

Biosynthesis of single-cell biomass from olive mill wastewater by newly isolated yeasts

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Abstract The aim of this study was to assess the potential of newly isolated yeast strains *Schwanniomyces etchellsii* M2 and *Candida pararugosa* BM24 to produce yeast biomass on olive mill wastewater (OMW). Maximum biomass yield was obtained at 75 % (v/v) OMW, after 96 h of incubation at 30 °C and 5 % (v/v) inoculum size. The optimal carbon/nitrogen (C/N) ratio was in the range of 8:1 to 10:1, and ammonium chloride was selected as the most suitable nitrogen source. Under these conditions, a maximum biomass production of 15.11 and 21.68 g L⁻¹ was achieved for *Schwanniomyces etchellsii* M2 and *Candida pararugosa* BM24, respectively. Proteins were the major constituents of yeast cells (35.9–39.4 % dry weight), lipids were 2.8–5 % dry weight, and ash ranged from 4.8 to 9.5 % dry weight. Besides biomass production, yeast strains were also able to reduce toxicity and polluting parameter levels of the spent OMW-based medium. The practical results presented show that pH rose from initial value of 5.5 to 7.24–7.45 after fermentation. Approximately 23.1–41.4 % of the chemical oxygen demand (COD) and 15.4–19.2 % of the phenolic compounds were removed. The removal of phenolic compounds was associated

with their biodegradation and their partial adsorption on yeast cells.

Keywords Olive mill wastewater · *Schwanniomyces etchellsii* · *Candida pararugosa* · Yeast biomass · Protein · Toxicity

Introduction

Tunisia is the world's fourth largest olive oil producer behind Mediterranean giants Spain, Italy, and Greece. Olive oil production is unfortunately associated with the generation of large quantities of by-products, such as olive mill wastewater (OMW), which are produced at a rate above 7×10^5 m³ year⁻¹ (Hachicha et al. 2006). The difficulty of disposing OMW includes its high biochemical oxygen demand (BOD), chemical oxygen demand (COD), and phenolic compounds that may exhibit antimicrobial, ecotoxic, and phytotoxic properties (Lanciotti et al. 2005). However, OMW also contains valuable resources such as a high organic matter concentration and a variety of assimilable carbon sources that could be recycled and used as a substrate for the culture of microorganisms. The organic fraction includes sugars, tannins, polyphenols, polyalcohols, pectins, and lipids (Papanikolaou et al. 2008). The value of these by-products for food supply fortification or for consumption as single-cell protein (SCP) can be an alternative way to both reduce the pollutant load and solve the problem of worldwide protein shortage. Currently, SCP is produced from many species of microorganisms, including algae, fungi, and bacteria. The production of these high protein content cells can be continuous and not dependent on environmental conditions (Lee and Lee 1996). Yeast was the first microorganism whose importance as animal food supplement was recognized almost a century ago. It is suitable for SCP because of its high

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nutritional quality (Nasseri et al. 2011). Yeasts such as *Candida utilis* (Torula yeast), *Candida lipolytica*, *Candida tropicalis*, *Candida novellas*, *Candida intermedia*, and *Saccharomyces* spp. (Gharsallah 1993) are used as SCP producers to convert effluents from olive mill into microbial biomass protein.

The present investigation aimed to extend the possibilities of using OMW as a substrate for SCP production. Thus, the main purposes of this work were (a) to evaluate the biomass production from OMW using yeast isolates following enrichment, isolation, and identification, (b) to improve cell growth yield, and (c) to decrease the OMW acute toxicity.

Material and methods

Isolation and identification of yeasts

Yeast strains were isolated from different biotopes such as soil irrigated with OMW, sludge from OMW evaporating ponds, and fresh OMW collected from traditional mills located in Sfax-Tunisia. For yeast isolation, 1 g of each sample was suspended in 9 mL sterilized physiological water. The suspension was incubated at 30 °C, 150 rpm for 2 h, and 1 mL was serially (1:10) diluted with sterilized physiological water. Of each dilution, 0.1 mL was spread on Sabouraud Glucose Agar (peptone 5 g L⁻¹, glucose 20 g L⁻¹, agar 20 g L⁻¹, pH 5.5) containing 50 mg L⁻¹ chloramphenicol to inhibit bacterial growth. The plates were incubated for 48 h at 30 °C. Colonies with distinct morphological differences such as color, shape, and size were picked and purified by streaking at least three times on yeast peptone glucose agar (YPGA) (yeast extract 5 g L⁻¹, peptone 5 g L⁻¹, glucose 10 g L⁻¹, and agar 20 g L⁻¹) medium. The purified isolates were stored on YPGA plates at 4 °C. The selected strains were identified based on their internal transcribed spacer (ITS) sequences. The ITS region was amplified by PCR using ITS1 and ITS4 primers (forward ITS 1-5' TCC GTA GGT GAA CCT GCG G 3' and Reverse ITS 4-5' TCC TCC GCT TAT TGA TAT GC 3') and was directly sequenced (White et al. 1990). The obtained sequences were compared to ITS sequences available in GenBank.

