Elsevier Editorial System(tm) for Science of the Total Environment

Manuscript Draft

Manuscript Number: STOTEN-D-16-05747R1

Title: A sustainable use of Ricotta Cheese Whey for microbial biodiesel production

Article Type: VSI: ISEB2016

Keywords: Ricotta Cheese Whey, Cryptococcus laurentii, oleaginous yeasts, biodiesel, single cell oil

Corresponding Author: Professor Alessandro D'Annibale, PhD

Corresponding Author's Institution: University of Tuscia

First Author: Eleonora Carota

Order of Authors: Eleonora Carota; Silvia Crognale, PhD; Alessandro D'Annibale, PhD; Anna M Gallo; Silvia R Stazi, PhD; Maurizio Petruccioli, Professor

Abstract: The increasing demand of plant oils for biodiesel production has highlighted the need for alternative strategies based either on non-food crops or agro-industrial wastes that do not compete with food and feed production. In this context, the combined use of wastewater and oleaginous microorganisms could be a valuable production option. Ricotta cheese whey (RCW), one of the major byproducts of the dairy industry, is produced in very high and steadily increasing amounts and, due to its high organic load, its disposal is cost-prohibitive.
In the present study, in order to assess the adequacy of RCW as a growth medium for lipid production, 18 strains of oleaginous yeasts were investigated in shaken flask for their growth and lipid-producing capabilities on this substrate. Among them, Cryptococcus curvatus NRRL Y-1511 and Cryptococcus laurentii UCD 68-201 adequately grew therein producing substantial amounts of lipids (6.8 and 5.1 g L-1, respectively). A high similarity between the percent fatty acid methyl esters (FAME) composition of lipids from the former and the latter strain was found with a predominance of oleic acid (52.8 vs. 48.7%) and of total saturated fatty acids (37.9 vs. 40.8%). The subsequent scale transfer of the C. laurentii UCD 68-201 lipid production process on RCW to a 3-L STR led to significantly improved biomass and total lipid productions (14.4 and 9.9 g L-1, respectively) with the biodiesel yield amounting to 32.6%. Although the C. laurentii FAME profile was modified upon process transfer, it resembled that of the Jatropha oil, a well established feedstock for biodiesel production. In conclusion, C. laurentii UCD 68-201, for which there is very limited amount of available information, turned out to be a very promising candidate for biodiesel production and wide margins of process improvement might be envisaged.

Response to Reviewers: Reviewer #1
Suggestion: Discussion should be enriched by using papers recently published in the international literature. E.g. substrates of high interest for several EU countries, such as OMW, biodiesel derived

glycerol, lignocellulosic materials, food wastes etc. have been used by several authors for SCO production. Please consider related papers.
Answer: The suggestion of Reviewer #1 has been accepted and the text modified by making explicit mention to second generation biodiesel production on other low-cost feedstocks. Changes have been done at the second paragraph of the Discussion section. Four further references have been added.

