Combined yeast and microalgal cultivation in a pilot-scale raceway pond for urban wastewater treatment and potential biodiesel production

F. Iasimone, G. Zuccaro, V. D’Oriano, G. Franci, M. Galdiero, D. Pirozzi, V. De Felice and F. Pirozzi

ABSTRACT
A mixed culture of oleaginous yeast Lipomyces starkeyi and wastewater native microalgae (mostly Scenedesmus sp. and Chlorella sp.) was performed to enhance lipid and biomass production from urban wastewaters. A 400 L raceway pond, operating outdoors, was designed and used for biomass cultivation. Microalgae and yeast were inoculated into the cultivation pond with a 2:1 inoculum ratio. Their concentrations were monitored for 14 continuous days of batch cultivation. Microalgal growth presented a 3-day initial lag-phase, while yeast growth occurred in the first few days. Yeast activity during the microalgal lag-phase enhanced microalgal biomass productivity, corresponding to 31.4 mgTSS m⁻² d⁻¹. Yeast growth was limited by low concentrations in wastewater of easily assimilated organic substrates. Organic carbon was absorbed in the first 3 days with a 3.7 mgC L⁻¹ d⁻¹ removal rate. Complete nutrient removal occurred during microalgal linear growth with 2.9 mgN L⁻¹ d⁻¹ and 0.96 mgP L⁻¹ d⁻¹ removal rates. Microalgal photosynthetic activity induced high pH and dissolved oxygen values resulted in natural bactericidal and antifungal activity. A 15% lipid/dry weight was measured at the end of the cultivation time. Fatty acid methyl ester (FAME) analysis indicated that the lipids were mainly composed of arachidic acid.

INTRODUCTION
The current demand for alternative energy sources to meet the growing global energy needs continues to rise. Non-renewable energy sources, such as oil, are projected to be mostly depleted in less than 50 years. Moreover, the extensive use of petroleum, coal and natural gas has caused a number of environmental concerns, i.e. climate change resulting from global warming effects. For this reasons, alternative solutions that are renewable, economical, and less harmful to the environment need to be widely implemented. One such alternative is the use of biodiesel, whose production is based on the transesterification of long chain triglycerides from renewable sources using methanol (Reyna-Martínez et al. 2015). Biodiesel contributes no net carbon dioxide or sulfur to the atmosphere and emits less gaseous pollutants than normal diesel (Lang et al. 2001).

Biofuels produced from plants have the potential to replace a portion of fossil fuel consumption with a renewable alternative. However, the use of food crops for biodiesel and other renewable fuels may be an uneconomic long term solution (Meng et al. 2009; Demirbas 2011). As a feasible solution, microbial oils, produced by oleaginous microorganisms, can be used as potential alternative feedstock for biodiesel production, due to their high growth rate, non-use of arable agriculture land and fatty acid profiles similar to those derived from vegetable oils (Tapia et al. 2012; Yen et al. 2015). Moreover, oleaginous microorganisms are able to use wastes as a source of nutrients, which makes their cultivation economically sustainable and environmentally friendly (Reyna-Martínez et al. 2015). Oleaginous microorganisms, including bacteria, yeasts,
molds and algae, are defined as microbial species with microbial lipid content higher than 20% (Li et al. 2007).

Yeasts can use a vast variety of organic materials, accumulating high amount of lipids, up to 70% of their dry weight (Cheirsilp et al. 2011). Microalgae are considered an attractive source for biodiesel production due to their high lipid content, photosynthesis efficiency and CO₂ reduction ability (Olguín 2012). Recent studies showed that the combined cultivation of microalgae and yeasts could significantly enhance biomass and lipid production (Zhang et al. 2014; Yen et al. 2015). In mixed cultures, microalgae produce the oxygen used by yeast respiration, yeasts provide the CO₂ consumed by microalgal photosynthesis, and both carry out lipid production.

