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

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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.

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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., *Leptinula 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; Dhoub 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.,

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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. chevalerie*, *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. cylindrica*, *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 $T = 5 \pm 1^\circ\text{C}$. 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; $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ (Merck), 1.5; $\text{CaCl}_2 \times 2\text{H}_2\text{O}$ (Fluka), 0.15; $\text{FeCl}_3 \times 6\text{H}_2\text{O}$ (Mallincrodt), 0.15; $\text{ZnSO}_4 \times 7\text{H}_2\text{O}$ (Prolabo), 0.02; $\text{MnSO}_4 \times \text{H}_2\text{O}$ (Fluka), 0.06; $(\text{NH}_4)_2\text{SO}_4$ (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 (8000g, 10 min) in a Heraeus (Bonne, Germany) centrifuge. O.M.Ws of two batches having different phenolic compound contents (3.5 ± 0.2 and $6.4 \pm 0.4 \text{ g l}^{-1}$, respectively, expressed as gallic acid equivalent) were used. Both O.M.Ws contained small amounts of sugars ($5.5 \pm 0.5 \text{ g l}^{-1}$, expressed as glucose equivalent) and negligible quantities of oil ($0.3 \pm 0.1 \text{ g l}^{-1}$ -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 ($3.5 \pm 0.5 \text{ g l}^{-1}$), malic acid ($1.5 \pm 0.3 \text{ g l}^{-1}$) and acetic acid ($2.5 \pm 0.5 \text{ g l}^{-1}$).

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 $50 \pm 1 \text{ ml}$ of growth medium, inoculated with 1 ml of exponential pre-culture (carried out in the synthetic medium with $\text{Glucose} = 25 \text{ g l}^{-1}$). Flasks were incubated in an orbital shaker (New Brunswick Scientific, USA) at an agitation rate of $180 \pm 5 \text{ rpm}$ and incubation temperature $T = 28 \pm 1^\circ\text{C}$. 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 7000g/20 min and washed once with distilled water. Cell concentration (X) was determined from dry weight ($90 \pm 5^\circ\text{C}$ 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

conducted in a Jenway 3020 pH-meter. Determination of reducing sugars concentration was conducted according to D.N.S. method (Miller, 1959) and was expressed as glucose. Phenolic compounds concentration was determined according to Folin-Ciocalteu method and was expressed as gallic acid. Organic acids were analysed in a H.P.L.C. apparatus (Waters Association 600E) equipped with a U.V. detector (Waters 486). The column used was Aminex HPX-87H (Bio-Rad). The mobile phase used was H_2SO_4 at 0.005 M and the column temperature was 65°. Organic acids were determined at 290 nm. Citric acid and iso-citric acid were not sufficiently separated and the reported concentration corresponds to the sum of these acids, expressed as total citric acid. In order to proceed with a more precise determination of iso-citric acid, an enzymatic method, based on the measurement of the NADPH_2 produced during conversion of the iso-citric to α -ketoglutaric acid, reaction catalysed by the iso-citrate dehydrogenase, was employed. Iso-citric acid represented a quantity of 5–8% (wt/wt) of total citric acid produced, regardless of the culture conditions employed. Ammonium was determined in the culture medium after biomass removal, by an ammonia-selective electrode (Hach 95-12, Germany). Total cellular lipids were extracted with a mixture of chloroform and methanol 2:1 (v/v). Solvents were removed at reduced pressure and lipid was determined gravimetrically. Lipids were converted to methyl-esters by a two-step reaction with methanolic sodium and hydrochloric methanol (A.F. NOR, 1984) and analysed in a Fisons 8000 series G.L.C. as previously described (Papanikolaou et al., 2002).

The toxicity of untreated and fermented O.M.Ws was evaluated against *Lepidium sativum* seeds by using the parameter of Germination Index (G.I.) (Tsioulpas et al., 2002):

$$\text{G.I.} = \frac{\text{rootlet's length in O.M.W.}}{\text{rootlet's length in water}} \times \frac{\text{germination in O.M.W.}}{\text{germination in water}} \times 100$$

Decolourisation of the treated residue was measured according to Sayadi and Ellouz (1995). Fermentation samples were 30-fold diluted, the pH was adjusted in the range between 6.0 and 6.3 and the absorbance was measured at 395 nm.

2.4. Notation

X is the biomass (g l^{-1}), Glc is the glucose (g l^{-1}); Cit is the total citric acid (citric plus iso-citric acid) (g l^{-1}), L is the total lipid (g l^{-1}), N-NH_4^+ (g l^{-1}), $Y_{X/\text{Glc}}$ is the biomass yield on glucose consumed (g formed per g of sugar consumed), $Y_{X/\text{N}}$ is the biomass yield on nitrogen (g formed per g of NH_4^+ consumed), $Y_{\text{Cit}/\text{Glc}}$ is the total citric acid yield on glucose (g formed per g of glucose consumed), μ is the specific growth rate, h^{-1} , q_{Cit} is the specific rate of citric acid production (g of citric acid per g of biomass per h).

Subscripts 0, f and max indicate the initial, final and maximum quantities, respectively, of the components, in the kinetics performed.

3. Results

3.1. Growth parameters of *Y. lipolytica* on diluted O.M.Ws enriched with commercial glucose

In order to investigate the effect of O.M.Ws upon growth of *Y. lipolytica*, kinetic studies were carried out in media containing the synthetic medium enriched with commercial glucose (a low-added-value material), in which O.M.W. in various concentrations was added. Low-phenolic-content O.M.W. (initial phenolic compounds at $3.5 \pm 0.2 \text{ g l}^{-1}$) was used, and Glc_0 was at 25 g l^{-1} . Fermentations were carried out in media in which O.M.W. was added in different ratios: 0% (v/v) (control experiment without O.M.W. addition), 2% (v/v), 4% (v/v), 10% (v/v), 20% (v/v), 30% (v/v), 40% (v/v), 50% (v/v).

