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Assessment of olive-mill wastewater as a growth medium for lipase production by *Candida cylindracea* in bench-top reactor

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ABSTRACT

Olive-mill wastewater (OMW) was investigated for its suitability to serve as a medium for lipase production by *Candida cylindracea* NRRL Y-17506. The OMW that best supported enzyme production was characterized by low COD and low total sugars content. In shake flask batch cultures, OMW supplementation with 2.4 g l^{-1} NH_4Cl and 3 g l^{-1} olive oil led to an enzyme activity of about 10 U ml^{-1}. The addition of glucose or malt extract and supplements containing organic N (e.g., peptone, yeast extract) either depressed or did not affect the enzyme production. Further experiments were then performed in a 3-l stirred tank reactor to assess the impact of medium pH and stirring speed on the yeast enzyme activity. The lipase activity was low (1.8 U ml^{-1}) when the pH was held constant at 6.5, significantly increased (18.7 U ml^{-1}) with uncontrolled pH and was maximum (20.4 U ml^{-1}) when the pH was let free to vary below 6.5. A stirring regime, that varied depending on the dissolved oxygen concentration in the medium, both prevented the occurrence of anoxic conditions during the exponential growth phase and enabled good lipase production (i.e., 21.6 U ml^{-1}) and mean volumetric productivity (i.e., 123.5 U l^{-1} h^{-1}).

1. Introduction

The mechanical process of olive oil extraction produces a dark-coloured effluent, termed olive-mill wastewater (OMW), the incorrect disposal of which may cause serious and large-scale environmental problems, particularly in the Mediterranean area where it is largely produced (up to 30×10^6 m^3 per year) (Crognale et al., 2006).

OMW can be regarded also as a possible resource containing simple and complex sugars, lipids, residual oil, proteins, mineral elements and phenols that could all be either directly recovered by chemical extraction and subsequent purification or utilized for fermentative production processes (Lanciotti et al., 2005; Crognale et al., 2006; Ertugrul et al., 2007). Particularly interesting is the residual oil that OMW contains in variable quantities, depending on the extraction process efficiency, thus making this waste a potentially suitable liquid growth medium for lipolytic microorganisms (Asses et al., 2009). In fact, lipolytic fungi are among the primary colonizers of OMW during its storage both in tanks and aerated lagoons (Millan et al., 2000).

Due to the great industrial interest and the ever increasing number of applications of lipases (glycerol ester hydrolases E.C. 3.1.1.3), aim of the present study was to assess the OMW suitability as a growth medium for the microbial production of these enzymes (Jaeger and Eggert, 2002). However, OMW is characterized by large variations in its chemical composition mainly depending on olive cultivar, harvesting period and extraction system. In this study, therefore, we used the strain *Candida cylindracea* NRRL Y-17506 (synonymous of *Candida rugosa* ATCC 14830) that was found to grow well on different OMW typologies and to produce higher lipase activities than other well known fungal lipase producers (D'Annibale et al., 2006a). A proper formulation of the OMW-based medium was first obtained in shake flask batch cultures. The yeast was then cultivated in a 3-l bench-top stirred tank reactor (STR) to determine the impact of the stirring regime and the pH control strategy, in view of a preliminary assessment of the process scale transfer feasibility.

2. Methods

2.1. Microorganism

Candida cylindracea NRRL Y-17506 was obtained from the North Regional Research Laboratory (NRRL, Peoria Illinois) culture collection. During the study, the strain was maintained at 4 °C on Potato Dextrose Agar (PDA; Difco, Detroit, MI, USA) and sub-cultured every month.

2.2. Olive-mill wastewater and culture media

OMW samples, the composition of which is shown in Table 1, were taken from different olive oil extraction plants that used

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either conventional or modified (low water consumption) three-phase extraction technology and stored at −20 °C until use.

