Technical Note

Received: 17 May 2011 Revised: 16 December 2011 Accepted: 18 December 2011 Published online in Wiley Online Library: 20 February 2012

(wileyonlinelibrary.com) DOI 10.1002/jctb.3738

Fed-batch fermentation of olive mill wastewaters for lipase production

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Abstract

In the Mediterranean basin countries, huge amounts of olive mill wastewaters (OMW) are produced by the olive oil industry. It constitutes a serious environmental problem, nevertheless its composition turns OMW into a potential growth medium to lipolytic microorganisms. The aim of this work was to study lipase production as well as OMW degradation in fed-batch cultures of *Candida cylindracea* CBS 7869, *Candida rugosa* CBS 2275 and *Yarrowia lipolytica* W29 (ATCC 20460). Besides the improvement of lipase production, the fed-batch approach enhanced the effluent degradation, since it led to good COD and lipids reduction, both higher than 50%. *C. rugosa* achieved the highest value of lipase productivity (130 U L^{-1} h^{-1}), in parallel with highest lipids reduction (77%). This study demonstrates that OMW are becoming a competitive and valuable growth medium in fermentation processes with lipolytic microorganisms. The fed-batch strategy used proved to be an efficient approach to enhance lipase production from OMW and to reduce significantly the final organic load of the medium.

Keywords: *Candida* species; *Yarrowia lipolytica*; lipase; olive mill wastewater; fed-batch fermentation

INTRODUCTION

Olive oil is one of the most important elements in the traditional Mediterranean diet. Mediterranean countries are known to have favourable conditions for olive oil production. Spain, Italy and Greece are the most significant olive oil producers with 47%, 16% and 11% of the 2009/2010 world's production, respectively. Portugal was responsible for 2% (58 700 tonnes) of the world's production in 2009/2010, and 67 500 tonnes were expected for 2010/2011.[1] Huge amounts of olive mill wastewaters (OMW) are produced; typically, reaching 1.3 m^3 of OMW per ton of olives processed.[2] The OMW content of simple and complex sugars, residual oil (lipids), proteins, mineral elements and phenols, turns this effluent into a renewable resource, as its components can be extracted and purified or used for fermentative production processes.[3–5] In addition, residual oil in OMW turns this waste into a potential growth medium for lipolytic microorganisms.[2,6] Although the use of OMW as a growth medium to produce lipase has been studied by several authors,[2,4,5,7,8] it has been mostly conducted using batch operation, even though many reports revealed that, with synthetic media, the highest lipase production was achieved with a fed-batch strategy.[9–11]

The aim of this work was to study the performance of three yeast species in fed-batch cultures using OMW-based medium, with the purpose of optimizing lipase (EC 3.1.1.3) production as well as OMW degradation. Batch cultures were performed as comparison.

MATERIALS AND METHODS

Microorganisms

Candida cylindracea CBS 7869, *Candida rugosa* CBS 2275 and *Yarrowia lipolytica* W29 (ATCC 20 460) were maintained in YPDA plates (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose, 30 g L^{-1} 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), before being used for the OMW medium inoculation.

Olive mill wastewater

OMW samples were collected from three-phase olive oil mills in the northern region of Portugal, during the olive oil production campaign of 2009/2010. They were stored at −20 °C, on the same day of collection.

OMW was characterized in terms of pH, chemical oxygen demand (COD), total nitrogen (TN), total organic carbon (TOC), total solids (TS), total volatile solids (TVS), total suspended solids (TSS), reducing sugars, total phenols, total lipids and long chain fatty acids (LCFA). The characterization of raw OMW is shown in Table 1. The main free LCFA present in the OMW collected were palmitic acid (C16 : 0), palmitoleic acid (C16 : 1), stearic acid (C18 : 0) and oleic acid (C18 : 1). COD, TN and TOC were determined using test kits (Hach Lange). Solids (total, volatile and dissolved) were assessed according to standard methods.[12] Total phenols and reducing sugars were assessed by the Folin–Ciocalteau method (Commission Regulation (EEC) No. 2676/90) and by a DNS-adapted method, respectively.[13] LCFA were determined as described elsewhere.[14] Lipids (total fat) content was extracted with diethyl ether, in a Soxtec System HT2 1045-extraction unit, after samples lyophilization.[15]