A numerical model capable of quantifying the production of biomass by *Y. lipolytica* was developed. The equations of the model are presented below:

$$\text{Biomass production rate: } \frac{dX}{dt} = \mu \cdot X \quad (1)$$

$$\text{Glucose consumption rate: } -\frac{d\text{Glc}}{dt} = \mu \cdot X \cdot \left(\frac{1}{Y_{X/\text{Glc}}} \right) \quad (2)$$

Specific growth rate (μ), expressed according to

$$\text{Verhulst equation as: } \mu = \mu_{\max} \cdot \left(1 - \frac{X}{X_{\max}} \right) \quad (3)$$

Differential equations were integrated by using the Runge–Kutta fourth order integration method, while parameters were optimised using the least squares method. The Marquardt iterative search algorithm (initial $\lambda = 10^{-3}$) was used to determine the parameter values that minimised the residual sum of squares. Optimised-predicted parameter values were found to be close to the ones calculated from the experimental data (Table 1). Additionally, with imposition of various constraints, the model converged always towards the predicted parameter values for all runs. Biomass production (expressed as X_{\max} and $Y_{X/\text{Glc}}$) was almost unaffected by the addition of O.M.W. and the subsequent presence of phenolic compounds into the culture medium (Table 1). Furthermore, in all cases, insignificant sugar quantities remained unconsumed into the medium at the end of fermentation. Regardless of the initial quantity of O.M.W. added into the medium, the microorganism reached almost simultaneously in all cultures at the kinetics plateau (data not presented), and presented similar maximum specific growth rate μ_{\max} values (Table 1).

In all cultures, aeration of the growth medium was very satisfactory, given that in all fermentations, D.O. level corresponded to oxygen saturation of 55–85% (v/v) for all growth steps. In the fermentations carried out in this section, pH value of the medium was not corrected; in all

Table 1
Data of *Yarrowia lipolytica*, originated from kinetics and model application, in media containing commercial glucose and various initial O.M.W. concentrations

O.M.W. added (v/v)	Initial phenolics (g l ⁻¹)	Glc ₀ (g l ⁻¹)	Glc _f (g l ⁻¹)	X _{max} (g l ⁻¹) experimental	X _{max} (g l ⁻¹) predicted	Y _{X/Glc} (g g ⁻¹) experimental ^b	Y _{X/Glc} (g g ⁻¹) predicted	μ _{max} (h ⁻¹) experimental ^a	μ _{max} (h ⁻¹) predicted
0	–	28.1	0.1	7.3	6.7	0.26	0.24	0.18	0.29
2	0.03	29.3	0.0	6.4	6.1	0.22	0.21	0.17	0.30
4	0.12	29.8	0.1	6.4	6.1	0.22	0.21	0.17	0.30
10	0.35	30.3	1.0	6.2	5.9	0.21	0.21	0.16	0.26
20	0.65	28.8	2.1	5.9	5.5	0.22	0.24	0.16	0.31
30	1.02	29.0	1.5	6.0	5.5	0.22	0.26	0.17	0.26
40	1.35	28.4	0.2	6.0	5.0	0.21	0.21	0.15	0.23
50	1.81	32.5	2.1	6.6	6.0	0.22	0.22	0.18	0.26

Representation of initial phenolic compounds in the culture medium, initial and remaining glucose (Glc₀ and Glc_f), maximum biomass concentration (X_{max}) experimentally measured or predicted by the model, biomass yield on glucose consumed (Y_{X/Glc}) experimentally measured or predicted by the model and maximum specific growth rate (μ_{max}) experimentally measured or predicted by the model. Culture conditions: incubation temperature T = 28 ± 1 °C, growth on flasks, initial pH 6.0, final pH 2.2 ± 0.4 values lower than the initial value, oxygen saturation 55–85% (v/v) for all growth phases. Three lots of independent cultures were conducted by using different inocula. In all of the determinations, standard error calculated was less than 15%.

^a μ_{max} was calculated by fitting the equation $\ln\left(\frac{x}{x_0}\right) = f(t)$ within the early exponential growth phase.

^b Y_{X/Glc} values were generated by the slope of the curve of biomass produced per remaining glucose.

experiments, after 45 ± 10 h of inoculation, a gradual and constant drop of the pH value in the medium occurred suggesting accumulation of organic acids into the medium. This pH drop was significant since the lower pH value obtained for all fermentations was approximately 2.2 ± 0.4 units lower compared with the initial one. Cit was the main organic acid produced but, despite the remarkable drop of the medium pH value, its production was low (Cit_{max} from 3.0 to 5.5 g l⁻¹).

Literature suggests that enhancement of Cit production by yeast strains occurs only in nitrogen-limited media in which pH is maintained in values higher than 4.5. Values between 5.0 and 6.0 are considered as optimal (see for instance Papanikolaou et al., 2002; Morgunov et al., 2004; Anastassiadis and Rehm, 2005; Kamzolova et al., 2005). Therefore, cultures in a more controlled mode concerning pH evolution into the medium (see Section 2) were performed. A trial with Glc₀ at 29 ± 2 g l⁻¹ without O.M.W. addition and a daily pH correction was carried out and it was compared (as regards X and Cit production) with the control experiment presented in Table 1 (culture in which no O.M.W. was added and no pH correction occurred). In media in which pH was maintained within the range of 5.0 and 6.0, Cit_{max} was 10.7 g l⁻¹ (around 2.5-fold times higher compared with the respective fermentation with no pH correction). Biomass concentration presented comparable values in both cultures (X_{max} values around 6.0 and 7.0 g l⁻¹). As in all previous fermentations, in the culture in which pH was corrected, oxygen saturation presented high values [D.O. > 65% (v/v)] in all growth steps.

3.2. Citric acid production by *Y. lipolytica* growing on glucose/O.M.W. media

The microorganism under investigation has been successfully used in the production of citric acid when it was

flask-cultured on industrial glycerol utilised as substrate in nitrogen-limited experiments (Papanikolaou et al., 2002). Furthermore, in the above experiments (see paragraph 3.1.), some quantities of citric acid were extra-cellularly produced when *Y. lipolytica* was grown on O.M.W./Glc mixtures, while it has been demonstrated that controlled conditions relative to pH changes in the culture medium, favour the production of citric acid. In the present experiment, we have tried to valorise the O.M.W. as liquid medium in citric acid fermentation process. O.M.W. of the second batch (initial phenolic compounds at 6.4 ± 0.4 g l⁻¹) was added into the culture medium at 30% (v/v). O.M.W. used in this section contained higher amounts of phenolic compounds compared with that of the first trial (see Section 2). In the previous experiments (paragraph 3.1.), maximum phenolic compounds concentration in the presence of which growth was carried out without any inhibition was near 2.0 g l⁻¹ [see Table 1 – utilisation of a low-phenolic compounds O.M.W. added at 50% (v/v), maximum phenolic compounds concentration in the medium at 1.81 g l⁻¹]. In the present experiment, it was desirable to add into the synthetic medium the high-phenolic-content O.M.W. in such a quantity so as to have an initial phenolic compounds concentration of approximately 2.0 g l⁻¹. Indeed, the medium with 30% (v/v) of the second O.M.W. added, contained initial phenolic compounds concentration of 2.05 g l⁻¹. Furthermore, in order to enhance citric acid production, *Y. lipolytica* was cultivated on a mixture of O.M.W. and the mineral nitrogen-limited medium with commercial glucose, supplied at a relatively high initial concentration (Glc₀ = 65 g l⁻¹). The control experiment was carried out in the mineral medium with Glc₀ = 65 g l⁻¹ without O.M.W. addition. In both trials, pH was corrected daily (see Section 2). Despite the relatively high carbon concentration in the medium, both fermentations were conducted again under highly aerated

conditions [D.O. between 45% and 70% (v/v) in all fermentation steps]. The kinetics of citric acid accumulation into the medium and ammonium nitrogen uptake showed that the onset of citric acid production occurred when NH_4^+ disappeared from the medium (approximately 45 ± 5 h after inoculation).