Reviewer#2
Remark #1: In table 1: Yx/s and Yp/s of C. curvatus NRRL Y-1511 is 0.84 and 0.63, respectively. According to the authors' description in P8, line 183 and 184, 0.84 g biomass will be obtained when 1 g substrate (lactose) is consumed, and 0.63 g lipid will be obtained when 1 g substrate (lactose) is consumed. There may be a serious error, it is well known that biomass yield (Yx/s) of yeast on the glucose is about 0.5 g/g, and theoretical yield of lipid on the glucose is 0.31 g/g (Ratledge C. Biochemistry, stoichiometry, substrate and economics. In: Moreton R S. Single Cell Oil. London: Longman, 1988: 33-70). So, the yield of biomass (lipid content reached to 20-60% of biomass) should be 0.3-0.4 g biomass/g substrate. It is not possible to have such a high biomass yield (0.84) and lipid yield (0.63). The same problem has also been found in table 2. There is a very serious problem, which needs to be addressed by the authors.
Answer: We are very grateful to Reviewer #2 for his remarks to Tables 1 and 2 regarding the presence of uneven values of some yields (YX/S and YP/S) in C. curvatus and C. laurentii cultures.
With regard to Table 1, the culture supernatants, which were stored at -80 °C, were analyzed again and it was found that the residual concentrations of total sugars, and thus the extents of their consumption, were underestimated. As a consequence, recalculations of YX/S and YP/S have led to values equal to 0.50 and 0.37 which have been incorporated at row #4, columns #4 and #6, respectively, of Table 1.
With reference to the Remarks of the Reviewer, we have done further changes:
In the footnote of Table 1, it has been specified that YP/S and YP/X values were calculated with reference to total lipids determined according to Izard and Limberger (2003).
Figure 1 has been modified by adding biodiesel yields and the amounts of FAME derived from transesterification; the latter parameter has been referred to original unit volume of culture broth. These parameters are shown in Figure 1B. The biodiesel yield has been defined at the end of the Subsection 2.5.
In Table 2, we have reported biodiesel yield and, on this basis, the production yields (YP/S and YP/X) and productivity (rp) have been recalculated. This has been clearly specified both in the caption and in the footnote of Table 2. This choice is coherent with the title of the Ms. which refers to biodiesel (i.e., namely to a mixture of fatty acid methyl esters) and not to lipids.
As a consequence of these modifications, the text has been changed in the subsections 3.1 and 3.2 and in the section 4. As a consequence of these modifications, the YP/S value, obtained in Stirred tank reactor, is in line with both literature data and the aforementioned threshold suggested by Ratledge (1988). With regard to YX/S, instead, due to an incorrect setting of the spreadsheet cells, the biomass at time zero was not subtracted but after this correction, this parameter is still higher than the theoretical threshold of 0.5. In this respect, it is worth mentioning that this threshold had been sometimes exceeded by oleaginous yeasts on carbohydrate-containing liquid media. For instance, YX/S of 0.7 and 0.6

were observed in Rhodosporidium toruloides cultures in 10-L and 150-L reactors, respectively, on a sucrose-based medium (sugarcane juice added with urea) under batch conditions (Soccol et al., Bioresour. Technol. 2017, 223: 259–268). In another study, conducted with Apiotrichum curvatum (syn. Cryptococcus curvatus) on a similar medium to that of the present study (deproteinized whey), YX/S amounted to 0.58 (Davies et al., Appl Microbiol Biotechnol, 1990; 33:569-573). Similarly, on a glucose-based medium with a C/N ratio similar to that of our medium (50 vs. 55), the YX/S in batch C. curvatus cultures was 0.58 (Hassan et al, Process Biochem. 31, 355-361, 1996).

Remark #2: P 5, line 110: "reactor" not "rector"
Answer: Corrected

Remark #3: P 6, line 145: most performing oleaginous?
Answer: “most performing oleaginous” has been changed to “best”

Remark#4: P7, line 147: "mL" not "ml"
Answer: Changed here and elsewhere as requested

Remark #5: P14, line 335-336: unclear
Answer: The sentence in question has been changed to “In particular, when nitrogen depletion takes place, the residual carbon in the medium is readily converted to storage lipids.”

Remark #6: There is no data to support the conclusion in the manuscript that "Last but not least, the non-seasonal availability of RCW makes this byproduct a valuable feedstock ensuring a continuous supply".
Answer: Although we are sure that dairy plants where cheese whey is routinely converted into ricotta ensure a continuous supply of RCW, we have not statistical figures supporting this statement. For this reason, we have deleted that sentence as suggested by the Reviewer.

DIBAF
DIPARTIMENTO PER LA INNOVAZIONE NEI SISTEMI BIOLOGICI AGROALIMENTARI E FORESTALI

Dear Editor,

I am sending you the manuscript titled "*A sustainable use of Ricotta Cheese Whey for microbial biodiesel production*" which has been revised according the suggestions/remarks of the reviewers. Following your indications, responses to Reviewers' suggestions/criticism have been done on a point by point basis. A clear identification of the added modifications can be retrieved from an additional Ms. text file where changes have been tracked (i.e., Carota_Manuscript_R1_annotated_version.doc).

Yours sincerely

Alessandro D'Annibale

Alessandro D'Annibale, Ph.D DIC
Department of Innovation in Biology and Forestry Systems (DIBAF)
University of Tuscia
Via San Camillo de Lellis snc
I-01100 Viterbo (Italy) Phone: +39-(0)761357368 Fax +39-(0)761-357242
E-mail: dannib@unitus.it

Reviewer #1

Suggestion: Discussion should be enriched by using papers recently published in the international literature. E.g. substrates of high interest for several EU countries, such as OMW, biodiesel derived glycerol, lignocellulosic materials, food wastes etc. have been used by several authors for SCO production. Please consider related papers.