OMW was used either as such or after dilution (1:1) with deionized water (Table 1) and, from here onwards, the diluted OMW-based media are referred to as d-OMW. Unless otherwise indicated, OMW was centrifuged (3800 *g*, 20 min) and added with NH_4Cl (2.4 g l^{-1}) (D'Annibale et al., 2006a). The pH of OMW-based media was adjusted to 6.1 with 1.0 N NaOH before and after sterilization (121 °C for 20 min) for shake flask batch and bioreactor cultures, respectively.

The influence of oil content was evaluated by adding olive oil to d-OMW-2 at the following concentrations (g l^{-1}): 0, 1.0, 3.0, 5.0 and 10.0. The effect of supplement addition to d-OMW-2 added with olive oil (3.0 g l^{-1}) was assessed using, alone or in different combinations, Tween 80 (0.5 g l^{-1}), glucose (5.0 g l^{-1}), malt extract (1.0 g l^{-1}; Difco), yeast extract (0.5 g l^{-1}; Difco) and peptone (1.0 g l^{-1}; Difco). The effect of glucose was investigated both in the presence and in the absence of added olive oil (3.0 g l^{-1}).

2.3. Inoculum preparation

Five-day-old PDA-slant cultures were suspended in 5.0 ml of sterile deionised water and 1.0 ml of the suspension used as the inoculum for pre-cultures. Incubations were carried out at 28 °C for 72 h under orbital shaking (180 rpm) in Erlenmeyer flasks (500 ml) containing 99 ml of d-OMW-1 to yield a microbial density of about 5.0×10^8 cells ml^{-1}. The cell suspension was then used as the inoculum for both shake flask batch (approx. 0.2 ml) and reactor cultures (approx 4.0 ml) to give an initial cell density of 1.0×10^6 cells ml^{-1}.

2.4. Shake flask batch cultures

OMW-based media (100 ml) in baffled Erlenmeyer flasks (500 ml) were sterilized (121 °C, 20 min) and, after cooling at room temperature, inoculated as above. Cultures were incubated at 28 °C for 240 h under orbital shaking (180 rpm). Samples (2.0 ml each) were taken every 24 h starting from the 48th h of fermentation, centrifuged (3800 *g*, 10 min) and the supernatants used for all tests. All experiments were performed in triplicate.

2.5. Bench-top stirred tank reactor cultures

Experiments were conducted in 3-l jacketed bench-top stirred tank reactors (STR) (Applikon Dependable Instruments, Schiedam, NL) filled with 2.0 l of d-OMW-2 medium added with 3.0 g l^{-1} olive oil and inoculated as above. The STR was equipped with a top stirrer bearing two six-blade Rushton-type turbines. The following probes were installed on the top plate: dissolved oxygen sensor (Ingold, CH), double reference pH sensor (Phoenix, AZ), PT 100 temperature sensor. Unless otherwise indicated, standard conditions were as follows: initial cell concentration, 1.0×10^6 cells ml^{-1}; impeller speed, 500 rpm (tip speed = 118 cm s^{-1}); aeration rate, 1.0 vvm; temperature, 28 °C; initial pH, 6.1; initial dissolved oxygen concentration, 100% of saturation. Fermentation parameters were monitored by an adaptive/PID digital controller, ADI 1030 (Applikon Dependable Instruments, Schiedam, NL). Each condition was tested in triplicate.

Influence of the medium pH was studied following three different strategies: (i) uncontrolled pH, the pH was allowed to fluctuate freely; (ii) pH 6.5, the pH was automatically maintained at the set value by controlled addition of either 1.0 N NaOH or HCl; (iii) pH < 6.5, the pH was allowed to fluctuate freely but only below the threshold value of 6.5 by the controlled addition of 1.0 N HCl. The effect of stirring was assessed either at constant impeller speeds (i.e., 300, 500 and 700 rpm) or at speed let free to vary from 300 to 800 rpm (variable regime) to automatically maintain the concentration of dissolved oxygen (DO) ⩾ 20% saturation.