Optimization of biomass production by yeast species

Yeast isolates were individually inoculated in YPG medium at 30 °C on a rotary shaker at 150 rpm for 24 h. The precultures were used to inoculate 50 mL of water-diluted OMW (25 %, v/v) with 1 mL of a mid-exponential growth phase containing 4×10^8 cells mL⁻¹ (v/v), and then, the cultures were incubated at 30 °C in a shaker at 150 rpm for 48 h. OMW media were supplemented with ammonium chloride at a concentration of 2 g L⁻¹. All media were sterilized by autoclaving at 121 °C for 20 min.

Cell growth was monitored by dry weight of yeast biomass in 10 mL culture following centrifugation at 8000 rpm for 15 min. Dry cell matter was determined after evaporating its water content at 105 °C until a constant mass.

Yeast strains showing the highest biomass growth yield on OMW were further studied. To investigate the effect of initial OMW concentrations on biomass production, several experiments were conducted with different proportions of OMW ranging between 25 and 100 %. OMW at the desired concentration was prepared by mixing raw OMW with distilled water and was supplemented with ammonium chloride at a concentration of 2 g L⁻¹. In order to study the nitrogen requirements of the yeast strains, various organic nitrogen sources (yeast extract, soy peptone, and a mixture of yeast extract/soy peptone (1:1)) and inorganic nitrogen sources (ammonium sulfate, sodium nitrate, and ammonium chloride) were added to OMW-based media (at the desired concentration) to produce carbon/nitrogen (C/N) ratios of 10. The effect of C/N on the biomass production was tested using ammonium chloride as nitrogen source at different C/N ratios (4:1, 6:1, 8:1, 10:1, 12:1, 15:1, 20:1). Inoculum sizes ranging from 2 to 10 % were added to OMW basal medium. Different pH levels (4, 4.5, 5, 5.5, 6, and 7) were set for the experiment. The effect of temperature on the growth of yeast strains was also tested using different temperatures (25, 30, and 37 °C).

Chemical characterization of OMW

COD, solids (total, volatile, and dissolved), and nitrogen (Kjeldahl) were determined according to Standard Methods (APHA et al. 1989). Phosphorous content was determined by vanadomolybdophosphoric acid method (American Public Health Association 1992). pH was measured using a combination electrode and pH meter. Total phenols were assessed by the Folin-Ciocalteu method (Wolfe et al. 2003) using gallic acid as a standard. Reducing sugars were measured by DNS method (Miller 1959). The concentration of the following minerals (Na, K, Mg, Ca, Cl, and Fe) has been determined by atomic absorption spectrometry.

To avoid spontaneous fermentation, OMW was stored at -20 °C. Before use, OMW was centrifuged at 8000 rpm for 15 min at 4 °C to remove solid particles and the supernatant was used for further fermentation studies.

Characterization of yeast biomass and spent OMW-based medium

Yeast cells were separated from the spent OMW-based medium by centrifugation (8000 rpm for 15 min). The yeast biomass was washed, then freeze-dried, and analyzed for crude protein content by the Kjeldahl method ($N \times 6.25$) and for crude lipid content according to the method of Folch et al.

(1957). The ash content was determined after combusting samples at 550 °C for 2 h.

Spent OMW-based medium was characterized with regard to its pH, COD, phenolic compounds, and reducing sugars as mentioned above.

Phytotoxicity tests

Phytotoxicity was assessed using the germination index of *Lycopersicon esculentum* defined by Zucconi et al. standard method (1981).

Statistical analysis

All experiments were conducted in duplicate. Data were expressed as means±standard error and were statistically analyzed by one-way analysis of variance (ANOVA) followed by the Tukey post-test. Values of $p < 0.05$ were considered significant.

Results and discussion

OMW characterization

Table 1 lists the characteristics of OMW sample. It can be seen that OMW contains high levels of organic matter expressed in term of COD content (214.3 g L^{-1}) and phenolic compounds (11.8 g L^{-1}), which are toxic. It is an acidic effluent with a high nutrient content which offers a selective environment for yeast growth. In contrast, the total nitrogen concentration was extremely low, at 0.1 g L^{-1} . Thus, addition of a nitrogen source is needed, generally in the form of ammonium salts, aqueous ammonia, soluble proteins, or urea (Gutiérrez et al. 2012).

Isolation, screening, and identification of yeast strains for biomass production

A total of 11 yeast strains named M1, M2, M3, M31, M32, M33, BM1, BM22, BM24, SM10, and SM14 were isolated from different OMW-contaminated biotopes such as soil irrigated with OMW, sludge from OMW evaporating ponds, and fresh OMW. Yeast strains were identified relying on their ITS region, and the sequences obtained were compared with those available in the GenBank. The results are presented in Table 2.

