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ScienceDirect
Bioresource Technology 99 (2008) 2419–2428
BIORESOURCE TECHNOLOGY

Citric acid production by *Yarrowia lipolytica* cultivated on olive-mill wastewater-based media

Seraphim Papanikolaou [a,*], Maria Galiotou-Panayotou [a], Stylianos Fakas [a], Michael Komaitis [a], George Aggelis [b]

[a] *Department of Food Science and Technology, Agricultural University of Athens, 75 Iera Odos, 11855 Athens, Greece*
[b] *Department of Biology, Division of Genetics, Cell and Development Biology, University of Patras, 26504 Patras, Greece*

Received 6 June 2006; received in revised form 2 May 2007; accepted 5 May 2007
Available online 2 July 2007

Abstract

Yarrowia lipolytica ACA-DC 50109 cultivated on olive-mill wastewater (O.M.W.)-based media, enriched with commercial-industrial glucose, presented an efficient cell growth. Parameters of growth were unaffected by the presence of O.M.Ws in the growth medium. In diluted O.M.Ws enriched with high glucose amounts (initial sugar concentration, 65 g l^{-1}), a notable quantity of total citric acid was produced (28.9 g l^{-1}). O.M.W.-based media had a noteworthy stimulating effect on the production of citric acid, since both final citric acid concentration and conversion yield of citric acid produced per unit of sugar consumed were higher when compared with the respective parameters obtained from trials without added O.M.W. Adaptation of the strain in O.M.W.-based media favoured the biosynthesis of cellular unsaturated fatty acids (principally of oleic and palmitoleic acids). Additionally, a non-negligible decrease of the phenolic compounds in the growth medium [up to 15% (wt/wt)], a slight decrease of the phyto-toxicity, and a remarkable decolourisation of the O.M.W. were observed. All these results suggest the potentiality of O.M.Ws utilisation in the fermentation process of citric acid production.

Keywords: *Yarrowia lipolytica*; Fermentation; Olive-mill wastewater; Citric acid

1. Introduction

The manufacturing process of olive oil production yields a liquid fraction, which is called olive-mill wastewater (O.M.W.). This important residue of the olive oil industry is one of the most difficult to treat wastes because of its high content in phenolic compounds (Gharsallah et al., 1999; Garcia Garcia et al., 2000; Aggelis et al., 2003; Ammar et al., 2005). Besides the presence of phenolic substances, O.M.Ws cause serious environmental problems due to their potentially high concentration in sugars, tannins, pigments and emulsified oil that result in increased C.O.D. values, and their black colour that stains indelibly the soil (Scioli and Vollaro, 1997; Tsioulpas et al., 2002; Fadil et al., 2003). To reduce pollution in O.M.Ws, chemical or biological processes have been used (Mantzavinos and Kalogerakis, 2005; Crognale et al., 2006). One principal strategy involves the use of moulds (e.g. *Phanerochaete* spp., *Pleurotus* spp., *Panus tigrinus*, *Geotrichum* spp., *Lentinula edodes*, *Trametes versicolor* or *Aspergillus* spp.), which have been found capable of reducing C.O.D. values and breaking down phenolic compounds of the O.M.Ws treated (Sayadi and Ellouz, 1992, 1995; Gharsallah et al., 1999; Garcia Garcia et al., 2000; Tsioulpas et al., 2002; Aggelis et al., 2003; Fenice et al., 2003; D'Annibale et al., 2004; Ayed et al., 2005; Dhouib et al., 2006). Likewise, prokaryotic microorganisms (e.g. *Pseudomonas putida*, *Klebsiella oxytoca*, *Lactobacillus plantarum*, *Citrobacter diversus*) have the capability to degrade phenolic compounds, in the presence or absence of sugars in the growth media (Seker et al., 1997; Lamia and Moktar, 2003; Ammar et al.,

* Corresponding author. Tel./fax: +30 210 5294700.
E-mail address: spapanik@aua.gr (S. Papanikolaou).

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doi:10.1016/j.biortech.2007.05.005

2005). In some cases, yeasts or yeast-like species have been used in the valorisation of O.M.Ws in order to produce single-cell protein (S.C.P.) together with other metabolites (e.g. alcohol or enzymes). In most of the cases, the yeast strains employed belonged to the genera *Torulopsis* (Bambalov et al., 1989), *Cryptococcus* (*C. albidus*) (Federici et al., 1988), *Saccharomyces* (*S. norbensis*, *S. oleaceus*, *S. chevalierie*, *S. rouxii*) (Bambalov et al., 1989; Gharsallah, 1993), *Yarrowia* (*Y. lipolytica*) (De Felice et al., 1997; Scioli and Vollaro, 1997; Lanciotti et al., 2005) and *Candida* (*C. krusei*, *C. cylindricae*, *C. tropicalis*) (Gharsallah, 1993; Fadil et al., 2003; Ettayebi et al., 2003; D'Annibale et al., 2006).

