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ARTICLE

Microbial Conversion of Olive Oil Mill Wastewaters into Lipids Suitable for Biodiesel Production

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Lipomyces starkey were able to survive and proliferate in the presence of olive oil mill wastewaters (OMW), a medium difficult to process by biological treatments, due to the antimicrobial activities of their phenolic components. The microorganisms were grown in the presence of undiluted OMW, without external organic supplements, producing a significant reduction of both the total organic carbon (TOC) and the total phenols content. The OMW treated by *L. starkey* showed a significant increase of the germination index. The preliminary dilution of OMW enhanced the reduction of polluting components of OMW, leading to a complete TOC removal, as well as to lower levels of residual phenols. The activities of extracellular lipases and esterases significantly increased in the course of the OMW fermentation. A significant increase in lipid yield was observed in *L. starkey* in the course of the OMW treatment, particularly enhanced when the feedstock was preliminarily diluted. The fatty acid distribution showed a prevalence of oleic acid, demonstrating the potential of *L. starkeyi* as a source of lipids to be used as a feedstock for the synthesis of II generation biodiesel.

KEYWORDS: Yeasts; lipids; biodiesel; olive oil mill wastewaters

INTRODUCTION

Biodiesel is attracting increasing interest as a substitute for petroleum-based diesel, due to the negative environmental effects of fossil fuels combustion and the concerns about petroleum supplies. As a matter of fact, biodiesel is a biodegradable, nontoxic, and clean biofuel that can be obtained from renewable sources.

Unfortunately, the starting materials traditionally used for biodiesel synthesis, namely, vegetable oils, animal fats, and (more recently) waste cooking oils, cannot realistically satisfy the demand for biodiesel at the current rate of consumption (*1*, *2*). In addition, the cost of the biodiesel, which is mainly due to (70−85%) the vegetable oils used as feedstocks, still exceeds that of the mineral diesel. Alternative sources of triacylglycerols (TAGs) are also necessary to reduce the social cost of biodiesel production, as the increase of the latter is leading to significant increases in the price of vegetable oils, a basic food in many underdeveloped countries, as well as to the deforestation of large areas.

Thus, new cheaper sources of lipids are needed for biodiesel to be a competitive and sustainable fuel, and the development of nontraditional processes for the production of TAGs, to be used as feedstock for biodiesel production, is presently targeted by a growing number of research work (*1*−*3*).

Oleaginous microorganisms, which have the ability to produce more than 20% of their weight in the form of lipids, are attracting increasing interest as a potential source of TAGs (*4*−*8*). The basic physiology of lipid accumulation in such microorganisms has been well-studied (*9*−*12*). It is known that lipid production requires nitrogen-limiting conditions.

An important advantage offered by the application of the oleaginous microorganisms stems from their ability to produce aerobically lipids from residual organic matters. Consequently, to optimize the cost of the process, as well as to increase its environmental benefit, residual materials have been tested as possible nutrients for the oleaginous microorganisms, such as sewage sludge (*8*), lignocellulosic materials (*7*), and hydrolyzed tomato waste (*13*).

In this study, we demonstrate that the oleaginous yeast *Lipomyces starkeyi* can be grown in the presence of olive oil mill wastewaters (OMW). The objective of this work was to investigate the conversion of OMW into microbial lipids as an alternative feedstock for the synthesis of biodiesel.

Olive oil production is a significant agricultural activity with great economic importance, particularly in Mediterranean countries. However, it generates high amounts of waste waters derived from the olive mill process (OMW). This waste causes disposal problems because of its highly polluting properties, which are documented by high chemical oxygen demand (COD) and biological oxygen demand (BOD) values (*14*).

Different studies have been so far devoted to the application of biological treatments for the reduction of the high organic carbon contents of the OMW (*15*). Most of these studies describe treatments based on the use of yeasts (*16*, *17*) or white rot fungi (*18*−*20*). It has been shown (*21*) that the phenolic components of OMW may inhibit the growth of microorganisms, limiting the efficiency of the digestion processes. As far as we know, no previous scientific papers have focused on the biological synthesis of lipids starting from OMW-based media.