The use of combined yeast and microalgal cultures is still in its early stages and the data available in the literature are lacking. The principal aim of studies based on this topic and available in the literature has been to investigate the possibility of achieving higher lipid accumulation in mixed yeasts–microalgae cultures. They have been conducted at laboratory-scale conditions using synthetic growth medium (Zhang et al. 2014; Reyna-Martínez et al. 2015; Yen et al. 2015) or enriched urban wastewater (Chi et al. 2011; Ling et al. 2014). The studies have confirmed the possibility of obtaining a synergistic effect in combined yeasts–algal cultivations with different growth substrates and using different oleaginous species. In this work, the combined yeast–microalgal cultivation was conducted in a 400 L outdoor raceway pond using raw urban wastewater as the growth substrate. A native wastewater microalgal culture was used as the inoculum in order to reduce the initial time of adaptation to the medium (Su et al. 2011). Lipomyces starkeyi was added as the oleaginous yeast since it shows characteristics of high interest: the ability to accumulate lipids, high flexibility in carbon source utilization and culture conditions, and a fatty acid composition highly similar to vegetable oils (Tapia et al. 2012). Several physiological studies, relating to growth and lipid production by Lipomyces starkeyi, have been reported in the literature but urban wastewater has never been tested as a growth medium for this microorganism (Wild et al. 2010; Liu et al. 2013; Tsakona et al. 2014).

The aim of this work is to investigate the synergistic effect of mixed yeasts–microalgal cultures to enhance microbial lipid accumulation using urban wastewater as a growth substrate. The mixed biomass growth was monitored and the dissolved nutrient concentrations were measured during the cultivation period. A microbial evaluation was also conducted in order to understand the evolution in time of the microbial community in the water pond. Indeed, in the last decade, microalgae have been found to produce antibiotics: a large number of microalgal extracts and extracellular products have shown antibacterial, antifungal, antiprotozoal and antiplasmodial functions. The antimicrobial activity of microalgae has been attributed to several chemical compounds, such as indoles, terpenes, acetogenins, phenols, fatty acids and volatile halogenated hydrocarbons (Amaro et al. 2011; Jyotirmayee et al. 2014).

**MATERIALS AND METHODS**

**Strains**

*Lipomyces starkeyi* DBVPG 6195 was used as the oleaginous yeast, purchased from the Culture Collection of the Dipartimento di Biologia Vegetale of Perugia University (Italy). The strain was maintained at 5 °C on a yeast extract peptone dextrose (YPD) solid medium with the following composition (g/L): yeast extract (10), peptone (20), D-glucose (20), agar (20). Prior to fermentation, the yeast was grown in a 100 mL Erlenmeyer flask with an initial volume of 50 mL which contained (g/L): KH₂PO₄ (5.0), Na₂HPO₄ (5.0), yeast extract (5.0), glucose (10.0), peptone (5.0). The pre-culture broth was sterilized at 121 °C for 21 min prior to inoculation. Cultures for lipid production were inoculated with 5% v/v of the pre-culture media. The incubation of the pre-culture was carried out at 30 °C, 160 rpm for 48 hours (Minitron HT Infors, Switzerland).

The microalgal polyculture was obtained from a native wastewater biomass. It was collected from the effluent channel of a secondary clarifier located in the urban wastewater treatment plant in Pesche (Isernia, Italy). Afterwards, the biomass was maintained in laboratory controlled conditions using Bold basal medium (Richmond 2004) as the growth substrate. The cultivation was conducted in a 1 L flask equipped with a magnetic stirring bar (150 rpm) which maintained the biomass in suspension. The culture was kept under a homogeneous and continuous light of 1500 Lux (Cool White Fluorescent Lamps) with the environmental temperature of 25 ± 2 °C. Optical microscope analysis showed that the resulting biomass was composed of cyanobacteria, diatoms and microalgae (mostly *Scenedesmus* sp. and *Chlorella* sp.).

