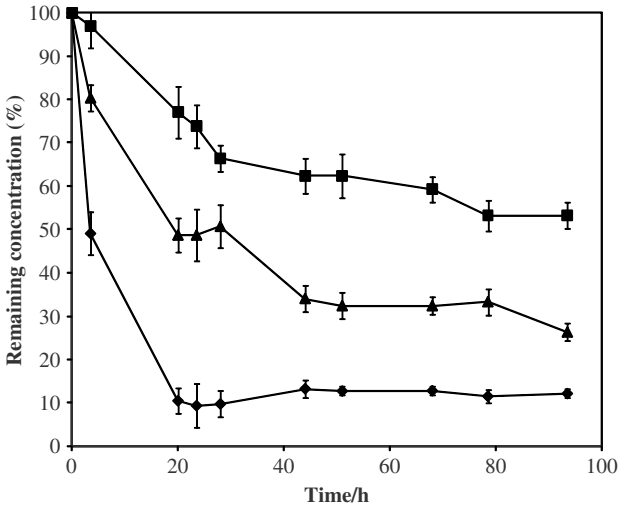

Figure 3. Degradation of reducing sugars (♦), COD (▲) and phenols (■), obtained with *Y. lipolytica* W29 cultures in OMW supplemented with 6 g L^{-1} of ammonium sulphate, 1 g L^{-1} of yeast extract and 1 g L^{-1} of Tween 80. The values are the replicates mean ± standard deviation.

without modification. According to the Table 3 data, this strain is also able to degrade COD and phenols without the need for OMW supplementation. The low nitrogen content of the medium (Table 1) and the lack of some vitamins may, however, limit the lipase production.

Surfactant addition to the supplemented OMW medium improved organic load degradation, but had a different effect on the total phenols reduction. Figure 3 shows the variation of sugars, phenols and COD during cultivation time in the presence of Tween 80. Identical profiles were obtained for other conditions, showing in all cases that the first substrates consumed were sugars and the most difficult to consume were the phenolic compounds. The high percentage (around 70%) of total phenols degradation achieved, at the same conditions that also favoured the lipase production, indicates that the OMW use by *Yarrowia lipolytica* has a high potential of OMW valorisation and degradation, since the effluent detoxification through the phenols content reduction is a crucial step for further treatment in biological plants.

CONCLUSIONS

The results of this study confirmed the potential application of the yeast *Y. lipolytica* for OMW valorisation by its use as culture medium for biomass and enzymes production. The strain W29 showed better performance in this hostile medium than the wild type strain IMUFRJ 50 682. This result confirms the wide range of application in bioprocesses development of the W29 strain, particularly in media with lipidic components.[22] 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 in effluent treatment.

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