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Table 1. Chemical Composition of OMW

	concentration (g/L)
water	965.40
nonaqueous components	4.70
sugars	12.79
phenols	9.14
proteins	3.41
lipids	0.72
dry weight (105 °C)	47.20
mineral residue (550 °C)	11.00
Fe	0.01
Mg	0.19
Ca	0.26
K	2.85
Na	0.65
Cu	0.002
Zn	0.003

The natural habitat of *L. starkeyi* is soil and ensilage (*22*), where the microorganisms degrade carbohydrates using extracellular carbohydrolases and contribute to the biodegradation of herbicides (*23*). *L. starkeyi* have been proved to store large amounts of lipids (*9*), showing only a minimal reutilization of the stored lipids (*10*). Lipid accumulation is affected by the concentration of some ions like Zn^{2+} and Mn^{2+} lipids (*11*, *12*). So far, sewage sludge has been the only residual material tested as a culture medium for *L. starkeyi* growth (*8*).

MATERIALS AND METHODS

Microorganisms and Culture Medium. All of the oleaginous yeasts used within the present work (*L. starkeyi*, *Cryptococcus curvatus*, *Rhodotorula glutinis*, and *Rhodosporidium toruloides*) were obtained by the collection of the Dipartimento di Biologia Vegetale of the Perugia University (Italy). The microorganisms were kept on potato dextrose agar (Sigma) at $T = 5 \pm 1$ °C and cultivated in a synthetic N-limiting medium, containing (g/L): KH_2PO_4 (Serva), 1.0; $MgSO_4 \cdot 7H_2O$ (BDH), 0.5; $(NH_4)_2SO_4$ (Carlo Erba), 2.0; yeast extract (Fluka), 0.5; and glucose, 70.0. The growth was carried out under aerobic conditions at 30 °C on a rotary shaker at 160 rpm (Minitron, Infors HT, Switzerland).

OMW. OMW was obtained from the Casa Olearia Italiana (Monopoli, Italy). Samples were immediately frozen at −20 °C until further use. Before each experimental test, OMW samples were defrozen, and the solids were removed by centrifugation (4000 rpm, 30 min, 20 °C) in a thermostatic centrifuge (Rotanta 460R, Hettich, United States). The pH of OMW after centrifugation was 4.68. The composition of the OMW is given in **Table 1**.

Fermentation in OMW. The fermentation tests were carried out using a fixed volume (150 mL) of OMW (both raw or diluted), without external organic supplement, in a 500 mL conical flask. A preliminary centrifugation of OMW (2000 rpm, 10 min) was carried out before each test. The liquid medium was inoculated with 2 mL of microorganism suspension, obtained by dissolving 5 loops of solid culture in 8 mL of physiological solution. The flasks were incubated in a rotary shaker at an agitation rate of 160 ± 5 rpm and an incubation temperature of $T = 30 \pm 1$ °C.

Lipid Extraction and Measurement. Methanol (5.0 mL) and chloroform (2.5 mL) were added to 200 mg of dry biomass and vortexed for 5 s. Subsequently, the cells were disrupted for 12 min in an Ultrasonic Homogenizer (Omni Ruptor 250, United States) at 50% power and 90% pulser. The cells were then filtered off with Whatman no. 1 filter paper, and the solvent−lipid mixture was placed in a 50 mL tube fitting with centrifuge racks. The layers were separated by centrifugation for 10 min at 2000 rpm in a thermostatic centrifuge (Rotanta 460R, Hettich) at 20 °C. The lower layer was then transferred to a pear-shaped flask with a Pasteur pipet. Again, 10 mL of 10% (v/v) methanol in chloroform was added to the residue, a new centrifugation was carried out, and the lower phase was added to that from the first extraction. The solvent in the pear-shaped flask was evaporated to dryness (BÜCHI Rotavapor R-200, Switzerland), and the extracted weight was finally recorded after drying at 105 °C for 1 h.