Preliminary experiments were conducted to test the potential of diluted OMW (at the rate of 25 % (v/v)) in supporting yeast growth. All tested strains were significantly able to grow in OMW-based medium (Table 2). Among all the isolated strains, *Schwanniomyces etchellsii* M2, *Candida pararugosa* BM24, *Candida pararugosa* BM22, *Candida diddensiae* SM10, *Candida diddensiae*

Table 1 Physical and chemical characteristics of olive mill wastewater used in this study

Characteristics	Data
pH	5.3
COD (g L^{-1})	214.3 ± 14.5
BOD ₅ (g L^{-1})	66.3 ± 3.8
Total dry matter (g L^{-1})	111.2 ± 0.2
Suspended solids (g L^{-1})	12.5 ± 0.5
Mineral matter (g L^{-1})	26.4 ± 1.0
Organic matter (g L^{-1})	84.8 ± 0.4
Lipids (%)	4.0 ± 0.6
Total nitrogen Kjeldhal (g L^{-1})	0.1 ± 0.4
Reducing sugars (g L^{-1})	17.7 ± 0.5
Total sugars (g L^{-1})	52.5 ± 0.5
Total phenolic compounds (g L^{-1})	11.8 ± 1.7
Phosphates (g L^{-1})	3.8 ± 0.5
Na (g L^{-1})	1.0 ± 0.1
Cl (g L^{-1})	1.7 ± 0.5
K (g L^{-1})	9 ± 0.8
Ca (g L^{-1})	1.3 ± 0.1
Fe (mg L^{-1})	31.4 ± 1.6
Mg (mg L^{-1})	154.0 ± 0.5

Values are the means±standard errors of three determinations

SM14, and *Debaryomyces etchellsii*, BM1 showed the highest biomass production ($p < 0.05$). However, there was no significant difference on cell mass within the abovementioned strains ($p > 0.05$). To the best of our knowledge, *Schwanniomyces etchellsii* M2 and *Candida pararugosa* BM24 have not yet been reported for SCP production. Therefore, they were tested throughout this study for SCP production.

Schwanniomyces etchellsii also known as *D. etchellsii*, *Pichia etchellsii*, *Torulaspora etchellsii*, *Kloeckera faecalis*, and *Pichia faecalis* can be found in many habitats such as soil, vegetables, and foods and rarely in clinical samples (Kurtzman et al. 2011). This strain exhibits high levels of cell wall-bound β -glucosidases, an important property responsible for the hydrolysis of natural glucosides and influencing food aroma (Wallecha and Mishra 2003).

Candida pararugosa, formerly known as *Cryptococcus aggregates* and *Torulopsis aggregata*, not only was first isolated from human sources (Nakagawa et al. 2004 and Giammanco et al. 2004), but also was recovered in other food ecosystems such as dairy products (Spanamberg et al. 2004), a low-alcohol grain-based beverage (Botes et al. 2007), and commercial red wines (Jensen et al. 2009) and recently from dried meat (Matsheka et al. 2014). According to Pennacchia et al. (2008), this strain exhibits a satisfactory survival percentage in intestinal fluid (>70 %) after preexposure to gastric juice

Table 2 Identification and cell mass production of isolated yeast strains in diluted OMW (25 %, v/v)

Yeast strain	Origin	Identification	Accession number	Biomass production (g L ⁻¹)
M1	Fresh OMW	<i>Candida ethanolica</i>	KP895586	1.6±0.7 a
M2	Fresh OMW	<i>Schwanniomyces etchellsii</i>	KP895587	2.9±0.4 b
M3	Fresh OMW	<i>Pichia</i> sp.	KP895590	1.4±0.5 a
M31	Fresh OMW	<i>Pichia</i> sp.	KP895588	1.7±0.0 a
M32	Fresh OMW	<i>Saccharomyces cerevisiae</i>	KP895589	1.8±0.1 a
M33	Fresh OMW	<i>Candida molendinolei</i>	KP895592	2.0±0.2 a
BM1	Sludge from OMW evaporating ponds	<i>Debaryomyces etchellsii</i>	KP699722	2.9±0.3 b
BM22	Sludge from OMW evaporating ponds	<i>Candida pararugosa</i>	KP895609	2.9±0.5 b
BM24	Sludge from OMW evaporating ponds	<i>Candida pararugosa</i>	KP895591	3.1±0.4 b
SM10	Soil irrigated with OMW	<i>Candida diddensiae</i>	KP895594	2.9±0.6 b
SM14	Soil irrigated with OMW	<i>Candida diddensiae</i>	KP895593	2.9±0.2 b

Means without a common letter differ ($p < 0.05$)

demonstrating a high tolerance to bile salts and pancreatic exposure. On the other hand, it showed considerable lipolytic and proteolytic activities, two important properties in the event of using this strain in food fermentation processes.

In the recent literature, there are no reports of human diseases related to these species. Therefore, they could be used as new supplements in foods or pharmaceutical preparations.

Effect of initial OMW concentrations

In order to test the effect of initial OMW concentration on the growth kinetics of these two yeast species, experiments were performed in the presence of increasing concentrations of OMW: 25, 50, 75, and 100 % (v/v) in distilled water to which 2 g L⁻¹ NH₄Cl was added (Fig. 1). It can be seen that both strains were able to grow in all tested OMW concentrations. However, in the case of strain M2, an inhibition effect was observed for undiluted OMW since the quantity of biomass produced was only 1.49 g L⁻¹. In spite of the high organic load of OMW, higher dilutions were disadvantageous for BM24 and the lower biomass production (3.27 g L⁻¹) was shown at 25 % of OMW. Both strains grew better at 75 % of OMW, and the maximum cell mass production was 6.69 and 7.63 g L⁻¹ for M2 and BM24, respectively, after 96-h incubation period. Despite the high content of phenolic compounds in 75 % OMW, no inhibition growth was observed during the yeast strain cultivation. Similar results were reported by Gharsallah (1993) for *Saccharomyces rouxii* who showed that the majority of OMW phenolic compounds did not affect the growth of *Saccharomyces rouxii* except cinnamic acid and vanillic acid. Compared to other

microorganisms, yeasts are more adapted to high concentrations of phenolic compounds and low pH values of mill wastes allowing them to grow in this stressful environment (Ben Sassi et al. 2006).