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Table 1. Characterization of the OMW used. Data are the replicates ($n = 6$) mean value ±95% confidence interval

Parameter		Value
pH		4.5
COD/(g L^{-1})		261 ± 25
TN/(mg L^{-1})		198 ± 10
TOC/(g L^{-1})		45.6 ± 0.0
TS/(g L^{-1})		155 ± 1
TVS/(g L^{-1})		116 ± 6
TSS/(g L^{-1})		55.4 ± 8.8
Reducing sugars/(g L^{-1})		68.5 ± 1.18
Phenols/(g L^{-1})		7.9 ± 1.9
Lipids/(g L^{-1})		31.9 ± 15.1
LCFA/(mg L^{-1})	C16:0	2446 ± 120
	C16:1	*N**
	C18:0	733 ± 71
	C18:1	2390 ± 76

N * Negligible

OMW-based medium

The OMW-based medium is composed of OMW (Table 1), without dilution and supplemented with ammonium chloride, in order to obtain a C/N ration of 15; and yeast extract, in a ratio of 2 : 5 relative to the NH_4Cl added, to assure a minimum amount of vitamins. Before sterilization, the pH was adjusted to 7.2, with NaOH, and six drops of silicon antifoam agent were added.

Batch and fed-batch trials

Batch and fed-batch trials were performed in a 2 L bioreactor (Biolab, B. BRAUN) using the following conditions: pH 7.2, aeration rate 1.5 L min^{-1} and agitation 500 rpm. pH was automatically adjusted with NaOH 1N and HCl 1N. In batch experiments, the reactor was filled with 1.3 L of OMW-based medium after sterilization; it was inoculated with an amount of yeast cells, previously grown in YPD medium, to attain an initial cell concentration of 1×10^8 cells mL^{-1}. Fed-batch experiments were initiated with a batch phase of 24 h, after which OMW-based medium was used to feed the bioreactor. The feeding was performed with two pulses per day of 10% (v/v) of the medium volume inside the reactor, each. Thus, fed-mode operation was approximated by a repeated-batch operation. The dilution rate was approximately 0.015 h^{-1}. The fed-batch was stopped when the reactor was filled to 1.5 L, with 1 L of OMW-based medium, achieving 9 days of fermentation. The cells were grown within the bioreactor (with 400 mL of YPD) and the OMW-based medium was fed afterwards.

The time course of cell density (determined by microscopic cell counting), total phenols, reducing sugars and lipase activity were followed throughout the experiments. However, chemical oxygen demand (COD), total nitrogen (TN) and total organic carbon (TOC) were assessed only at the beginning and the end point of the experiments.

Enzymatic assays

Extracellular lipase was measured in the samples supernatant, using *p*-nitrophenyl-butyrate (*p*-NPB) in sodium acetate buffer 50 mmol L^{-1} (pH 5.6) as 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 assay conditions.[16] Protease in cell-free samples was quantified using 0.5% (w/v) azocasein as substrate in acetate buffer at pH 5.0, 37 °C for 40 min. One unit of activity was defined as the amount of enzyme per minute that causes an increase of 0.01 of absorbance, relative to the blank, under assay conditions.

RESULTS AND DISCUSSION

The aim of this study was to investigate the performance of three yeast species in batch and fed-batch cultures in OMW-based medium, with the purpose of improving lipase production as well as OMW degradation. Figure 1 depicts the results obtained in batch mode experiments. All strains presented similar cellular growth profiles on OMW medium, showing a remarkable cell adaptation to the undiluted OMW, since no cellular growth inhibition was observed for the three strains. Reducing sugars consumption profiles were quite similar between strains and overall sugars degradation was 49%, 43% and 54% for *C. cylindracea, Y. lipolytica* and *C. rugosa*, respectively. Most of this consumption (around 85%) was achieved in the early hours, especially for *C. cylindracea* CBS 7869, which presented highest extracellular lipase productivity (30 U L^{-1} h^{-1}), compared with *C. rugosa* (20 U L^{-1} h^{-1}) and *Y. lipolytica* (7 U L^{-1} h^{-1}). The sugar consumption profile is indicative of a short lag phase for all yeasts under study, which corroborates the good adaptability of these strains to OMW based medium. Brozzoli *et al.*[2] also obtained highest initial rates of total sugars consumption by *Candida cylindracea* NRRL Y-17 506, grown in a 3 L bench-top stirred tank reactor on OMW-based medium. In batch cultures of this study, *C. cylindracea* was the yeast with the best lipase productivity. Several authors had previously screened some yeast strains and other microorganisms for lipase production and *C. cylindracea* is often pointed out as the best lipase producer.[7,8] Regarding OMW degradation, other authors reported that COD concentration gradually decreases throughout the experiments,[3,7,19] however in the herein reported batch experiments, negligible COD degradation was obtained. In fact, the OMW used in this study presented an extremely high value of COD (261 g L^{-1}) while other authors generally use OMW with COD values below 100 g L^{-1}.[3,7,19,20,21] Although this concentration has not been toxic for the microorganisms, it did not allow COD degradation.