Biomass Analysis. The biomass concentration in the synthetic medium was measured by OD determination at 600 nm. When microorganisms were cultured in the OMW, the OD measurement could not be carried out due to the darkness of the medium. Consequently, the total count of microorganisms was carried out by sequential dilution and insemination in plate count agar medium (Difco Laboratories, Detroit, MI). The colonies were counted after 24 h of culture on agar medium. After each fermentation test, the biomass was recovered by centrifugation (3500 rpm for 10 min) and lyophilized (LYOBETA- 50, Spain), to enable the determination of the dry biomass and the lipid concentration measurement.

Fatty Acids Composition. The fatty acids composition was determined by GC analysis on a Shimadzu GC 17/3 gas chromatograph equipped with a flame ionization detector, following the method suggested by Li and co-workers (*6*). Briefly, wet cell pellets from 1 mL of culture were treated with 0.5 mL of a 5% KOH−methanol solution at 65 °C for 50 min. After the addition of 0.2 mL of BF_3 diethyl etherate and 0.5 mL of methanol, the mixture was refluxed for 10 min, cooled, diluted with distilled water, and extracted with petroleum ether. The organic layer was collected and washed with distilled water. The GC analysis of the fatty acid methyl esters was carried out using N_2 as the carrier gas (40 mL/min), an injection temperature of 230 °C, an oven temperature of 190 °C, and a detector temperature of 230 °C.

Chemical Analyses. The chemical analyses of OMW samples before and after treatment with microorganisms were performed according to the Rodier methods (*24*), and each test was performed in triplicate. In particular, the cations (Fe, Mg, Ca, K, Na, Cu, and Zn) were determined by atomic absorption spectrometry (Perkin-Elmer Analyst 700). The total sugar and protein contents were measured according to anthrone (*25*) and Bradford (*26*) methods, respectively, by using in the first analysis an equimolar standard solution of galactose and mannose (50% w/v) and, for the second, a bovine sieroalbumine solution as the standard. The total phenolic content was estimated according to the Folin method (*27*), using gallic acid as the standard.

The total organic carbon (TOC) measurements were carried out with a TOC-$V_{CSH/CSN}$ (Shimadzu, Japan), upon suitable dilution of a culture medium sample. The TOC values were obtained by subtracting the IC (inorganic carbon) value from the TC (total carbon) value.

Extracellular Lipase and Esterase Activity. The activity of lipases and esterases in the culture medium was measured after biomass removal by centrifugation, using *p*-nitrophenyl-butyrate (*p*NPB) in 10 mM sodium phosphate buffer, pH 7.0, at 37 °C. One unit of activity was defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol per minute.

Phytotoxicity Test. The OMW phytotoxicity was assessed on the seeds of *Lactuca sativa* species purchased by "La Semiorto Sementi" located in Sarno, South of Italy. The bioassays were carried out according to U.S. EPA procedures (*28*).

A suitable volume (5 mL) of OMW, both before or after treatment, with *L. starkeyi*, diluted (1:10 and 1:25) with deionized water, was added to 100 mm diameter Petri dishes containing a filter paper disk (Whatman no. 1, Ø 90 mm). Ten seeds were placed on each paper disk, and for each sample tested, three replicates were prepared. Controls with deionized water were also run. The plates were incubated in a growth chamber (Angelantoni HCT 120) in the dark at 23 °C, and after 72 h, the germinated seeds were counted, and the rootlet of each germinated seeds was measured with a ruler. Each experiment was repeated three times. A primary root >2 mm was considered as the end germination point. Seed germination and root elongation at the end of the bioassays were measured, and the relative index of germination (GI) was calculated according to the following formula:

$$\%GI = (Gs/Gc) \times (Ls/Lc) \times 100$$

where Gs and Gc are the number of germinated seeds in the sample and in the control, respectively, and Ls and Lc are the average root length of seedlings for the samples and for the control, respectively.

Statistical Analysis. All experiments have been carried out adopting a sample size of at least $n = 3$. The hypothesis tests for the GI data were carried out by a one-sided *t* tests (*29*), with significance levels of $\alpha = 0.01\%$.

RESULTS AND DISCUSSION

Fermentation in Synthetic Medium. Oleaginous yeasts accumulate lipids as storage materials only under N-limiting conditions.