**Culture media and conditions**

The culture medium consisted of raw urban wastewater, collected from the entrance of the wastewater treatment plant.
located in Pesche. The raw wastewater was half diluted with tap water for a total cultivation volume of 150 L. The dilution was performed in order to reduce odour emissions and liquid turbidity, resulting in better light penetration. Indeed, light penetration is one of the principal parameters that influence microalgal photosynthesis and it is principally affected by pond depth, and microalgal and suspended particulate concentrations in the medium (Sutherland et al. 2015). Physical and chemical characteristics of the raw wastewater are shown in Table 1. Pre-cultured wastewater native microalgae (3% v/v) were added along with pre-cultured Lipomyces starkeyi (1.5% v/v). The cultivation was carried out for 14 days in batch mode.

The experiment was conducted in outdoor conditions in July 2016, with an average natural light intensity of 600 μmol m⁻² s⁻¹ and natural light/dark cycles. The pilot-scale raceway pond was installed on the roof of the Department of Bioscience and Territory, University of Molise, Pesche. The pond was a single-loop open channel with semi-circular end-walls with 1 m² surface area and 0.4 m³ total volume. A four-blade paddle wheel, coupled with a motor engine working at 6 rpm, was used to mix the culture media and keep a constant mean surface velocity of 0.10 m/s.

Analytical methods

Total suspended solids (TSS) were considered to be an indicator of the total biomass concentration in the raceway pond (García et al. 2006; Matamoros et al. 2015). TSS measurements were conducted every day according to Standard Methods (APHA/AWWA/WEF 2012).

Dissolved oxygen (DO) and pH values were determined using a DO meter (YSI 550 DO) and a pH meter (HI 8424, Hanna) respectively. The biomass concentration was monitored with a Shimadzu UV 1601 spectrophotometer (Japan) and measuring the turbidity of liquid samples at 600 nm and 680 nm. These readings are proportional to yeast and microalgal concentrations respectively, according to the scientific literature (Tapia et al. 2012; Sacristán de Alva et al. 2013).

Yeast and microalgal cell growth was monitored by cell counting. Moreover, chlorophyll a (Chl a) measurements were used as a further indicator of microalgal growth. A fluorometer (AquaFluorTM; Handheld Fluorometer/Turbidimeter; Turner Designs) was used to measure the content of in vivo Chl a in raw samples.

Biomass production, for both yeasts and microalgae, was evaluated according to a first order Monod law (Equation (1)); the biomass production rate (μ, d⁻¹) was calculated according to Equation (2), where X is the biomass concentration (cells/mL) and t is the cultivation time (days).

\[
\frac{dX}{dt} = \mu X
\]

\[
\mu = \frac{\ln (X_t/X_0)}{(t-t_0)}
\]

The biomass productivity (P, mg L⁻¹ d⁻¹) was calculated according to the following Equation (3):

\[
P = \frac{TSS_t - TSS_0}{t-t_0}
\]

where TSS₀ (mg/L) is the biomass concentration at time t₀ (days) and TSSₜ (mg/L) is the biomass concentration at any time t (days) subsequent to t₀.

Dissolved nutrient quantities were determined using liquid ion chromatography (Dionex, ICS 1000) as NH₄⁺, NO₂⁻, NO₃⁻, PO₄³⁻ concentrations. Chemical oxygen demand (COD) measurements were conducted for raw samples and supernatant after centrifugation (3,000 rpm, 10 min), according to Standard Methods (APHA/AWWA/WEF 2012). The removal rate of relevant substrates, Rᵢ (mg L⁻¹ d⁻¹, i = phosphate-P, ammonia-N, organic carbon-C), was calculated using Equation (4).