In recent investigations, *Yarrowia lipolytica* strain ACA-DC 50109 has been cultivated on raw glycerol utilised as the sole substrate in nitrogen-limited cultures, and produced remarkable quantities of citric acid (Papanikolaou et al., 2002). Moreover, this process has been successfully simulated with the aid of various numerical models (Papanikolaou and Aggelis, 2003). The aim of the present investigation was to valorise O.M.Ws by producing citric acid in batch cultures using the aforementioned microorganism. The study had a double orientation, to both valorise and detoxify the effluent.

2. Methods

2.1. Microorganism and media

Yarrowia lipolytica was used in the present study. This microorganism was isolated and identified in the Laboratory of General and Agricultural Microbiology – Agricultural University of Athens and obtained the culture code ACA-DC 50109 (formerly LGAM *S*(7)1). The microorganism was kept on potato dextrose agar (Fluka) at $T = 5 \pm 1$ °C. The salt composition of the medium in which the microorganism was cultivated contained ($g\,l^{-1}$): KH_2PO_4 (Fluka), 7.0; Na_2HPO_4 (Merck), 2.5; $MgSO_4 \times 7H_2O$ (Merck), 1.5; $CaCl_2 \times 2H_2O$ (Fluka), 0.15; $FeCl_3 \times 6H_2O$ (Mallincrondt), 0.15; $ZnSO_4 \times 7H_2O$ (Prolabo), 0.02; $MnSO_4 \times H_2O$ (Fluka), 0.06; $(NH_4)_2SO_4$ (Fluka), 0.5; yeast extract (Fluka) 0.5.

O.M.Ws were obtained from two three-phase decanter manufactures of the Prefectures of Fthiotida and East Attiki (central Greece). Samples were immediately transported to the laboratory and kept at −20 °C until further use. In order to be used in the experiments, O.M.Ws were de-frozen and the solids were removed after a filtration through a Whatman no. 1 filter and a subsequent centrifugation (8000*g*, 10 min) in a Heraeus (Bonne, Germany) centrifuge. O.M.Ws of two batches having different phenolic compound contents (3.5 ± 0.2 and $6.4 \pm 0.4\,g\,l^{-1}$, respectively, expressed as gallic acid equivalent) were used. Both O.M.Ws contained small amounts of sugars ($5.5 \pm 0.5\,g\,l^{-1}$, expressed as glucose equivalent) and negligible quantities of oil ($0.3 \pm 0.1\,g\,l^{-1}$-determination of oil conducted after a triple extraction with hexane). In both batches, organic acids were also present in small quantities. The principal organic acids detected were citric acid ($3.5 \pm 0.5\,g\,l^{-1}$), malic acid ($1.5 \pm 0.3\,g\,l^{-1}$) and acetic acid ($2.5 \pm 0.5\,g\,l^{-1}$).

Given that the aim of the present study was to valorise O.M.Ws as process water in the fermentation of citric acid, commercial glucose was added into the effluent, since O.M.Ws of both batches used did not contain sufficient quantities of sugars in order to support considerable citric acid production. Commercial glucose is the main industrial low-value material utilised in confectionary industries having 95% purity [impurities composed of maltose (2%, wt/wt), malto-dextrines (0.5%, wt/wt), water (1.5%, wt/wt) and salts (0.5%, wt/wt)]. The initial concentration of glucose in the fermentations carried out was either 25 or $65\,g\,l^{-1}$.

2.2. Culture conditions

All experiments were performed in 250-ml conical flasks, containing 50 ± 1 ml of growth medium, inoculated with 1 ml of exponential pre-culture (carried out in the synthetic medium with $Glc_0 = 25\,g\,l^{-1}$). Flasks were incubated in an orbital shaker (New Brunswick Scientific, USA) at an agitation rate of 180 ± 5 rpm and incubation temperature $T = 28 \pm 1$ °C. In all experiments carried out and given that nitrogen-limited conditions were employed, quantities of organic acids (mainly citric acid) were gradually accumulated into the culture medium, reducing, thus, the pH value of the medium. In some of the fermentations, the pH of the culture medium was not corrected and the final pH value was 2.2 ± 0.4 units lower than that of the initial one (final pH value approximately 4.0). In some other trials and in order to carry out experiments in a more controlled mode, the medium pH was maintained in the range between 5.0 and 6.0 by adding (periodically and aseptically) small quantities (e.g. 500–600 μl) of 5 M KOH into the flasks (see Papanikolaou et al., 2002). The exact volume of KOH solution needed for pH correction was evaluated by measuring the volume of KOH solution required for pH correction in one (at least) flask (collected daily). Then the appropriate volume of KOH solution was aseptically added in the remaining flasks and the value of pH reached was verified to be in the range of 5.0–6.0.

2.3. Analytical methods

Cells were harvested by centrifugation (Heraeus Sepatech Suprafuge-22 apparatus) at 7000*g*/20 min and washed once with distilled water. Cell concentration (X) was determined from dry weight (90 ± 5 °C until constant weight). Dissolved oxygen (D.O.) concentration was determined by a selective electrode (oxi200 Sensodirect, Lovinbod). Before harvesting, the shaker was stopped and the probe was placed into the flask. Then, the shaker was switched on and the measurement was taken after D.O. equilibration (usually within 10 min). pH measurement was