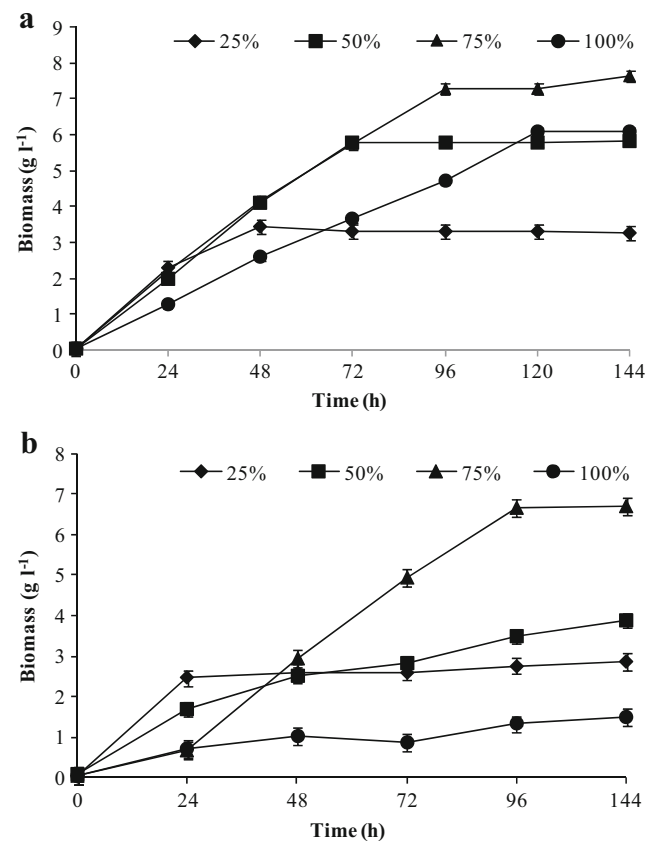


Fig. 1 Growth of strains **a** BM24 and **b** M2 in the presence of increasing concentrations of OMW (v/v): 25, 50, 75, and 100 % (v/v). Experimental conditions are as follows: 2 % inoculum size, 2 g L⁻¹ NH₄Cl, 30 °C, 150 rpm, and 150-mL working volume

Optimization of medium composition and culture conditions

Effect of nitrogen source on biomass production

Nitrogen is the basic nutrient for yeast growth and should be adjusted to provide a C/N ratio in the range of 7:1 to 10:1 to favor high protein content (Halasz and Lasztity 1990). Thus, to improve yeast cell production in 75 % of OMW, several organic nitrogen sources (yeast extract, soy peptone, and yeast extract + soy peptone) and inorganic nitrogen sources (ammonium sulfate, sodium nitrate, or ammonium chloride) were added to get a C/N ratio of 10:1. Results showed that biomass production varied considerably depending on the types of nitrogen source used (Fig. 2). The amount of yeast biomass produced in the fermentation medium, containing only OMW as sole carbon and nitrogen sources, was lower than those obtained under nitrogen supplementation. Biomass production was significantly enhanced ($p < 0.05$) by both ammonium salts reaching 16.61 and 15.56 g L⁻¹ with NH₄Cl and 15.29 and 13.57 g L⁻¹ with (NH₄)₂SO₄ for BM24 and M2, respectively. According to Halasz and Lasztity (1990), ammonium salts are suitable nitrogen sources for yeasts of interest in SCP production. The results of the present study also agree with that of Choi and Park (2002) who showed that when cultured in juice extracted from cabbage waste, the addition of ammonium sulfate increased the cell mass of *Pichia stipitis*. It is water soluble and does not produce any toxic effect toward the majority of microbial enzymes. Bamforth (2005) reported that nitrogen in ammonium salt is readily assimilated by yeast; its replacement with organic nitrogen sources resulted in poor biomass production.

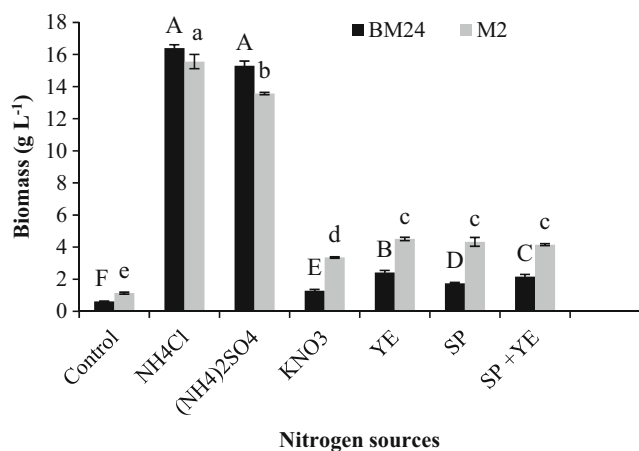


Fig. 2 Effect of organic (yeast extract (YE), soy peptone (SP), and yeast extract + soy peptone (YE + SP)) and inorganic (KNO₃, NH₄Cl, and (NH₄)₂SO₄) nitrogen source addition on biomass production of strains BM24 and M2. Experimental conditions are as follows: 75 % OMW (v/v), 2 % inoculum size, 30 °C, 150 rpm, 4-day incubation, and 50-mL working volume. *a–e* and *A–F* Means without a common letter differ ($p < 0.05$)