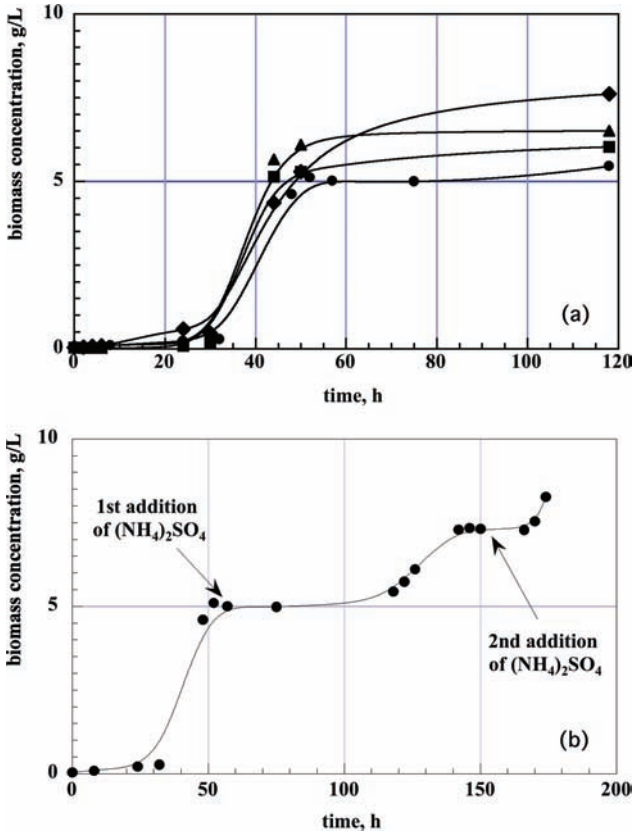

Figure 1. (**a**) Growth kinetics of four oleaginous yeasts using a N-limiting synthetic medium in batch cultures: *C. curvatus* (▲), *R. glutinis* (■), *R. toruloides* (♦), and *L. starkeyi* (●). Operating conditions: $T = 30$ °C and 160 rpm. The medium composition is as in the Materials and Methods. (**b**) Growth of *L. starkeyi* using a N-limiting synthetic medium in batch cultures, under multiple additions of the nitrogen source. Operating conditions: $T = 30$ °C and 160 rpm. The medium composition is as in the Materials and Methods.

Consequently, different oleaginous microorganisms (*L. starkeyi*, *C. curvatus*, *R. glutinis*, and *R. toruloides*) were preliminary cultured in a synthetic medium (described in the Materials and Methods) with a C/N ratio = 58. The growth profiles reported in the **Figure 1a** demonstrate that the growth kinetics are substantially similar. The lipid yield obtained with *L. starkeyi* after 120 h of growth (12.4%) was higher as compared to these pertaining to the other microorganisms. In addition, *L. starkeyi* has been proved to store large amounts of lipids, showing only a minimal reutilization of the stored lipids (*10*). For these reasons, *L. starkeyi* was selected for the subsequent tests.

Figure 1b describes the growth profile of *L. starkeyi* under multiple additions of fixed amounts (3 g each) of the nitrogen source $(NH_4)_2SO_4$ after a stationary phase was established. The experimental data show that, after each addition of $(NH_4)_2SO_4$, a new exponential phase starts, leading to an increase in the biomass concentration obtained under N-limiting conditions, although the increases in the biomass concentration are progressively reduced. The lipid yields obtained after the first and the second additions of $(NH_4)_2SO_4$ (see **Figure 1b**) were 14.1 and 15.5, respectively, showing that operation under N-limiting conditions also allows an increase in the lipid fraction of the biomass.