A numerical model was used in order to quantify the production of biomass and citric acid by *Y. lipolytica*. Again a Verhulst-type model (see Eq. (3) in paragraph i) was used. Other equations of the model are presented below:

$$\text{Specific citric acid production rate } (q_{\text{Cit}})q_{\text{Cit}} = \alpha \quad (4)$$

Glucose consumption rate:

$$-\frac{d\text{Glc}}{dt} = \mu \cdot X \cdot \frac{1}{Y_{X/\text{Glc}}} + q_{\text{Cit}} \cdot X \cdot \frac{1}{Y_{\text{Cit}/\text{Glc}}} \quad (5)$$

$$\text{Nitrogen consumption rate: } -\frac{dN}{dt} = \mu \cdot X \cdot \frac{1}{Y_{X/N}} \quad (6)$$

$$\text{Citric acid production rate: } \frac{d\text{Cit}}{dt} = q_{\text{Cit}} \cdot X \quad (7)$$

As indicated by the fermentation data and as shown in Eq. (4), citric acid production is a non-growth coupled process. Models were fitted well on the experimental data (see experimental values and theoretical curves in the fermentation with O.M.W. presented into the medium, Fig. 1). Comparison between the optimised-predicted parameter values and those calculated from the experimental data was satisfactory, indicating the validity of the proposed model (see Table 2). The maximum total citric acid concentration achieved during growth on O.M.W./Glc media was 28.9 g l^{-1} , while, as indicated by parameter values and experimental results, the presence of O.M.W. into the culture medium increased both Cit_{max} and $Y_{\text{Cit}/\text{Glc}}$ values (Table 2). q_{Cit} was slightly lower in the medium supplemented with O.M.W. while in both cases X_{max} and μ_{max} were unaffected by the concentration of phenolic compounds into the medium (Table 2). As it was previously stated, the initial concentration of phenolic compounds into the medium was rather high (at 2.05 g l^{-1}). Other organic acids produced in smaller quantities were α -ketoglutaric acid (1.5 – 2.5 g l^{-1}) and acetic acid (1.0 – 2.0 g l^{-1}). Furthermore, the impact of phenolic compounds of the residue upon the composition of fatty acids of *Y. lipolytica* cellular lipids has been investigated. Total lipid was extracted and in all growth steps did not exceed the quantity of 6–10% (wt/wt) in dry matter, suggesting that lipid accumulation did not occur in the present culture conditions. Fatty acid composition showed a predominance of oleic acid ($^{\Delta 9}\text{C18:1}$) in all growth steps and in both cultures. However, the presence of O.M.W. into the culture medium seemed to favour the synthesis of a cellular lipid that contained somehow higher quantities of unsaturated fatty acids, in comparison with the culture in which no O.M.W. has been added; ratios $^{\Delta 9}\text{C16:1}/\text{C16:0}$ and $^{\Delta 9}\text{C18:1}/\text{C18:0}$ presented clearly

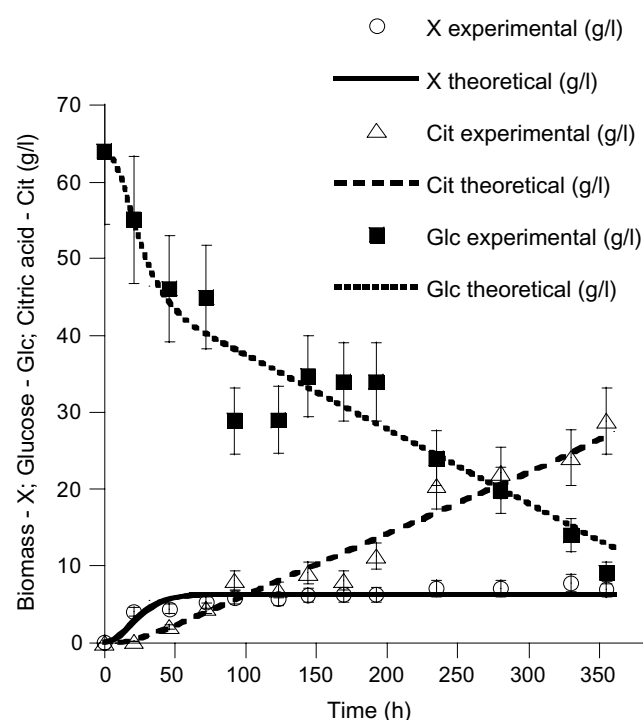


Fig. 1. Biomass (X , g l^{-1}), glucose (Glc , g l^{-1}) and total citric acid (Cit , g l^{-1}) evolution during growth of *Yarrowia lipolytica* on O.M.W.-based medium enriched with glucose. Representation of experimental points and theoretical curves. Culture conditions: growth on flasks, $T = 28 \pm 1^\circ$, $\text{Glc}_0 = 65 \text{ g l}^{-1}$, agitation rate $180 \pm 5 \text{ rpm}$, pH 5.0–6.0, initial phenolic compounds concentration 2.05 g l^{-1} , oxygen saturation 45–70% for all growth phases. Three lots of independent cultures were conducted by using different inocula. Bars represent standard errors which, in all of the determinations, were less than 15%.

higher values in media in which O.M.W. was used as co-substrate (increment of 2.0–2.5-fold times for both ratios). As for the ratio of $^{\Delta 9,12}\text{C18:2}/^{\Delta 9}\text{C18:1}$, although it presented a slight decrease with the presence of O.M.W. into the medium, this decrease was mainly attributed to the increment of cellular $^{\Delta 9}\text{C18:1}$, given that the concentration of cellular $^{\Delta 9,12}\text{C18:2}$ was almost unaffected by the addition of the wastewater into the medium (Table 3).