Effect of C/N ratio on biomass production

To determine the effect of C/N ratio on the growth of yeast strains M2 and BM24 on OMW-based medium, ammonium chloride as nitrogen source was added to give media with the following C/N ratios (4:1, 6:1, 8:1, 10:1, 12:1, 15:1, 20:1) (Fig. 3). It can be seen that for both strains, the optimal range for C/N ratio is 8–10 with a maximum biomass production of 15.48 and 16.6 g L⁻¹ for strains M2 and BM24, respectively. The above differences in cell mass values are indeed statistically significant at $p < 0.05$ when different C/N ratios were applied. An optimal C/N ratio is the key factor to enhance microbial biomass protein (Zheng et al. 2005a). A C/N ratio of 10:1 or less favors high protein contents, while higher C/N ratios favor intracellular lipid or carbohydrate accumulation, which are undesirable in the final product (Harder and Brooke 1990).

Effect of inoculum size on biomass production

Inoculum size is one of the key factors in starting the fermentation process. The effect of different inoculum sizes on biomass production is shown in Fig. 4. In the case of the strain BM24, the addition of 2 to 5 % inoculum from a mid-exponential growth phase preculture containing 4×10^8 cells mL⁻¹ led to maximum biomass production ($p < 0.05$) after 4 days of incubation. Besides, 7 % inoculum caused a drop in yeast biomass to approximately 28 % as compared to biomass obtained with 5 % inoculum size. Similarly, a slight decrease (10 %) of the biomass production of M2 was observed when the inoculum size exceeds 7 % (v/v). However, there was no significant difference in biomass production of this strain ($p > 0.05$), when different inoculum sizes were tested. These findings indicated that with large inoculum sizes (> 7 % (v/v)), the nutrients in the growth medium might be rapidly consumed which results in low rate of yeast growth

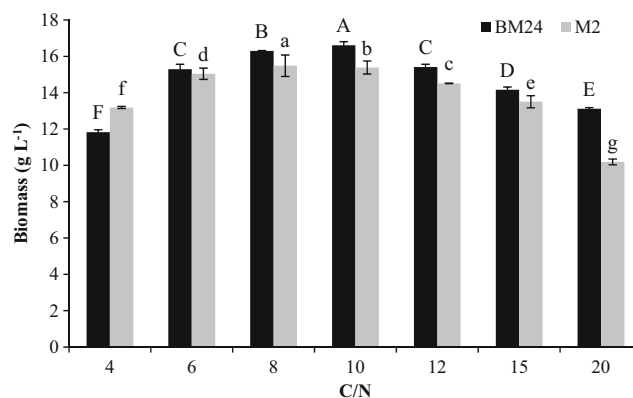


Fig. 3 Effect of different C/N ratios on biomass production of strains BM24 and M2. Experimental conditions are as follows: 75 % OMW, 30 °C, 150 rpm, and inoculum size 2 % nitrogen source NH₄Cl, 4 days of incubation, and 50-mL working volume. *a–g* and *A–F* Means without a common letter differ ($p < 0.05$)

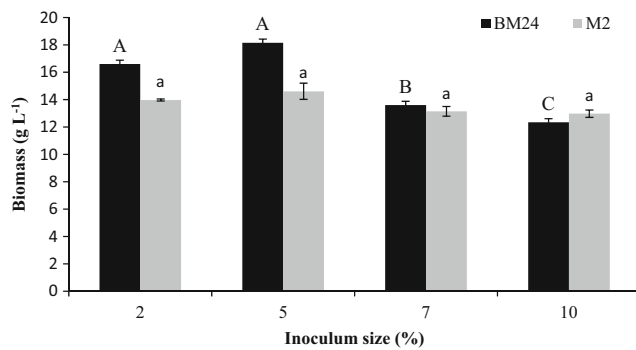


Fig. 4 Effect of inoculum size on biomass production of strains BM24 and M2. Experimental conditions are as follows: 75 % OMW, 30 °C, 150 rpm, C/N=10, 4 days of incubation, and 50-mL working volume. *a* and *A–C* Means without a common letter differ ($p < 0.05$)

and cell survival. According to Reade and Gregory (1975), the increase of initial inoculum size may possibly favor autolysis. Therefore, additional nutrient is needed to maintain the cell survival. However, lower concentrations of inoculum size may not be enough for initiating of the cell growth.