Other modes of operation have proven to be useful to increase lipase production in synthetic media.[9–11] Montesinos *et al.*[11] studied the interaction between medium components, microorganisms and the operational mode, to produce lipase by *Candida rugosa* ATCC 14 830 in synthetic media, and found that the specific productivity in continuous culture was slightly higher than in batch cultures. Furthermore, Gordillo *et al.*[9] also studied lipase production from *Candida rugosa* (ATCC 14 830) in synthetic media and reported that the best operating mode tested was fed-batch, with controlled specific growth rate, which increased the productivity 10-fold, compared with batch operation. Thus, fed-batch experiments were performed using the three yeast strains. Figure 2 shows the time course of cell growth, reducing sugars consumption and lipase activity of the culture broth for the three yeasts.

Results showed, in general, higher cell growth (more than 1 log units), sugar consumption and lipase production (Fig. 2). The fed-batch culture strategy led to overall better results, especially for *C. rugosa*.

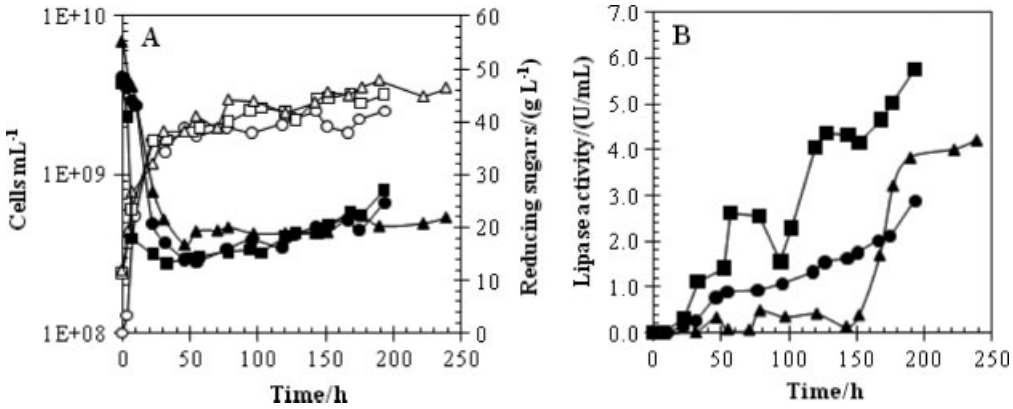

Figure 1. (A) Time course of cell growth (open symbols), reducing sugars consumption (closed symbols); and (B) lipase production, by *C. cylindracea* (■), *Y. lipolytica* W29 (●) and *C. rugosa* (▲) in batch experiments.