3.3. Phenol and colour removal by *Y. lipolytica* growing on glucose/O.M.W. media

Besides the production of citric acid, the potential decolourisation and detoxification of the residue by *Y. lipolytica* treatment was evaluated. The microorganism, even at the early fermentation steps, remarkably removed the colour of the employed effluent (decolourisation achieved around $36 \pm 3\%$) (Fig. 2). Growth of *Y. lipolytica* on O.M.W./Glc mixture was also accompanied by a reduction of the concentration of phenolic compounds into the medium but in a lesser extent compared to decolourisation degree [approximate phenolic compounds reduction $15 \pm 3\%$ (wt/wt), Fig. 2]. Removal of colour and phenolic compounds was attributed to the microbial activity and

Table 2

Data of *Yarrowia lipolytica*, originated from kinetics and model application, in media with $Glc_0 = 65 \text{ g l}^{-1}$, and the presence or absence of O.M.W. from the medium

	$Glc_0 = 65 \text{ g l}^{-1}$, O.M.W. at 30% (v/v), predicted values	$Glc_0 = 65 \text{ g l}^{-1}$, O.M.W. at 30% (v/v), experimental values	$Glc_0 = 65 \text{ g l}^{-1}$, absence of O.M.W., predicted values	$Glc_0 = 65 \text{ g l}^{-1}$, absence of O.M.W., experimental values
$\mu_{\max} (\text{h}^{-1})^a$	0.19 ± 0.01	0.17	0.085 ± 0.005	0.13
$q_{\text{Cit}} [\text{g (g h)}^{-1}]$	$1.33 \times 10^{-2} \pm 7.1 \times 10^{-4}$	n.d.	$2.12 \times 10^{-2} \pm 1.0 \times 10^{-3}$	n.d.
$Y_{\text{X/Glc}} (\text{g g}^{-1})^c$	0.32 ± 0.02	0.30	0.28 ± 0.03	0.26
$Y_{\text{Cit/Glc}} (\text{g g}^{-1})^b$	0.82 ± 0.04	0.53	0.72 ± 0.03	0.45
$Y_{\text{X/N}} (\text{g g}^{-1})^c$	67.72 ± 3.03	87.77	47.72 ± 4.04	59.18
$X_{\max} (\text{g l}^{-1})$	6.23 ± 0.31	7.76	5.91 ± 0.42	6.5
$Cit_{\max} (\text{g l}^{-1})$	— ^d	28.9 ^d	— ^d	27.6 ^d
χ^2	2.54×10^2		3.30×10^2	
$\chi^2/\text{D.F.}$	6.60		8.25	

Culture conditions: growth on flasks, $T = 28 \pm 1^\circ$, $Glc_0 = 65 \text{ g l}^{-1}$, agitation rate $180 \pm 5 \text{ rpm}$, pH 5.0–6.0 with application of pH-stat system, initial phenolic compounds concentration 2.05 g l^{-1} , initial ammonium nitrogen $115 \pm 10 \text{ ppm}$, oxygen saturation 45–70% (v/v) for all growth phases. Three lots of independent cultures were conducted by using different inocula. In all of the determinations, standard error calculated was less than 15%. \pm Values are the ones that are estimated from the model in the optimised-predicted parameter values.

n.d.: Non-determined.

^a Experimental μ_{\max} values were calculated by fitting the equation $\ln\left(\frac{X}{X_0}\right) = f(t)$ within the early exponential growth phase.

^b Experimental values were generated by the slope of the curve of citric acid produced per remaining glucose.

^c Biomass yield on nitrogen consumed $Y_{\text{X/N}}$ was expressed as g of dry biomass produced per per g of NH_4^+ consumed—experimental values were generated by the slope of the curve of biomass produced (X) per NH_4^+ consumed.

^d Maximum quantity of total citric acid produced (Cit_{\max}) was not amongst the parameters to be predicted, and, hence, was evaluated only on the basis of experimental data.

^e Evaluated in the logarithmic growth phase—experimental values were generated by the slope of the curve of biomass produced per remaining glucose during this phase.

Table 3

Composition of fatty acids in the total cellular lipid (% wt/wt) of *Yarrowia lipolytica* cultivated on glucose or mixture of glucose and O.M.W.

Time (h)	C16:0	$\Delta^9\text{C16:1}$	C18:0	$\Delta^9\text{C18:1}$	$\Delta^{9,12}\text{C18:2}$	$\Delta^9\text{C16:1/C16:0}$	$\Delta^9\text{C18:1/C18:0}$	$\Delta^{9,12}\text{C18:2}/\Delta^9\text{C18:1}$
<i>Growth on glucose ($Glc_0 = 65 \text{ g l}^{-1}$), no O.M.W. as co-substrate</i>								
75	16.5	4.9	10.7	44.9	19.9	0.30	4.20	0.44
180	18.2	6.0	9.9	46.1	18.9	0.33	4.65	0.41
319	16.4	6.1	8.9	46.0	19.1	0.37	5.17	0.41
<i>Growth on mixture of glucose and O.M.W. (30% v/v) ($Glc_0 = 65 \text{ g l}^{-1}$)</i>								
72	16.9	8.9	4.8	52.2	17.9	0.52	10.88	0.34
192	17.5	9.5	5.8	51.2	15.9	0.54	8.83	0.31
330	16.4	9.9	5.2	48.1	19.9	0.60	9.25	0.41

Each experimental point presented in the table is the mean value of two determinations. Culture conditions are same as in Table 2.

not due to the agitation, given that in the control experiment (agitation of O.M.W./Glc mixture for 250 h without cultivation of microorganisms), insignificant changes in both colour and phenolic compounds concentration of the residue were observed. Furthermore, the toxicity of the treated and untreated residue was evaluated on the basis of G.I. Non-fermented and fermented samples were tested before and after dilutions. In undiluted media [100% (v/v) medium and 0% (v/v) water] or samples in which low dilutions were performed [i.e., 75% (v/v) medium – 5% (v/v) water or 50% (v/v) medium – 50% (v/v) water], G.I. was equal to 0.0% regardless of the treatment or non-treatment of the residue. When higher dilution was employed [25% (v/v) medium – 75% (v/v) water] it has been observed that the treated samples presented a G.I. that gradually increased with the fermentation time

(initial G.I. value 18.3%, final G.I. value 330 h after fermentation 32.9% – Table 4).