pH and temperature effect on biomass production

As pH is important for yeast biomass production, different pH levels (4.0, 4.5, 5.0, 5.5, 6.0, and 7.0) were set for the experiments. The initial pH of the medium seemed to have a greater effect on biomass production. Indeed, both strains M2 and BM24 reached a maximum biomass at pH 5.5. Further increase in pH values resulted in a significant decrease of the biomass of BM24 ($p < 0.05$). However, no significant drop of the cell mass of M2 was observed ($p > 0.05$) (Fig. 5). Similar results were reported by Reiser and Gasperik (1995) and Vega et al. (2007) for *Saccharomycopsis fibuligera* and *Kluyveromyces cicerisporus*, respectively. The pH of the fermentation medium has an effect not only on the solubility of

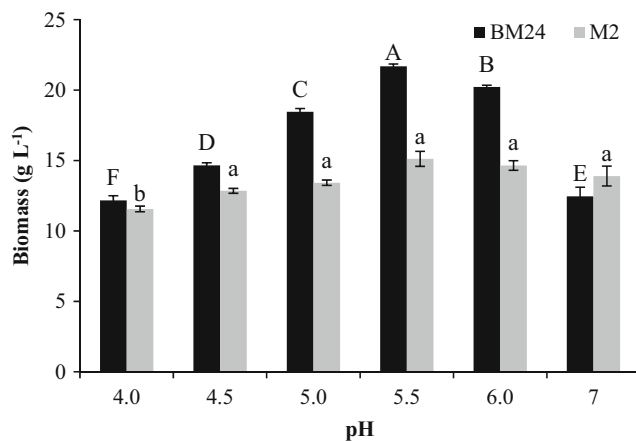


Fig. 5 Effect of pH on biomass production of strains BM24 and M2. Experimental conditions are as follows: 75 % OMW, 30 °C, 150 rpm, C/N=10, inoculum size=5 %, 4 days of incubation, and 50-mL working volume. *a*, *b* and *A–F* Means without a common letter differ ($p < 0.05$)

some nutrients but also on the permeability of cell membrane and, thus, the cell growth (Felipe et al. 1997).

Temperature also influenced the biomass production. Yeast biomass increased with temperature to reach its maximum at 30 °C for both strains (22.03 and 14.64 g L⁻¹ for BM24 and M2, respectively) (Fig. 6). However, at higher and lesser temperatures, biomass production decreased significantly ($p < 0.05$). Low temperature can inhibit nutrients to cross cell membrane, while high temperature may inactivate enzymes of metabolic pathway. This result was in good accordance with that of Torija et al. (2003) who reported that the growth of different strains of *Saccharomyces cerevisiae* was optimal at 30–35 °C.

Composition and nutritive value of single-cell biomass

The optimum OMW medium for single-cell biomass production of yeast strains M2 and BM24 was obtained with 75 % of OMW. Ammonium chloride was added as a nitrogen source to get a C/N ratio in the range of 8:1–10:1. Inoculum size was of 5 %. The pH was adjusted to 5.5, and the temperature was maintained at 30 °C. The chemical composition analyses of both strains M2 and BM24 used for biomass production are shown in Table 3.

Crude protein contents

Almost 35.9 and 39.4 % of crude protein contents were estimated in dried cell biomass of strains BM24 and M2, respectively. For both strains, the protein content was quite similar to that produced by *P. stipitis* CBS 5776 grown on cabbage juice medium (Choi and Park 2002). On the other hand, the values obtained were higher than those reported for *Candida utilis* which was only 26 % when cultivated in the presence of salad oil manufacturing wastewater (Zheng et al. 2005a) and 16 % when cultivated in silage effluent (Arnold et al. 2000).

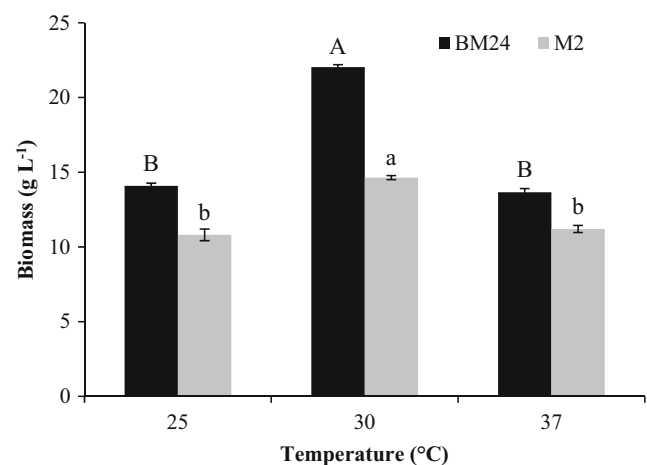


Fig. 6 Effect of temperature on biomass production of strains BM24 and M2. Experimental conditions are as follows: 75 % OMW, pH=5.5, 150 rpm, C/N=10, inoculum size=5 %, 4 days of incubation, and 50-mL working volume. *a*, *b* and *A*, *B* Means without a common letter differ ($p < 0.05$)

Table 3 Analyses of biomass composition of the yeast strains *Candida pararugosa* BM24 and *Schwanniomyces etchellsii* M2