Fermentation in the Presence of OMW. *L. starkeyi* was cultured in the presence of the OMW, without external organic supplement. Experimental tests were carried out in the presence of raw OMW (after a preliminary centrifugation), as well as in water mixtures containing 50 and 25% OMW, respectively. The biomass concentration was evaluated in terms of colony-forming

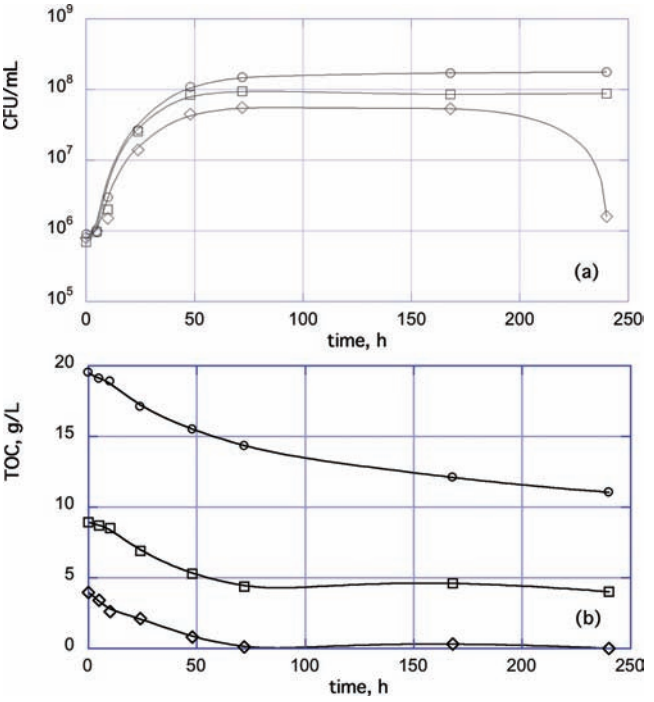

Figure 2. (**a**) Growth of *L. starkeyi* in batch cultures, in the presence of raw OMW (○), or in water mixtures containing 50% OMW (□) and 25% OMW (◇). Operating conditions: $T = 30$ °C and 160 rpm. The OMW composition is as in the Materials and Methods. (**b**) TOC measurements during the culture of *L. starkeyi* in batch cultures, in the presence of raw OMW (○), or in water mixtures containing 50% OMW (□) and 25% OMW (◇). Operating conditions: $T = 30$ °C and 160 rpm. The OMW composition is as in the Materials and Methods.

units (CFU), as OD measurements could not be carried out due to the darkness of the medium. Typical growth profiles are reported in **Figure 2a**.

The results show an initial increase of CFU limited to the first 3 days. The biomass growth was slightly slower when the yeasts were cultured in the presence of diluted OMW. Subsequently, *Lipomyces* cultured in 25% OMW were for about 170 h in a stationary phase, followed by a progressive reduction of CFU. *Lipomyces* cultured in the presence of 100 and 50% OMW survived at a constant biomass concentration for an even longer period.

The OMW are a very complex medium (see **Table 1**). Consequently, the biomass growth in OMW could be affected by different C sources (sugars, lipids, and phenols). Although it is likely that the microorganisms degrade preferentially the C sources that are more easy to metabolize (sugars), we first measured the degradative activity of the yeasts in terms of TOC levels, to evaluate subsequently the variation of the different classes of components. The TOC levels registered in the course of the same tests are reported in **Figure 2b**. A complete removal of the organic carbon was carried out only in the presence of 25% OMW. However, in all of the tests carried out, the OMW reduction was mostly achieved in the first 3 days, that is, in the period of the CFU growth, showing the use of the organic carbon as a primary carbon source (*30*).

Logistic Model for the Biomass Growth. To model the biomass production and the TOC abatement, a numerical model was built based on the experimental data obtained under different experimental conditions. The biomass production rate was obtained by a biomass balance:

$$\frac{dX}{dt} = \mu X \qquad (1)$$

Table 2. Comparison of Experimental Values of the Growth Parameters with the Theoretical Data Obtained with the Logistic Model, with Reference to the Culture of *L. starkeyi* in Batch Reactors, under Different Experimental Conditions