4. Discussion

Y. lipolytica presented notable cell growth when cultivated on O.M.W.-based media. X_{\max} , μ_{\max} and $Y_{\text{X/Glc}}$ values were almost completely unaffected by the addition of O.M.W. into the growth medium, although the concentration of phenolic compounds was in some of the trials indeed significant (up to 2.05 g l^{-1}). [In enriched with glucose O.M.Ws, a non-negligible decrease of the phenolic compounds ($15 \pm 3\%$, wt/wt) and a remarkable decolourisation ($36 \pm 3\%$) were observed]. Additionally, a somehow reduced toxicity of the treated residue against the untreated one, as evaluated with the aid of *Lepidium sativum* seeds,

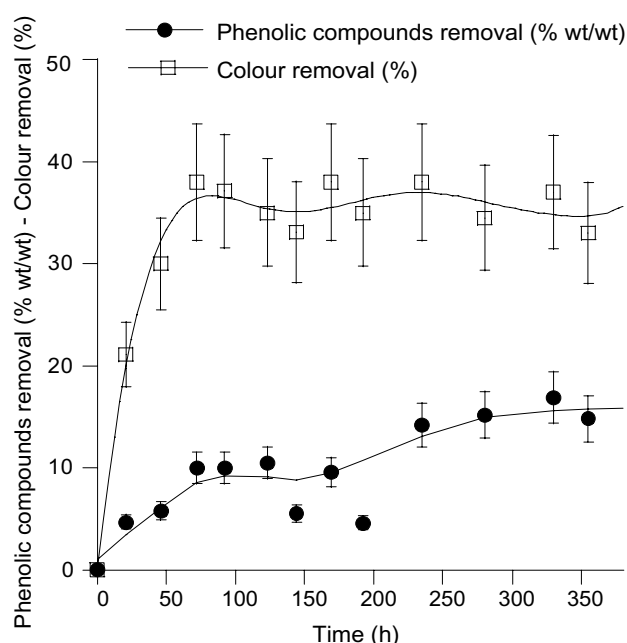


Fig. 2. Removal of phenolic compounds and decolourisation during growth of *Yarrowia lipolytica* on O.M.W.-based medium enriched with glucose. Culture conditions as in Fig. 1. Three lots of independent cultures were conducted by using different inocula. Bars represent standard errors which, in all of the determinations, were less than 15%.

was reported. In the literature, a certain confusion related with the removal of phenolic compounds by yeast cells growing on O.M.W.-based media, exists. In most cases, phenol removal from yeasts appears of being a strain-dependent process. Specifically, various *Saccharomyces* strains did not grow at all in effluents containing around 8 g l^{-1} of phenolic substances (Bambalov et al., 1989). De Felice et al. (1997) cultivated *Y. lipolytica* ATCC 20255 on O.M.W. rich in sugars (around 22 g l^{-1}) and olive oil (around 16 g l^{-1}) but presenting a low-phenolic-content (0.2 g l^{-1}) in batch-bioreactor experiments, and despite significant biomass ($X_{\max} = 23 \text{ g l}^{-1}$) and lipase (770 U l^{-1}) production, phenolic compounds concentration was not at all reduced. Lanciotti et al. (2005) reported growth of various *Y. lipolytica* strains on low-phenolic content undi-

luted O.M.Ws (phenolic compounds around 700 mg l^{-1}). Some of the used strains reduced significantly the phenolic-content of the treated residue [reduction around $18 \pm 3\%$ (wt/wt)-values similar to those obtained in the present study]. Though, other tested strains did not at all remove phenolic compounds from the medium (Lanciotti et al., 2005). In contrast, strains belonging to the species *Candida tropicalis*, have been reported to be capable of removing $51 \pm 5\%$ (wt/wt) of total phenolic compounds in diluted and enriched with various mineral nutrients O.M.Ws (Fadil et al., 2003). *Candida tropicalis* YMEC14 significantly detoxified O.M.Ws and reduced their polluting organic load, by directing microbial metabolism towards biodegradation pathways using hexadecane as co-substrate and by immobilising yeast cells in calcium alginate beads (69.2% and 55.3% removal of monophenols and polyphenols, respectively, after a 24-h fermentation cycle-Ettayebi et al., 2003). Likewise, a newly isolated *Trichosporon cutaneum* strain has been revealed capable of completely utilising phenolic compounds when these substances were used as the sole carbon and energy source at relatively low concentrations ($0.8\text{--}2.0 \text{ g l}^{-1}$), with the lag phase of the culture significantly increasing at the higher initial phenolic-content media. Using ethyl-acetate extracts of O.M.Ws as the sole carbon source, the above microorganism removed around 85% (wt/wt) of total phenolic compounds in media containing initial O.M.W. phenols at 6.0 g l^{-1} after a 6 days-treatment (Chtourou et al., 2004). Additionally, *Candida cylindracea* NRRL Y-17506 was flask-cultured on diluted O.M.Ws enriched with salts and olive oil and presented significant growth and lipase production with phenolic compounds decreasing around 36% (wt/wt) (D'Annibale et al., 2006). Besides the cases of the above-mentioned yeast strains that reduced phenolic compounds concentration when grown on O.M.W.-based media, literature indicates that a certain number of moulds (e.g. *Phanerochaete* spp., *Pleurotus* spp., *Panus tigrinus*, *Lentinula edodes*, *Aspergillus* spp., *Trametes versicolor*, *Geotrichum* spp. – 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

Table 4

Phenolic compounds content and phyto-toxicity of O.M.W.-based media treated with *Yarrowia lipolytica*

Fermentation time (h)	Dilution	Phenolic-content (g l^{-1})	G.I. (%)
0	100% Medium – 0% H_2O	2.05 ± 0.12	0.0
	25% Medium – 75% H_2O	0.53 ± 0.03	18.3 ± 2.6
46	100% Medium – 0% H_2O	1.91 ± 0.16	0.0
	25% Medium – 75% H_2O	0.48 ± 0.04	27.0 ± 3.0
92	100% Medium – 0% H_2O	1.89 ± 0.18	0.0
	25% Medium – 75% H_2O	0.46 ± 0.05	28.9 ± 2.1
192	100% Medium – 0% H_2O	1.98 ± 0.15	0.0
	25% Medium – 75% H_2O	0.49 ± 0.04	31.5 ± 1.9
330	100% Medium – 0% H_2O	1.75 ± 0.16	0.0
	25% Medium – 75% H_2O	0.44 ± 0.04	32.9 ± 1.5