Composition	Crude protein (% dry weight)	Crude lipid (% dry weight)	Ash (% dry weight)	Adsorbed ^a phenols (mg g ⁻¹)
<i>Candida pararugosa</i> BM24	35.9±0.9	2.8±0.5	4.8±0.1	43.8±0.3
<i>Schwanniomyces etchellsii</i> M2	39.4±0.6	5±0.2	9.5±0.2	38.4±0.2

^a Adsorbed phenols are expressed as milligrams of gallic acid/1 g of dry biomass

Nevertheless, crude proteins in strains M2 and BM24 were lower than those reported in previous studies for other yeast species and substrates. Nigam (1998) and Choi et al. (2002) produced cell biomass of *Candida utilis* with protein contents of 55.3 and 43 %, respectively. The presented results agree with those of Lee et al. (1993) and Petkov et al. (2002) who demonstrated that production of yeast biomass using fatty substrates resulted in 34–45 % protein concentration. Protein contents in dried cell biomass of strains M2 and BM24 indicate its potential use as a substitute of other traditional protein sources.

Crude lipid contents and fatty acid composition

As shown in Table 3, strains M2 and BM24 contained low amounts of lipids, 5 and 2.8 %, respectively. These results are in good accordance with those of Miller and Litsky (1976) who reported that yeast contains 2 to 6 % of lipids. However, the values obtained were lower than the 9 % value reported for *Candida utilis* grown in salad oil manufacturing wastewater effluent (Zheng et al. 2005a) and higher than 0.4 % reported on binary mixed yeast biomass (*Candida halophila* and *Rhodotorula glutinis*) from glutamate fermentation wastewater (Zheng et al. 2005b). Generally, the total lipid and protein contents in dry biomass depend on the composition of growth medium. Fatty acid analysis of lipids accumulated by the strains M2 and BM24 showed that the major cellular fatty acid produced is oleic acid (Δ^9 C18:1) followed by linoleic ($\Delta^{9,12}$ C18:2), palmitic (C16:0), and palmitoleic (Δ^9 C16:1) acids (Table 4). The high content of monounsaturated and polyunsaturated fatty acids (PUFAs) found in lipids extracted from strains M2 and BM24 is suitable when yeast biomass is used for nutritional purposes.

Ash contents

Ash or the mineral content constituted about 9.5 and 4.8 % of the yeast biomass for strains M2 and BM24, respectively. Brown et al. (1996) reported that ash content ranged from

4.7 to 13 % dry weight in yeast strains. Ash content of *Candida utilis* was 7 % of dry matter (Han et al. 1976), while ash content of *Yarrowia lipolytica* cultured in pure glycerol varied only from 3.47 to 4.7 % (Juszczak et al. 2013).

Adsorption of phenolic compounds to yeast cells

The uptake capacity of phenolic compounds by M2 and BM24 was found to be 38.4 and 43.8 mg g⁻¹, respectively. Both strains displayed a good adsorption performance when compared to *Saccharomyces cerevisiae* (26.95 mg g⁻¹) (Moyo et al. 2012). The adsorbed quantity of phenolic compounds may confer to the produced biomass several biological activities. In general, all phenolic compounds exhibit potential antioxidant activities which act through elimination of free radicals in cells and provide protection against oxidative stress in biomolecules (e.g., proteins, lipids, and DNA) (Boskou 2006). They also represent natural antibacterial, antiviral, and antifungal agents (Aziz et al. 1998; Koutsoumanis et al. 1998; Tassou and Nychas 1995; Vagelas et al. 2009). Schaffer et al. (2007) tested the effect of hydroxytyrosol-rich OMW extract (an *o*-diphenol) in vitro and ex vivo. No toxic effects have been shown when a dose of 100 mg of hydroxytyrosol per kilogram body weight was applied for 12 days. Interestingly, this treatment improved the resistance of dissociated brain cells to oxidative stress.

Characterization of the spent OMW-based medium

Organic pollution parameters

Spent OMW-based medium was characterized after the fermentation for a range of parameters such as pH, COD, and phenolics, which were chosen as indicators of pollution parameters of the effluent. OMW had an adjusted initial pH of 5.5 which increased after cultivation of both strains M2 and BM24 to pH 7.45 and 7.24, respectively. The substantial pH increases observed can be presumably due to ammonium from

Table 4 Fatty acid composition (%) of total lipids produced by *Candida pararugosa* BM24 and *Schwanniomyces etchellsii* M2

Strains	C16:0	C16:1	C18:0	Δ^9 C18:1	$\Delta^{9,12}$ C18:2	Others
<i>Candida pararugosa</i> BM24	10.6±0.9	5.7±0.3	6.0±0.4	67.8±0.2	7.6±0.6	2.2±0.3
<i>Schwanniomyces etchellsii</i> M2	16.9±0.1	9.2±0.3	4.7±0.4	52.4±0.3	13.6±0.0	3.2±0.2

Others: C10:0, C12:0, C14:0, C14:1, and $\Delta^{9,12,15}$ C18:3

protein degradation or to the degradation of COO^- and OH^- groups of phenolic compounds and organic acids such as lactic and acetic acids as suggested by several authors (Ercoli and Ertola 1983; El Hajjouji et al. 2008). It was also reported that at neutral or alkaline pH, the treated effluent becomes more economically friendly and less problematic (Arnold et al. 2000). The results given in Table 5 show a noticeable capacity for reducing the organic matter present in OMW. Reducing sugars decreased markedly after cultivation. This means that both yeast species are able to use sugars present in OMW as carbon sources.