\[
R_i = \frac{S_{0,i} - S_i}{t_0 - t}
\]

where S₀ᵢ (mg/L) is the initial concentration of substrate i and Sᵢ (mg/L) is the corresponding substrate concentration at time t (days).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Wastewater characterization</th>
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<tbody>
<tr>
<td>Urban wastewater physic-chemical characteristics</td>
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<tr>
<td>pH</td>
<td>7.5 ± 0.1</td>
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<tr>
<td>DO (mg/L)</td>
<td>5.4 ± 1.4</td>
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<tr>
<td>TSS (mg/L)</td>
<td>310 ± 13</td>
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<tr>
<td>COD (mg O₂/L)</td>
<td>480 ± 19</td>
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<tr>
<td>NH₄⁺ (mg/L)</td>
<td>18.0 ± 0.6</td>
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<tr>
<td>PO₄³⁻ (mg/L)</td>
<td>2.1 ± 0.4</td>
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<td>NO₂⁻ (mg/L)</td>
<td>&lt;0.1</td>
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<td>NO₃⁻ (mg/L)</td>
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Concentrations of organic acids, alcohols and carbohydrates were measured by HPLC (LC2010, Shimadzu, Japan) with a refractive index detector (RID-20A, Shimadzu, Japan). Samples were first centrifuged at 12,000 rpm for 15 min and then supernatants were filtered with 0.2 μm syringe filters. HPLC analysis were performed at a flow rate of 0.7 mL/min on a supelcogel, 300 × 7.8 mm (Supelco) column at a temperature of 60 °C. H₃PO₄ at 4 mM was used as the mobile phase. Total lipid content was determined by the sulfo-phospho-vanillin method (Mishra et al. 2014). For lipid composition analysis, the extraction was performed with a method adapted from the Bligh and Dyer protocol (Bligh & Dyer 1959). The samples were stirred in a CHCl₃/CH₃OH mixture (2:1 w/v) over 24 hours, and the oleaginous biomass was filtered off and washed with additional CHCl₃. The solvent was then removed by evaporation under N₂ stream. The extracted lipids were subjected to a transesterification reaction in a stirred container at 60 °C for 10 min, using NaOH (1% w/v) as the catalyst and methanol as the reagent. The samples were dried under N₂ stream and subsequently 1 mL of heptane was added for the analysis. The fatty acid compositions of the fatty acid methyl esters (FAME) were analyzed using a gas chromatograph (GC). The GC (GC-MS 2010, Shimadzu, Japan) was equipped with a flame ionization detector and an Omegawax 250 (Supelco) column (30 m × 0.25 mm I.D., 0.25 μm). Helium was used as a carrier gas (flow rate: 30 mL/min). The samples were initially dissolved in 1 mL of heptane and 1 μL of this solution was injected to the column. The temperature of the column was kept at 50 °C for 2 min, then heated to 220 °C at a rate of 4 °C/min, and finally kept constant for 2 minutes. Methyl decanoate was used as the internal standard. The peaks of each methyl ester were identified by comparing the retention time with the peak of the pure standard compound.

Microbial evaluation

Samples were imaged using a standard microscope equipped with a 40× objective (Nikon eclipse 80i). Samples, collected at 0 day, 1 day, 7 days and 14 days, were incubated overnight at 37 °C on a 300 rpm rotating agitator. The day after, 200 μL of each sample was inoculated into 10 mL of brain heart infusion (BHI) (Oxoid CM1135) liquid growth medium at 37 °C overnight as before. The microbiological growth curve was evaluated using optical density (OD) at 600 nm using a spectrophotometric method (Eppendorf BioPhotometer UV/vis Spectrophotometer mod. 6131) and compared to the respective controls.

Then, 10 μL of each samples was spread on BHI agar, MSA (Mannitol salt agar, bioMérieux 43671), MCK (MacConkey agar – bioMérieux 43141), SAB (Sabouraud glucose agar – Oxoid PO5001A) and SAB CG (Sabouraud supplemented with Chlormamphenicol and Gentamicin agar – bioMérieux 43651).