The cultivation was conducted in media enriched with 65 g l^{-1} with glucose, in which O.M.W. was at 30% (v/v). In the column “Dilution”, the fermentation medium was without dilution (100% medium – 0% H_2O) or it was fourfold diluted (25% medium – 75% H_2O). Data presented are mean values of three measurements. Culture conditions are same as in Table 2.

et al., 2004; Ayed et al., 2005; Dhouib et al., 2006; Crognale et al., 2006) or bacteria (e.g. *Pseudomonas putida*, *Klebsiella oxytoca*, *Lactobacillus plantarum*, *Citrobacter diversus* – Seker et al., 1997; Lamia and Moktar, 2003; Ammar et al., 2005) are capable of significantly reducing phenolic-content in O.M.W.-based media. The essential enzymes secreted and involved in the biodegradation of phenolic compounds during cultivation of various moulds on phenol-containing wastewaters are laccase, lignin-peroxidase and manganese-dependent peroxidase. The secretion of these enzymes is strain-dependent and influenced by various culture conditions (Sayadi and Ellouz, 1992, 1995; Tsioulpas et al., 2002; Fenice et al., 2003; D'Annibale et al., 2004; Ayed et al., 2005). Non-genetically modified *Y. lipolytica* strains are not capable of producing such type of enzymes, while heterologous expression and production of laccases from basidiomycetes (e.g. *Pycnoporus cinnabarinus* or *Trametes versicolor*) in *Y. lipolytica* strains has been recently achieved (Jolivald et al., 2005; Madzak et al., 2005). It could be supposed, hence, that the reduction of phenols observed in the present study could be due either to adsorption of these compounds in the yeast cell surface or to partial utilisation of phenolic compounds as carbon source by the microorganism.

The present investigation indicates that O.M.W. can become a competitive valuable liquid growth medium in fermentation processes. With its dilution and enrichment with commercial glucose (a low-added-value product) it was revealed as a promising substrate for the biotechnological production of citric acid ($Cit_{max} = 28.9 \text{ g l}^{-1}$). It should be noted that the addition of commercial glucose was carried out in the present investigation in order to enhance citric acid production, given that both O.M.Ws used, contained initial small quantities of fermentable sugars (around 5.5 g l^{-1} Section 2). A satisfactory production of citric acid can be achieved only when the carbon source used as substrate for this purpose (i.e., sugar, fat, ethanol etc.) is supplied in relatively high quantities (Anastassiadis et al., 2002; Morgunov et al., 2004; Anastassiadis and Rehm, 2005; Kamzolova et al., 2003, 2005; Soccol et al., 2006). Certainly, the cost of the process proposed in the present investigation can be further reduced by using O.M.Ws containing significantly higher quantities of sugars. In this case, the supplementation of the medium with commercial sugar can be avoided, at least partially. For instance, O.M.Ws obtained from three-phase extraction systems contain sugar quantities of approximately 40 g l^{-1} , while in O.M.Ws derived from press extraction systems, reducing carbohydrates, in even higher quantities, can be found (initial quantities of around 70 g l^{-1}) (Crognale et al., 2006). Utilisation, hence, of these O.M.Ws without or with low dilution and after addition of the necessary nutrients (e.g. yeast extract and phosphate salts) can result in relatively satisfactory citric acid production at remarkably low cost. Literature indicates that in at least one case undiluted O.M.Ws containing notable initial sugar quantities of around 70 g l^{-1} (concentration comparable with the

one of the present study used to enhance citric acid production, see Section 3) were used as sole carbon source in order to produce significant biomass and β -glucan concentrations by the mould *Botryosphaeria rhodina* (Crognale et al., 2003).

In general, the utilisation of O.M.Ws used as water in fermentation process is very limited in the literature and according to our knowledge, O.M.Ws have been used only in one case as liquid growth medium for the production of microbial lipases by various yeast or mould strains (Crognale et al., 2006; D'Annibale et al., 2006). It should also be noted that in the present study, interestingly, the presence of O.M.Ws, and, hence, phenolic compounds into the growth medium, presented a stimulating effect on the production of citrate by *Y. lipolytica*, compared with the culture in which no O.M.W. addition had been carried out (see Table 2). It is known that citric acid is produced by various *Candida* strains after nitrogen depletion from the culture medium (“overflow metabolism phenomenon”) as secondary anabolic activity (Wojtatowicz et al., 1991; Rane and Sims, 1993, 1994; Anastassiadis et al., 2002; Papanikolaou et al., 2002; Morgunov et al., 2004; Rymowicz and Cibis, 2006). It may be assumed, hence, that the presence of phenolic compounds into the culture medium provoked an activation of the secondary metabolism leading to higher citric acid secretion into the medium. Additionally, in the present study, citric acid production was influenced by the rapid pH decrease of the medium, with low quantities of citrate secreted at low pH values, presumably due to either increased intra-/extra-cellular citric acid ratio or high requirements of energy maintenance (and hence carbon losses as CO_2) (Papanikolaou et al., 2002; Anastassiadis and Rehm, 2005). The cultivation under controlled conditions as regards the evolution of pH into the medium favoured the production of citric acid. The same strain (ACA-DC 50109) has been successfully used in previous investigations for the production of relatively high citric acid quantities (around 35 g l^{-1}), when glycerol waste, discharged after bio-diesel production process, was used as the sole carbon source in flask and pH-controlled experiments (Papanikolaou et al., 2002). The quantity of Cit_{max} produced in the present study (28.9 g l^{-1}) was satisfactory, but lower when compared with fermentations reported in the literature by other *Y. lipolytica* or *Candida* strains [final concentrations of around $50\text{--}135 \text{ g l}^{-1}$ in various fermentation configurations with glucose, ethanol or fats used as substrates (Rane and Sims, 1993, 1994; Anastassiadis et al., 2002; Kamzolova et al., 2003, 2005; Rymowicz and Cibis, 2006)]. The highest total yield $Y_{Cit/Glc}$ achieved was around 0.53 g g^{-1} . This value was slightly lower compared with the maximum values reported by strains growing on sugars (values around $0.60\text{--}0.85 \text{ g g}^{-1}$, Wojtatowicz et al., 1991; Rane and Sims, 1993, 1994; Anastassiadis et al., 2002; Morgunov et al., 2004; Anastassiadis and Rehm, 2005), while significantly higher values of product yield, e.g., $0.85\text{--}1.55 \text{ g g}^{-1}$ have been reported for strains cultivated on ethanol or fatty materials (Kamzolova et al.,

2003, 2005). Additionally, specific citrate production rate (q_{Cit}) presented similar values with the ones reported by the same strain cultivated on raw glycerol (Papanikolaou and Aggelis, 2003), or by other *Y. lipolytica* and *Candida oleophila* strains growing on glucose (Wojtatowicz et al., 1991; Rane and Sims, 1993; Anastassiadis et al., 2002; Morgunov et al., 2004) in batch cultures. Nevertheless, notably higher q_{Cit} values were reported in the literature when growth of *Y. lipolytica* strain 187 was carried out on fats utilised as the sole substrate for citric acid production [q_{Cit} around $0.127 \text{ g (g h)}^{-1}$, Kamzolova et al., 2005].