						g/L		1/h		g/L		g/g	
experiment	OMW fraction (%)	dilution factor	pH control	addition of glucose	T (°C)	X_0	$[TOC]_0$	μ_{max} (exp)[a]	μ_{max} (pred)	X_{max} (exp)	X_{max} (pred)	$Y_{X/TOC}$ (exp)[b]	$Y_{X/TOC}$ (pred)
E1	25	4	no control	no addition	30	0.0512	3.95	0.181	0.190	3.20	2.81	1.32	1.30
E2	50	2	no control	no addition	30	0.0488	8.93	0.171	0.175	5.90	5.81	1.29	1.25
E3	100	1	no control	no addition	30	0.0576	19.5	0.128	0.132	10.4	10.0	1.20	1.28
E4	100	1	no control	C_{gluc} = 70 g/L	30	0.0539	20.1	0.127	0.136	11.1	10.8	1.31	1.22
E5	100	1	pH 5.5	no addition	30	0.0510	20.1	0.130	0.135	11.0	10.7	1.24	1.30
E6	100	1	pH 6.5	no addition	30	0.0491	20.0	0.127	0.134	10.6	10.5	1.30	1.27
E7	100	1	no control	no addition	35	0.0512	19.9	0.071	0.078	6.12	5.68	1.29	1.20
E8	100	1	no control	no addition	25	0.0522	20.1	0.099	0.097	11.0	10.3	1.34	1.31

[a] The experimental value of μ_{max} was calculated from the equation $\mu_{max} = \ln(X_2/X_1)/(t_2 - t_1)$, assuming a constant growth rate in the early exponential phase. [b] The experimental value of $Y_{X/TOC}$ was calculated from the slope of the curve of the biomass concentration (X) as a function of the TOC.

The specific growth rate was defined adopting the logistic model:

$$\mu = \mu_{max}\left(1 - \frac{X}{X_{max}}\right) \quad (2)$$

The TOC profiles were described adopting the hypothesis of proportionality between TOC reduction rate and biomass growth rate:

$$\frac{d[TOC]}{dt} = \mu X \frac{1}{Y_{X/TOC}} \quad (3)$$

where $Y_{X/TOC}$ is the ratio of the amount of biomass produced to the amount of TOC consumed (g biomass/g TOC).

Equations 1−3 were integrated using a fourth-order Runge−Kutta integration method. The least-squares method was used to obtain the parameter estimates. The model fitted the experimental data (see the results in the **Table 2**) with R^2 values higher than 0.95. **Figure 3** shows a graphic comparison between the model predictions (dashed line) and the experimental results of a typical test. Standard errors, reported in the **Figure 3**, never exceeded 14%.

Growth Parameters of *L. starkeyi* in the Presence of OMW. A detailed comparison between the experimental and the theoretical data is given in **Table 2**. As higher OMW concentration values are adopted (experiments E1, E2, and E3 in **Table 2**), the specific growth rate (μ_{max}) slightly reduces. This result agrees with previously reported data, concerning the bacterial growth in the presence of OMW (*30*), and is due to the higher concentration of the phenolic components of OMW. In principle, another component of OMW that may inhibit microbial growth is the olive oil. However, it was ascertained (**Table 3**) that the lipase activity of *L. starkeyi* in the presence of OMW increases significantly. Consequently, the yeasts should be able to metabolize olive oil residuals.

The maximum values of biomass concentration (X_{max}), reported in the **Table 2**, increase with the initial OMW concentration, with the maximum value of X_{max} obtained when using raw OMW (10.4 g/L). The biomass yield based on TOC consumption ($Y_{X/TOC}$) appears to be substantially constant, suggesting that changes in the OMW concentration do not cause a significant increase in the maintenance requirements.

Table 4 describes the reduction of sugars, proteins, and phenols concentration obtained during the *L. starkey* cultures at different concentrations of OMW (experiments E1, E2, and E3 in **Table 2**). The experimental data demonstrate that efficiency of the yeasts in metabolizing the phenols is higher as the preliminary dilution of OMW increases, yielding phenol removal efficiencies of 43, 47, and 53% in the presence of OMW volumetric fractions of 100, 50, and 25%, respectively. These results indicate the critical concentration of phenols in OMW, preventing the growth of *Lipomyces*, to be higher than 9 g/L.