In all cases, the COD removal after cultivation was approximately about 41.4 and 23.1 %, respectively, for M2 and BM24. Total phenol removals in 75 % of OMW were found to be only 15.4 and 19.2 % for M2 and BM24, respectively. Strain M2 seemed to be more efficient than strain BM24 for degrading the COD in OMW, but strain BM24 degraded better the phenolic compounds. Hainal et al. (2011) reported the ability of yeasts to degrade and to use polyphenolic compounds as a carbon source. More dilution before subjecting OMW to biodegradation or employing a stepwise cultivation technique for adaptation seemed to be necessary for high degree of dephenolization and COD removal. These results are in agreement with those of Jarboui et al. (2011) who reported that the reduction of phenols in OMW by *Rhodotorula mucilaginosa* decreased with increasing the initial phenol concentration. In the same line, Sayadi and Ellouz (1995) and Ahmadi et al. (2006) showed that high OMW dilutions should be used to reduce the initial organic and phenolic content. Fungal remediation of OMW has been tested using three types of organisms: white rot fungi, *Aspergillus* sp., and different yeasts. Several strains of yeasts have revealed interesting capacities for the removal of problematic OMW compounds. Many authors have examined the ability of *Y. lipolytica* to degrade OMW (Scioli and Vollaro 1997; Lanciotti et al. 2005; Lopes et al. 2009; Wu et al. 2009). *Y. lipolytica* appears quite effective, achieving removal rates as high as 80 % for COD and 68 % for phenolics (Lopes et al. 2009). Reduction of COD and phenol concentrations for more than 80 % was achieved using *Trichosporon cutaneum* (Chtourou et al. 2004). Various other yeast strains have already been used to reduce COD from OMW and to remove phenolic compounds, namely *Candida tropicalis* (Martinez-Garcia et al. 2007), *Trichosporon cutaneum* (Chtourou et al. 2004), and *Saccharomyces* sp. (Gharsallah 1993; Gonçalves et al. 2009).

Table 5 Overall results of organic matter degradation by *Candida pararugosa* BM24 and *Schwanniomyces etchellsii* M2

Strains	COD reduction (%)	Reducing sugar reduction (%)	Phenol reduction (%)
<i>Candida pararugosa</i> BM24	23.1±2.7	62±0.9	19.2±1.3
<i>Schwanniomyces etchellsii</i> M2	41.4±1.9	56±0.6	15.4±0.8

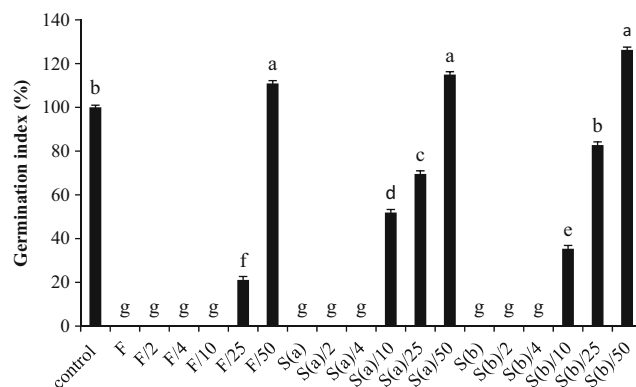


Fig. 7 Germination index of *Lycopersicon esculentum* on distilled water as control, spent 75 % OMW (S) and fresh 75 % OMW (F) by strains M2 (a) and BM24 (b) (number under the slash indicates the dilution number). a–g Means without a common letter differ ($p < 0.05$)

Phytotoxicity of the spent OMW-based medium

The effect of spent OMW-based medium (75 % OMW) and crude OMW 75 % on seed germination of *L. esculentum* was evaluated using the germination index (GI) as defined by Zucconi et al. (1981). The test was conducted on different OMW 75 % dilutions (1/1, 1/2, 1/4, 1/10, 1/25, and 1/50). The results showed that no germination was registered when untreated OMW dilution was lower than 1/10. On the other hand, data presented in Fig. 7 show that the treated OMW with both strains improved significantly GI even when diluted 1/10 was used. The GI increased up to 51.89 and 35.34 % for M2 and BM24, respectively. Treated OMW diluted to 1/10, 1/25, and 1/50 showed positive effects on germination ratio which was significantly higher than in untreated OMW at the same dilutions and control (distilled water). Therefore, many authors suggested that the phytotoxicity of OMW is principally due to the content of phenolic and organic acids present in untreated OMW at high concentrations (Komilis et al. 2005; Amaral et al. 2012).

Conclusions

The presented study suggests that the large amount of OMW generated from olive oil industries could be readily converted by selected yeast isolates into SCP without costly pretreatment. Furthermore, these strains were able to slightly reduce the pollution load of OMW. However, the degree of

purification was insufficient to allow direct discharge to a water course without supplementary treatment.

Further research, including the scale-up and the use of the produced yeast biomass as a major protein source in animal diets, is required in order to ascertain whether the results achieved by the current study can be turned into viable process.

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