Y. lipolytica grown in O.M.W.-based media did not accumulate significant lipid quantities inside its cell structures, while, compared with the trial in which no O.M.W. has been utilised as co-substrate, cellular lipids contained slightly higher quantities of oleic ($^{18}\text{C18:1}$) and palmitoleic ($^{18}\text{C16:1}$) acid and lower quantities of stearic (C18:0) acid. Ratios $^{18}\text{C16:1/C16:0}$ and $^{18}\text{C18:1/C18:0}$ (Table 3) suggest a significantly higher content of unsaturated fatty acids and, hence, a remarkable acyl- Δ^9 -desaturase activity in the presence of O.M.W. in the culture medium. This activation could be a result of the physiological response of the microorganism, due to microbial adaptation on the high-phenol concentration medium, since higher unsaturation of cellular lipids increases the selectivity and the fluidity of the microbial membrane in non-favourable environmental conditions.

In conclusion, *Y. lipolytica* demonstrated efficient growth on media containing mixtures of O.M.W. and commercial glucose. Maximum biomass produced, maximum specific growth rate and biomass yield on glucose consumed were almost completely unaffected by the presence of O.M.Ws into the growth medium. The fermentation resulted in a non-negligible decrease of the phenolic compounds, a slight decrease of the toxicity and a remarkable decolourisation of the residue. In nitrogen-limited diluted and enriched with high glucose quantity O.M.Ws, a noticeable amount (up to 28.9 g l^{-1}) of total citric acid was produced, suggesting the potentiality of valorisation of O.M.Ws as water in this fermentation process. The ability of *Y. lipolytica* to grow on relatively high-phenolic-content O.M.W.-based media and produce in notable quantities citric acid, make this non-conventional yeast worthy for further investigation. Specifically, of interest will be the utilisation of high-sugar content undiluted O.M.Ws as carbon source in order to avoid the supplementary addition of sugar and, hence, to reduce the overall process cost, as well as the study of a potential process scale-up.

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References

- A.F. NOR., 1984. Recueil des normes françaises des corps gras/grains oléagineux et produits dérivés. 3ème édition, 60–233, p. 95.
- Aggelis, G., Iconomou, D., Christou, M., Bokas, D., Kotzailias, S., Christou, G., Tsagou, V., Papanikolaou, S., 2003. Phenolic removal in a model olive oil mill wastewater using *Pleurotus ostreatus* in bioreactor cultures and biological evaluation of the process. *Water Res.* 37, 3897–3904.
- Ammar, E., Nasri, M., Medhioub, K., 2005. Isolation of phenol degrading Enterobacteria from the waste water of olive oil extraction process. *W. J. Microbiol. Biotechnol.* 21, 253–259.
- Anastassiadis, S., Aivasidis, A., Wandrey, C., 2002. Citric acid production by *Candida* strains under intracellular nitrogen limitation. *Appl. Microbiol. Biotechnol.* 60, 81–87.
- Anastassiadis, S., Rehm, H.J., 2005. Continuous citric acid secretion by a high specific pH dependent active transport system in yeast *Candida oleophila* ATCC 20177. *Electr. J. Biotechnol.* 8, 146–161.
- Ayed, L., Assas, N., Sayadi, S., Hamdi, M., 2005. Involvement of lignin peroxidase in the decolourization of black olive mill wastewaters by *Geotrichum candidum*. *Lett. Appl. Microbiol.* 40, 7–11.
- Bambalov, G., Israilides, C., Tanchev, S., 1989. Alcohol fermentation in olive oil extraction effluents. *Biol. Wastes* 27, 71–75.
- Chtourou, M., Ammar, E., Nasri, M., Medhioub, K., 2004. Isolation of a yeast: *Trichosporon cutaneum* able to use low molecular phenolic compounds: application to olive mill wastewater treatment. *J. Chem. Technol. Biotechnol.* 79, 869–878.
- Crognale, S., Federici, F., Petruccioli, M., 2003. β -Glucan production by *Botryosphaeria rhodina* on undiluted olive-mill waste waters. *Biotechnol. Lett.* 25, 2013–2015.
- Crognale, S., D’Annibale, A., Federici, F., Fenice, M., Quarantino, D., Petruccioli, M., 2006. Olive-mill waste water valorization by fungi. *J. Chem. Technol. Biotechnol.* 81, 1547–1555.
- D’Annibale, A., Casa, R., Pieruccetti, F., Ricci, M., Marabottini, R., 2004. *Lentinula edodes* removes phenols from olive-mill wastewater impact on durum wheat (*Triticum durum* Desf.) germinability. *Chemosphere* 54, 887–894.
- D’Annibale, A., Sermani, G.G., Federici, F., Petruccioli, M., 2006. Olive-mill wastewaters: a promising substrate for microbial lipase production. *Bioresour. Technol.* 97, 1828–1833.
- De Felice, B., Pontecorvo, G., Carfagna, M., 1997. Degradation of waste waters from olive oil mills by *Yarrowia lipolytica* ATCC 20255 and *Pseudomonas putida*. *Acta Biotechnol.* 17, 231–239.
- Dhouib, A., Ellouz, M., Aloui, F., Sayadi, S., 2006. Effect of bioaugmentation of activated sludge with white-rot fungi on olive mill wastewater detoxification. *Lett. Appl. Microbiol.* 42, 405–411.
- Ettayebi, K., Errachidi, F., Jamai, L., Ali Tahri-Jouti, M., Sendide, K., Ettayebi, M., 2003. Biodegradation of polyphenols with immobilized *Candida tropicalis* under metabolic induction. *FEMS Microbiol. Lett.* 223, 215–219.
- Fadil, K., Chahlaoui, A., Ouahbi, A., Zaid, A., Borja, R., 2003. Aerobic biodegradation and detoxification of wastewaters from the olive oil industry. *Int. Biodeter. Biodegr.* 51, 37–41.
- Federici, F., Montedoro, G.F., Servili, M., Petruccioli, M., 1988. Pectic enzyme production by *Cryptococcus albidus* var. *albidus* on olive oil vegetation waters enriched with sunflower calathide meal. *Biol. Wastes* 25, 291–301.
- Fenice, M., Sermani, G.G., Federici, F., D’Annibale, A., 2003. Submerged and solid-state production of laccase and Mn-peroxidase by *Panus tigrinus* on olive mill wastewater-based media. *J. Biotechnol.* 100, 77–85.
- Garcia Garcia, I., Jimenez Pena, P.R., Bonilla Venceslada, J.L., Martin Martin, A., Martin Santos, M.A., Ramos Gomez, E., 2000. Removal of phenol compounds from olive mill wastewater using *Phanerochaete*