The plates were then incubated at 37 °C and observed after 48 hours. All data are representative of biological triplicates.

RESULTS AND DISCUSSION

Biomass growth

Microalgae and yeast were both added to the cultivation water pond at time zero and their growth was monitored for 14 days (Figure 1(a) and 1(b)). The OD readings at the two different wavelengths of 600 nm and 680 nm showed the same variation trend during the cultivation time; consequently, the higher values (OD 680 nm) are shown in Figure 1(a) in order to show the mixed biomass growth.
with time. In the same Figure 1(a), the Chl-a concentrations are also shown in order to monitor the microalgal biomass production during the cultivation time. For the first 3 days, Chl-a concentrations were near to zero, corresponding to the initial microalgal lag-phase; then Chl-a values increased during the microalgal exponential growth phase. OD values were 0.3 abs for the first two days and decreased until day 6: the initial density could be linked to the presence of yeast, whose decline started after 3 days, contributing to explaining the decreasing OD values. The decreasing OD trend could be also associated with microalgal autoflocculation, which occurred in this period, just before the microalgal linear growth phase. This phenomenon was observed in a previous study (Iasimone et al. 2011) and is considered to be a microalgal physiological adaptation: the autoflocculation clarifies the culture liquid, promoting microalgal growth through higher light penetration. From day 6 (Figure 1(a)), OD values and Chl-a concentrations followed a similar growth trend, confirming the correlation between culture liquid density and microalgal concentration. However, in order to better understand the evolution in time of microalgae and yeast, their concentrations (cells/mL) are shown at logarithm scale in Figure 1(b).

Yeast growth occurred in the first day, while microalgal production occurred after an initial lag-phase. This result could be explained by the different metabolism of the two oleaginous microorganisms since microalgal growth is slower than yeast growth (Zhang et al. 2014). During the linear growth phase (days 3–9), the microalgal consortium showed the specific growth rate of 0.36 d⁻¹, calculated according to Equation (2). After 9 days of batch cultivation, microalgal growth reached a stationary phase, while yeast was not detected until the end of the test. These results could be principally explained by the high pH values reached after day 9 (Figure 3). At the end of the cultivation period, microalgal concentration was 1.4·10⁷ cells/mL and was mainly represented by *Scenedesmus* sp., as showed by microscopic analysis (Figure 2). A similar result was obtained by Park & Craggs (2011); indeed, *Scenedesmus* sp. and *Chlorella* sp. are particularly tolerant to wastewater conditions (Gouveia et al. 2016).

DO concentrations and pH were monitored during the cultivation time (Figure 3) since these parameters are crucial for microalgal and yeast growth (Ling et al. 2014; Yen et al. 2015). Yeast’s heterotrophic activity is responsible for oxygen consumption and CO₂ production (Wild et al. 2010), while microalgal photosynthetic activity produces DO and increasing pH (García et al. 2006). At time zero, the inoculum addition caused pH and DO reductions. During the first 3 days, low values of DO concentration and pH were measured, being 1 mg/L and 7.6 respectively. These values could be related to the presence of yeast, whose heterotrophic metabolism limited the DO concentration rise that generally occurs during microalgal pond start-up (Iasimone et al. 2017). By contrast, the microalgal photosynthetic
activity was responsible for DO and pH increasing after day 3. Finally, at day 9, pH values and DO concentrations showed little variation once the stationary phase of biomass growth was reached.

High pH and DO values, 10.9 and 15.4 mg/L respectively, were obtained at the end of the cultivation period. These conditions could explain the almost complete absence of fungus and bacteria found at the end of the cultivation period (Figure 4(a) and 4(b)). The presence of these species in the samples was evaluated by a double approach: (i) spectrophotometric absorbance (Figure 4(a)) and (ii) colony forming units on agar plates (Figure 4(b)). The resulting turbidimetric data were comparable to the microbial growth observed on the agar plates. A remarkable decrease in microorganism growth was observed for a corresponding increase in microalgal cell numbers, therefore a microbicial effect of the pond cultivation can be hypothesized.