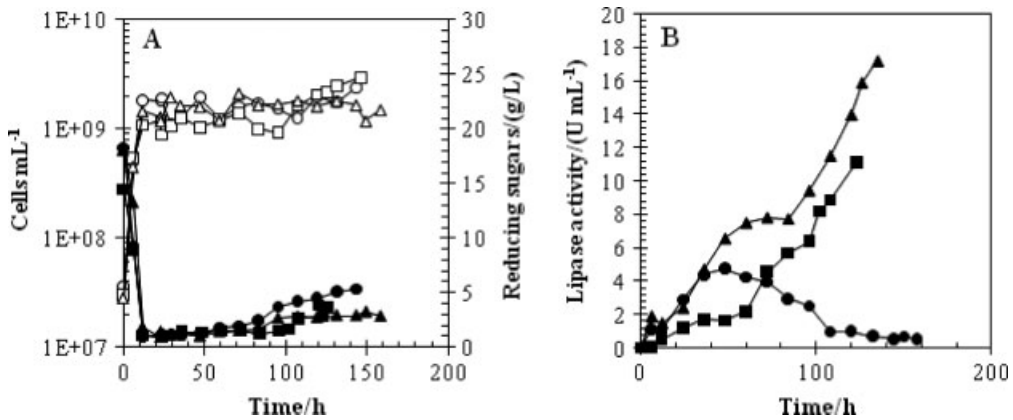

Figure 2. (A) Time course of cell growth (open symbols), reducing sugars consumption (closed symbols); and (B) lipase production, by *C. cylindracea* (■), *Y. lipolytica* (●) and *C. rugosa* (▲) for fed-batch cultures.

The exponential phase ends after 12 h, thus a long stationary phase occurred and higher values of lipase activity were obtained. In fact, a lipolytic activity of 17 U mL^{-1} was achieved by *C. rugosa*, the best lipase producer in these trials. *C. cylindracea* also reached good lipase activity values (11 U mL^{-1}) in these conditions. Typical lipase production kinetics of *Y. lipolytica*, with a decay phase after achieving a maximum value of lipase activity (5 U mL^{-1}), was observed (Fig. 2(B)). This kinetic of *Y. lipolytica* lipase production can be observed in several other works.[17,18,20,22,23] A possible reason for this behaviour is the presence of protease (Fig. 3), which can be responsible for lipase breakdown. Comparing lipase (Fig. 2(B)) and protease (Fig. 3) production profiles by *Y. lipolytica,* an inverse relation between both is observed. Some authors[23–25] also reported a sharp decrease in lipolytic activity caused by protease.

Global results of these experiments are summarized in Table 2 for COD, reducing sugars, phenolic compounds and lipids reduction. Besides the improvement of lipase production, the fed-batch approach enhanced the effluent degradation, since it led to good COD and lipids reduction, both higher than 50%. *C. rugosa* achieved the highest value of lipase productivity (130 U L^{-1} h^{-1}), in parallel with highest lipids reduction (77%). Freire *et al*.[24] observed the time course for lipase production and lipids consumption, by *Penicillium restrictum*, and found that the maximum lipolytic activity corresponded to the depletion of carbon source (lipids), which is in accordance with our results (Table 2). In these fed-batch experiments, 27% removal of phenolic compounds was reached using *C. rugosa*.

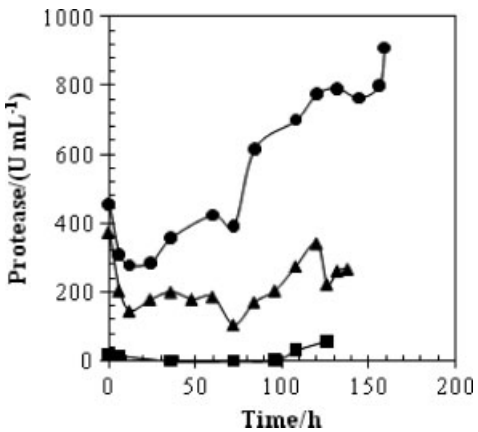

Figure 3. Time course of protease activity produced by *C. cylindracea* (■), *Y. lipolytica* (●) and *C. rugosa* (▲), for fed-batch cultures.

CONCLUSIONS

In conclusion, this study demonstrates that olive mill wastewaters are a competitive and valuable growth medium in fermentation processes with lipolytic microorganisms, since they allow

Table 2. Overall results of effluent degradation (COD, reducing sugars, phenolic compounds and lipids consumption) obtained for fed-batch cultures

Parameter	*C. cylindracea*	*C. rugosa*	*Y. lipolytica*
COD/(%)	58	64	50
Reducing sugars/(%)	52	66	54
Phenolics/(%)	4	27	1
Lipids/(%)	56	77	55

significant production of lipase. The fed-batch strategy used in the reported work proved to be an efficient approach to enhance lipase production from OMW by *Candida* species, such as *C. cylindracea* and *C. rugosa*, and to reduce significantly the final organic load of the medium. *C. rugosa* CBS 2275 was the strain that presented the best performance using this mode of operation.

ACKNOWLEDGEMENTS

The authors acknowledge the financial support provided by 'Fundação para a Ciência e Tecnologia' (Project PTDC/AMB/69379/2006; Grant SFRH/BD/27915/2006).

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