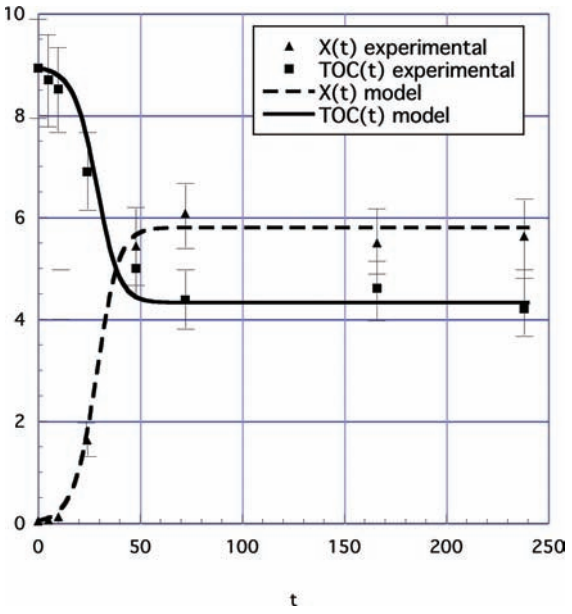

Figure 3. Comparison of experimental measurements of biomass concentration (X, g/L) and TOC (g/L) with the theoretical data obtained with the logistic model, with reference to the culture of *L. starkeyi* in batch cultures, in the presence of raw OMW. Operating conditions: T = 30 °C and 160 rpm. The OMW composition is as in the Materials and Methods. Standard error bars are reported.

Table 3. Extracellular Lipase Activity before and after *L. starkeyi* Culture in the Presence of OMW

		U/mL	
dilution factor	OMW fraction (%)	lipase activity before test	lipase activity after test
1	100	145	1040
2	50	134	827
4	25	122	710

The higher concentrations of OMW also reduce the removal of sugars and proteins (**Table 4**). Again, this detrimental effect is attributed to the higher initial concentration of phenols. The fraction of protein removed was in any case higher than 80%, reaching 98% in the presence of OMW volumetric fraction of 25%.

The highest efficiency of sugars removal was obtained with the most diluted OMW sample. In any case, the sugars removal was never complete, demonstrating that the break in the biomass

Table 4. Sugars, Proteins, and Phenols Content Removal (%) Obtained during the *L. starkeyi* Culture in the Presence of Undiluted and Diluted OMW as Compared with Untreated OMW

	%			
dilution factor	OMW fraction	removal of sugars content	removal of proteins content	removal of phenols content
1	100	48	82	43
2	50	54	87	47
4	25	86	98	53

Table 5. GI of *L. sativa* Seeds on Untreated and Treated Samples of Raw OMW

	GI	
dilution ratio	untreated OMW	*Lipomyces*-treated OMW
1	0	0
10	0	125.5
25	105.0	121.0

growth was not due to the exhaustion of sugars. This conclusion was confirmed by a further test: A glucose amount corresponding to a concentration of 70 g/L was added to raw OMW before the test beginning. In this case, the growth curves did not change significantly (experiment E4 in **Table 2**).

To check whether the growth of *L. starkeyi* in the presence of OMW was carried out under N-limiting conditions, supplementary additions (3 g) of the nitrogen source $(NH_4)_2SO_4$ were tried once a stationary phase was established during the culture in the presence of OMW (data not shown). In the latter tests, no further increases were observed in the biomass concentration, neither reductions in the TOC levels.

The initial pH of OMW (both raw or diluted) used in the experimental tests was between 4.7 and 5.0. A slow pH increase was observed in the course of the OMW fermentations, although the pH change in a single test was never higher than 1.2 pH units. To ascertain the actual effect of the pH, specific tests (experiments E5 and E6 in **Table 2**) were carried out, making every day pH adjustments to constant pH values (pH 5.5 and pH 6.5). The results reported in the **Table 2** show that the pH control does not affect appreciably the growth kinetics (i.e., μ_{max}) and stoichiometry ($Y_{X/TOC}$ and X_{max}).

Experimental tests (experiments E7 and E8 in **Table 2**) were carried out to evaluate the effect of the temperature. When the OMW fermentation was carried out at 35 °C, both μ_{max} and X_{max} were lower as compared to the corresponding values observed at 30 °C. The data obtained at a temperature of 25 °C showed a significantly lower value of μ_{max}, although the X_{max} value was close to that obtained at 30 °C.