Similar results were obtained by Craggs et al. (2004) for sunlight disinfection in a high rate pond, but this topic still needs further investigation.

TSS variation was monitored during the cultivation time and results are shown in Figure 5. The inoculum addition at time zero caused the TSS increase from 160 to 215 mg/L. A decreasing trend was monitored during first 6 days of cultivation, followed by an increasing trend until the end of the test. Initial TSS reduction was related to both yeast decline and microalgal autoflocculation, as explained before for the decreasing OD values obtained for the same period. On the other hand, TSS increasing from day 6 was principally linked to microalgal biomass production, since
other microorganism growth was inhibited by the high monitored values of pH and DO. Microalgal productivity was calculated according to Equation (3) and was 31.4 mgTSS m^{-2} d^{-1}. This value is comparable or even higher than the ones obtained for microalgal cultivation in wastewaters. Matamoros et al. (2015) found a TSS productivity of 24 mg/L and 13 mg/L for hydraulic retention times (HRTs) of 4 and 8 days respectively. García et al. (2006) found a TSS productivity of 12.7 mg/L and 14.8 mg/L for HRTs of 4 and 7 days respectively. Moreover, a previous study, conducted in similar experimental conditions but using only microalgal inoculum, showed a biomass productivity of 11.7 mgTSS m^{-2} d^{-1} (Iasimone et al. 2011) and an initial lag phase of 9 days. The last comparison evidenced that yeast activity during the initial microalgal lag-phase can enhance biomass production. This result could be explained by the CO2 gas enrichment of the culture liquid, which occurred during the microalgal lag-phase because of yeast respiration.

**Nutrient removal**

The organic carbon content was evaluated for both raw and clarified samples; measures were conducted during the cultivation time and results are shown in Figure 6. As clarified samples were considered to be the culture liquid without the suspended biomass, which is also constituted of organic carbon; as a consequence, the COD measures for raw samples were higher than the ones obtained for the clarified samples. The initial inoculum addition at time zero led to the COD increasing from 240 mgC/L to 455 mgC/L because of the high organic carbon concentration in yeast inoculum. Organic carbon was principally utilized during the first 3 days with a removal rate of 3.7 mgC L^{-1} d^{-1}, calculated according to Equation (4) for clarified samples. Its removal could be principally linked to both bacteria and yeast heterotrophic metabolism. On the other hand, the significant decrease in COD observed for raw samples after day 1 could be associated with the decline of yeast. After the initial 5 days, a carbon accumulation and stabilization occurred in the clarified medium, possibly related to the absence of heterotrophic activities. At the same time, increasing COD values (from 100 to 500 mg/L) were measured for raw samples starting from day 6. These results could be explained by the microalgal biomass production, which occurred in this time. By contrast, limited yeast metabolic activity was strictly related to low concentration of easily assimilated organic substrates (fermentable sugars), as indicated by HPLC analysis.

Dissolved nitrogen (NH4^+, NO3^-, NO2^-) and phosphorus (PO4^3-) were monitored during the cultivation time and results are shown in Figure 7. Dissolved oxidized nitrogen (NO2, NO3) maintained concentrations lower than 1 mg/L during the cultivation period, so their variation is not shown in Figure 7. The inoculum addition at time zero induced a phosphate increment from 0.9 to 12.2 mg/L and ammonium reduction from 8.9 to 4 mg/L, probably due to its complexation with ionic species contained into the inoculum medium. As shown in Figure 7, phosphate concentrations decreased during the microbial heterotrophic activity (days 0–2), then increased to reach the initial value of 12.2 mg/L at day 6. This variation could be associated with the decrease in the number of yeast cells, which induced the release of accumulated phosphorus. At the same time, for the first 2 days, ammonium concentrations were maintained at 4 mg/L and then increased towards the initial value. Also in this case, ammonium variations could be associated with yeast activity. These considerations could be confirmed by results obtained in previous experiments, conducted without yeast inoculum:

![Figure 6](image-url)  
*Figure 6* | Time monitoring of organic carbon in the cultivation media for raw and clarified samples (supernatant of centrifuged samples).