- chrysosporium*, *Aspergillus niger*, *Aspergillus terreus* and *Geotrichum candidum*. *Proc. Biochem.* 35, 751–758.
- Gharsallah, N., 1993. Production of single-cell protein from olive-mill wastewater by yeasts. *Environ. Technol.* 14, 391–395.
- Gharsallah, N., Labat, M., Aloui, F., Sayadi, S., 1999. The effect of *Phanerochaete chrysosporium* pretreatment of olive-mill wastewaters on anaerobic digestion. *Resour. Conserv. Recycl.* 27, 187–192.
- Jolival, C., Madzak, C., Brault, A., Caminade, E., Malosse, C., Mougin, C., 2005. Expression of laccase IIIb from the white-rot fungus *Trametes versicolor* in the yeast *Yarrowia lipolytica* for environmental applications. *Appl. Microbiol. Biotechnol.* 66, 450–456.
- Kamzolova, S., Shishkanova, N., Morgunov, I., Finogenova, T., 2003. Oxygen requirements for growth and citric acid production of *Yarrowia lipolytica*. *FEMS Yeast Res.* 3, 217–222.
- Kamzolova, S., Morgunov, I., Aurich, A., Perevoznikova, S., Shishkanova, N., Stottmeister, U., Finogenova, T., 2005. Lipase secretion and citric acid production in *Yarrowia lipolytica* yeast grown on animal and vegetable fat. *Food Technol. Biotechnol.* 43, 113–122.
- Lamia, A., Moktar, H., 2003. Fermentative decolorization of olive mill wastewater by *Lactobacillus plantarum*. *Proc. Biochem.* 39, 59–65.
- Lanciotti, R., Gianotti, A., Baldi, D., Angrisani, R., Suzzi, G., Mastrocola, D., Guerzoni, A., 2005. Use of *Yarrowia lipolytica* strains for the treatment of olive-mill wastewater. *Bioresour. Technol.* 96, 317–322.
- Madzak, C., Otterbein, L., Chamkha, M., Moukha, S., Asther, M., Gaillardin, C., Beckerich, J.M., 2005. Heterologous production of a laccase from the basidiomycete *Pycnoporus cinnabarinus* in the dimorphic yeast *Yarrowia lipolytica*. *FEMS Yeast Res.* 5, 635–646.
- Mantzavinos, D., Kalogerakis, N., 2005. Treatment of olive mill effluents, Part I organic matter degradation by chemical and biological processes – an overview. *Environ. Int.* 31, 289–295.
- Miller, G.L., 1959. Use of dinitrosalicylic reagent for determination of reducing sugar. *Anal. Chem.* 31, 426–428.
- Morgunov, I., Solodovnikova, N.Y., Sharychev, A.A., Kamzolova, S., Finogenova, T., 2004. Regulation of NAD⁺-dependent isocitrate dehydrogenase in the citrate producing yeast *Yarrowia lipolytica*. *Biochemistry (Moscow)* 69, 1391–1398.
- Papanikolaou, S., Muniglia, L., Chevalot, I., Aggelis, G., Marc, I., 2002. *Yarrowia lipolytica* as a potential producer of citric acid from raw glycerol. *J. Appl. Microbiol.* 92, 737–744.
- Papanikolaou, S., Aggelis, G., 2003. Modelling aspects of the biotechnological valorization of raw glycerol: production of citric acid by *Yarrowia lipolytica* and 1,3-propanediol by *Clostridium butyricum*. *J. Chem. Technol. Biotechnol.* 78, 542–547.
- Rane, K., Sims, K., 1993. Production of citric acid by *Candida lipolytica* Y 1095: Effect of glucose concentration on yield and productivity. *Enzyme Microb. Technol.* 15, 646–651.
- Rane, K., Sims, K., 1994. Oxygen uptake and citric acid production by *Candida lipolytica* Y 1095. *Biotechnol. Bioeng.* 43, 131–137.
- Rymowicz, W., Cibis, E., 2006. Optimization of citric acid production from glucose syrup by *Yarrowia lipolytica* using response surface methodology. *Electr. J. Polish Agric. Univ.* 9 (1), 20.
- Sayadi, S., Ellouz, R., 1992. Decolorization of olive mill waste-waters by the white-rot fungus *Phanerochaete chrysosporium*: involvement of the lignin degrading system. *Appl. Microbiol. Biotechnol.* 37, 141–146.
- Sayadi, S., Ellouz, R., 1995. Roles of lignin peroxidase and manganese peroxidase from *Phanerochaete chrysosporium* in the decolorisation of olive mill wastewaters. *Appl. Environ. Microbiol.* 61, 1098–1103.
- Scioli, C., Vollaro, L., 1997. The use of *Yarrowia lipolytica* to reduce pollution in olive mill wastewaters. *Water Res.* 31, 2520–2524.
- Seker, S., Beyenal, H., Salih, B., Tanyolac, A., 1997. Multi-substrate growth kinetics of *Pseudomonas putida* for phenol removal. *Appl. Microbiol. Biotechnol.* 47, 610–614.
- Soccol, C.R., Vandenberghe, L.P.S., Rondriques, C., Pandey, A., 2006. New perspectives for citric acid production and application. *Food Technol. Biotechnol.* 44, 141–149.
- Tsioulpas, A., Dimou, D., Ikononou, D., Aggelis, G., 2002. Phenolic removal in olive oil mill waste-water by strains of *Pleurotus* spp. in respect to their phenol oxidase (laccase) activity. *Bioresour. Technol.* 84, 251–257.
- Wojtatowicz, M., Rymowicz, W., Kautola, H., 1991. Comparison of different strains of the yeast *Yarrowia lipolytica* for citric acid production from glucose hydrol. *Appl. Biochem. Biotechnol.* 31, 165–174.