In the course of OMW fermentation, the extracellular activity of lipases and esterases increases significantly (**Table 3**), in agreement with the literature data (*31*, *32*). Consequently, a further potential benefit of the OMW treatment with *L. starkey* is related to the production of these enzymes. The lipase and esterase activities are higher as the OMW concentration increases, probably due to the induction produced by the TAGs (olive oil) contained in the OMW.

Phytotoxicity. The results on GI of *L. sativa* seeds of untreated and treated samples of raw OMW are reported in the **Table 5**. When no sample dilutions were carried out before the GI test, both the untreated OMW and the *Lipomyces*-treated OMW were completely phytotoxic. Consequently, further GI tests were carried out upon dilution of samples. When testing diluted samples (1:10 v/v), the untreated OMW remained completely phytotoxic, whereas the *Lipomyces*-treated OMW showed a significantly high GI (125.5).

Table 6. Lipid Concentration in *L. starkeyi* after 10 Days of Culture in OMW

sample	lipid yield (%)
L. starkeyi grown in synthetic medium	12.1
L. starkeyi grown in undiluted OMW	22.4
L. starkeyi grown in 50% OMW	28.6
L. starkeyi grown in 25% OMW	29.5

Table 7. Distribution of Fatty Acids in the Lipids Accumulated in the *L. starkeyi* Grown on OMW

fatty acids composition	%
myristic acid C14:0	<1
palmitic acid C16:0	19.1
palmitoleic acid C16:1	0.5
stearic acid C18:0	8.5
oleic acid C18:1	49.1
linoleic acid C18:2	18.8
linolenic acid C18:3	3.5
arachidonic acid C20:0	0.3
free fatty acids	14.5

To achieve a significant reduction of the phytotoxic activity of the untreated OMW, a higher dilution ratio (1:25) was required. The hypothesis that the GI of the OMW was actually increased in the course of the treatment was confirmed by a one-sided *t* test (*29*), with a significance level of $\alpha = 0.01\%$. As widely reported in the literature (*33*, *34*), the phenols are considered the compounds mainly responsible for the OMW phytotoxicity. Consequently, the increase of GI likely originated from the reduction of phenols (see **Table 5**).

Lipid Yield and Composition. The amounts of lipids extracted from *L. starkeyi* cultured in OMW are reported in **Table 6** in terms of lipid yield. The experimental data show that a 50% dilution of OMW results in a significant increase in the concentration of lipids (28.6 against 22.4%), although a further increase in wastewater dilution produces only a minimum improvement.

The fatty acids distribution in the lipids accumulated in the *L. starkeyi* grown in OMW is described in the **Table 7**. The composition indicates a clear prevalence of oleic acid, in agreement with results obtained using different oleaginous microorganisms (*6*, *35*, *36*). The total content of saturated fatty acids is low enough to allow an excellent cold behavior of biodiesel, reducing its tendency of crystallization or gelling at low temperatures.

In conclusion, the accumulation of lipids by *L. starkeyi* may occur in the presence of OMW with no preliminary treatments and without external organic supplement. The growth of the yeasts is associated with a significant reduction of both the TOC and the total phenols content. The increase of the GI of the OMW after the biological treatment demonstrates the environmental benefits that can be achieved by this process.

The use of diluted wastewaters increases the fraction of the organic compounds (TOC, total phenols, and proteins) removed, although the kinetics of the biomass growth is slower. The lipid concentration (between 20 and 30%) in the microorganisms increases in the course of the OMW treatment, particularly in the presence of the preliminary diluted feedstock. The fatty acids distribution demonstrates the usability of the lipids accumulated in the *L. starkeyi* as feedstock for biodiesel synthesis.

ABBREVIATIONS USED

t, time (h); TOC, total organic carbon (g/L); *X*, cell concentration (dry weight) (g/L); X_{max}, maximum value of cell concentration (dry weight) (g/L); $Y_{X/TOC}$, biomass yield on TOC consumed

(g of biomass formed per g of TOC consumed); μ, specific growth rate (h^{-1}); μ_{max}, maximum value of the specific growth rate obtained by the logistic model (h^{-1}).

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