2.6. Determination of oxygen uptake rate and volumetric oxygen transfer coefficient

Microbial oxygen uptake rate ($Q_{O2}X$) was estimated in the STR bioreactor according to the steady-state method described by Dunn et al. (2005) as follows:

$$Q_{O2}X(\text{mmol m}^{-3}\ s^{-1}) = (n_{in}Y_{O2}^{in} - n_{out}Y_{O2}^{out})/V \quad (1)$$

where, V is the broth volume (m^3), n_{in} and n_{out} (mmol s^{-1}) are the inlet and outlet molar gas flow rates, Y_{O2}^{in} and Y_{O2}^{out} are the oxygen molar fractions in the inlet and outlet gaseous streams, respectively. Microbial carbon dioxide production rate ($Q_{CO2}X$) was determined by Eq. (1) where oxygen molar fractions in the inlet and outlet gaseous streams (Y_{O2}^{in} and Y_{O2}^{out}, respectively) were replaced by carbon dioxide (Y_{CO2}^{in} and Y_{CO2}^{out}) ones.

The n_{in} value was calculated, converting the input volumetric air flow rate, determined by a 102-05 S-flowmeter (Aalborg Instruments Controls, Orangenburg, NY), according to the Clapeyron's gas law. The n_{out} was calculated from the nitrogen mass balance in the gas phase by the following equation (Dunn et al., 2005):

$$n_{out} = Y_{N2}^{in} \times n_{in}/(1 - Y_{O2}^{out} - Y_{CO2}^{out}) \quad (2)$$

where Y_{N2}^{in} is the nitrogen molar fraction in the inlet gaseous stream found to be equal to 0.79, as determined by the following equation:

$$Y_{N2}^{in} = 1 - Y_{O2}^{in} - Y_{CO2}^{in} \quad (3)$$

The respiratory quotient (RQ) was calculated from the $Q_{CO2}X/Q_{O2}X$ ratio. The outlet carbon dioxide and oxygen molar fractions were monitored during the fermentation using a BINOS® 100 2 M Dual-Channel Gas Analyzer (Emerson Process Management, Rosemount Analytical Solon, OH 44139 USA). The analyzer was previously calibrated using nitrogen-based gas mixtures containing oxygen (20.03% v/v) and carbon dioxide (4.97% v/v) at an optimal volumetric flow rate of 1.0 l min^{-1} as recommended by the manufacturer.

The volumetric oxygen transfer coefficient (K_La) was determined in the STR by the static method of gassing out of Wise

Table 1
Characteristics of the OMW used in the present study*.

OMW[A] (no.)	COD (g l^{-1})	Total sugar (g l^{-1})	COD/Total sugar	Total phenols (g l^{-1})	pH	Lipids (g l^{-1})	Uronic acid (g l^{-1})
1	87.27 ± 0.96[a]	28.17 ± 1.26[a]	3.10 ± 0.12[a]	7.44 ± 0.25[b]	5.46 ± 0.33[a]	0.27 ± 0.02[a]	2.35 ± 0.53[a]
2	50.56 ± 2.65[b]	15.75 ± 0.35[b]	3.21 ± 0.16[a]	4.94 ± 0.05[a]	5.46 ± 0.33[a]	1.49 ± 0.03[b]	1.86 ± 0.12[a]
3	48.99 ± 0.14[b]	4.87 ± 0.31[c]	10.06 ± 0.60[b]	5.18 ± 0.29[a]	4.65 ± 0.28[a]	0.12 ± 0.02[c]	1.35 ± 0.03[a]
4	55.24 ± 1.32[b]	16.75 ± 0.35[b]	3.30 ± 0.07[a]	5.68 ± 0.28[a]	5.40 ± 0.32[a]	1.04 ± 0.04[d]	1.81 ± 0.23[a]

* Data are means ± standard deviations of three determinations. Column data followed by the same superscript letter were not significantly different ($P \leqslant 0.05$; by Tukey test).

[A] OMW-2, -3 and -4 came from a traditional three-phase extraction system; OMW-1 came from a modified three-phase system with low-water-consumption. With the exception of OMW-3, that was withdrawn from storage tanks, the other samples were taken from the decanter during the manufacturing process.