![Figure 7](image-url)  
*Figure 7* | Time monitoring of dissolved ammonia and phosphates in the cultivation media.
during the microalgal lag-phase, phosphate concentrations remained constant while ammonium quantities decreased because of heterotrophic bacteria activity (Iasimone et al. 2017). As consequence, since ammonium was not consumed in this case, nitrifying bacteria metabolism could be inhibited by yeast competition.

At day 6, corresponding to the microalgae increasing in number, the depletion of both ammonium and phosphate occurred because of the combined effects of microalgal nutrient absorption and pH rise (Garcia et al. 2000). Nutrient removal rates were 2.9 mgN L$^{-1}$ d$^{-1}$ and 0.96 mg mgP L$^{-1}$ d$^{-1}$. They were measured until the microalgae declined, using Equation (4). At the end of the cultivation period, both ammonium and phosphates were depleted. Cheirsilp et al. (2011) measured COD and nitrate removal rates for industrial wastes by Rhodotorula glutinis and Chlorella vulgaris mixed cultures, which gave higher results than the ones obtained using unmixed cultures of yeasts or microalgae.

**Lipid accumulation**

Biomass lipid accumulation was monitored during the cultivation period and results are shown in Figure 8 as lipids/dry weight. At time zero, the inoculum addition led to the lipid concentration increasing from 5.2 to 8.8 mg/L (from 3.3% to 4.1% as lipids/dry weight). The first peak of 7% lipids/dry weight in the graph was registered at day 2, possibly due to yeast lipid accumulation. The second and highest peak of 15% lipids/dry weight was measured at the end of the cultivation time and could be principally related to the microalgal lipid content. Indeed, similar lipid percentages were obtained by Sacristán de Alva et al. (2013) for Scenedesmus acutus growth in wastewaters with low nutrient content (7.3 mg/L of orthophosphate, 27.7 mg/L of organic nitrogen and ammonia). Moreover, the higher lipid content, measured at the end of the cultivation period, agrees with previous studies (Li et al. 2008), which highlighted that high lipid accumulation occurs when the cells are under physiological stress conditions (such as nitrogen depletion) or if they are in a stationary growth state. The composition of lipids extracted from the mixed yeast/microalgal culture was principally long-chain fatty acids with 16 and 20 carbon including palmitic acid (3%) and arachidic acid (97%).

**CONCLUSIONS**

The preliminary results obtained for the combined yeasts–microalgal biomass, cultivated outdoors using urban wastewater as the growth substrate, highlighted that yeasts and microalgae showed different growth phases. Indeed, when the mixed biomass was inoculated at the same time, yeast growth started just after the inoculation, while microalgal growth occurred only after a lag-phase. Moreover, yeast growth was poor because of the low concentration of fast-assimilated organic carbon in urban wastewater. These findings suggest that lipid accumulation, for the tested experimental conditions, could be enhanced by adding the yeast inoculum during the microalgal exponential growth and increasing the easily assimilated organic substrates in urban wastewater or using dairy wastewater. The first solution would lead to the synergistic growth of the combined species, while the second solution would enhance yeast growth. On the other side, yeast activity during the initial microalgal lag-phase seemed to enhance the microalgal biomass production.

Complete nutrient removal was feasible in the combined yeast–microalgal cultivation, even if it was principally associated with microalgal activity.

Finally, the disinfection capability, related to the high pH and DO values (induced by microalgal photosynthetic activity) or linked to microalgal metabolites, is of relevant interest for further investigations.

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