Answer: The suggestion of Reviewer #1 has been accepted and the text modified by making explicit mention to second generation biodiesel production on other low-cost feedstocks. Changes have been done at the second paragraph of the Discussion section. Four further references have been added.

Reviewer#2

Remark #1: In table 1: Yx/s and Yp/s of C. curvatus NRRL Y-1511 is 0.84 and 0.63, respectively. According to the authors' description in P8, line 183 and 184, 0.84 g biomass will be obtained when 1 g substrate (lactose) is consumed, and 0.63 g lipid will be obtained when 1 g substrate (lactose) is consumed. There may be a serious error, it is well known that biomass yield (Yx/s) of yeast on the glucose is about 0.5 g/g, and theoretical yield of lipid on the glucose is 0.31 g/g (Ratledge C. Biochemistry, stoichiometry, substrate and economics. In: Moreton R S. Single Cell Oil. London: Longman, 1988: 33-70). So, the yield of biomass (lipid content reached to 20-60% of biomass) should be 0.3-0.4 g biomass/g substrate. It is not possible to have such a high biomass yield (0.84) and lipid yield (0.63). The same problem has also been found in table 2. There is a very serious problem, which needs to be addressed by the authors.

Answer: We are very grateful to Reviewer #2 for his remarks to Tables 1 and 2 regarding the presence of uneven values of some yields ($Y_{X/S}$ and $Y_{P/S}$) in *C. curvatus* and *C. laurentii* cultures.

With regard to Table 1, the culture supernatants, which were stored at -80 °C, were analyzed again and it was found that the residual concentrations of total sugars, and thus the extents of their consumption, were underestimated. As a consequence, recalculations of $Y_{X/S}$ and $Y_{P/S}$ have led to values equal to 0.50 and 0.37 which have been incorporated at row #4, columns #4 and #6, respectively, of Table 1.

With reference to the Remarks of the Reviewer, we have done further changes:

In the footnote of Table 1, it has been specified that $Y_{P/S}$ and $Y_{P/X}$ values were calculated with reference to total lipids determined according to Izard and Limberger (2003).

Figure 1 has been modified by adding biodiesel yields and the amounts of FAME derived from transesterification; the latter parameter has been referred to original unit volume of culture broth. These parameters are shown in Figure 1B. The biodiesel yield has been defined at the end of the Subsection 2.5.

In Table 2, we have reported biodiesel yield and, on this basis, the production yields ($Y_{P/S}$ and $Y_{P/X}$) and productivity (r_p) have been recalculated. This has been clearly specified both in the caption and in the footnote of Table 2. This choice is coherent with the title of the Ms. which refers to biodiesel (i.e., namely to a mixture of fatty acid methyl esters) and not to lipids.

As a consequence of these modifications, the text has been changed in the subsections 3.1 and 3.2 and in the section 4. As a consequence of these modifications, the $Y_{P/S}$ value, obtained in Stirred tank reactor, is in line with both literature data and the aforementioned threshold suggested by Ratledge (1988). With regard to $Y_{X/S}$, instead, due to an incorrect setting of the spreadsheet cells, the biomass at time zero was not subtracted but after this correction, this parameter is still higher than the theoretical threshold of 0.5. In this respect, it is worth mentioning that this threshold had been sometimes exceeded by oleaginous yeasts on carbohydrate-containing liquid media. For instance, $Y_{X/S}$ of 0.7 and 0.6 were observed in *Rhodosporidium toruloides* cultures in 10-L and 150-L reactors, respectively, on a sucrose-based medium (sugarcane juice added with urea) under batch conditions (Soccol et al., Bioresour. Technol. 2017, 223: 259–268). In another study, conducted with *Apiotrichum curvatum* (syn. *Cryptococcus curvatus*) on a similar medium to that of the present study (deproteinized whey), $Y_{X/S}$ amounted to 0.58 (Davies et al., Appl Microbiol Biotechnol, 1990;

33:569-573). Similarly, on a glucose-based medium with a C/N ratio similar to that of our medium (50 vs. 55), the $Y_{X/S}$ in batch *C. curvatus* cultures was 0.58 (Hassan et al, Process Biochem. 31, 355-361, 1996).

Remark #2: P 5, line 110: "reactor" not "rector"
Answer: Corrected

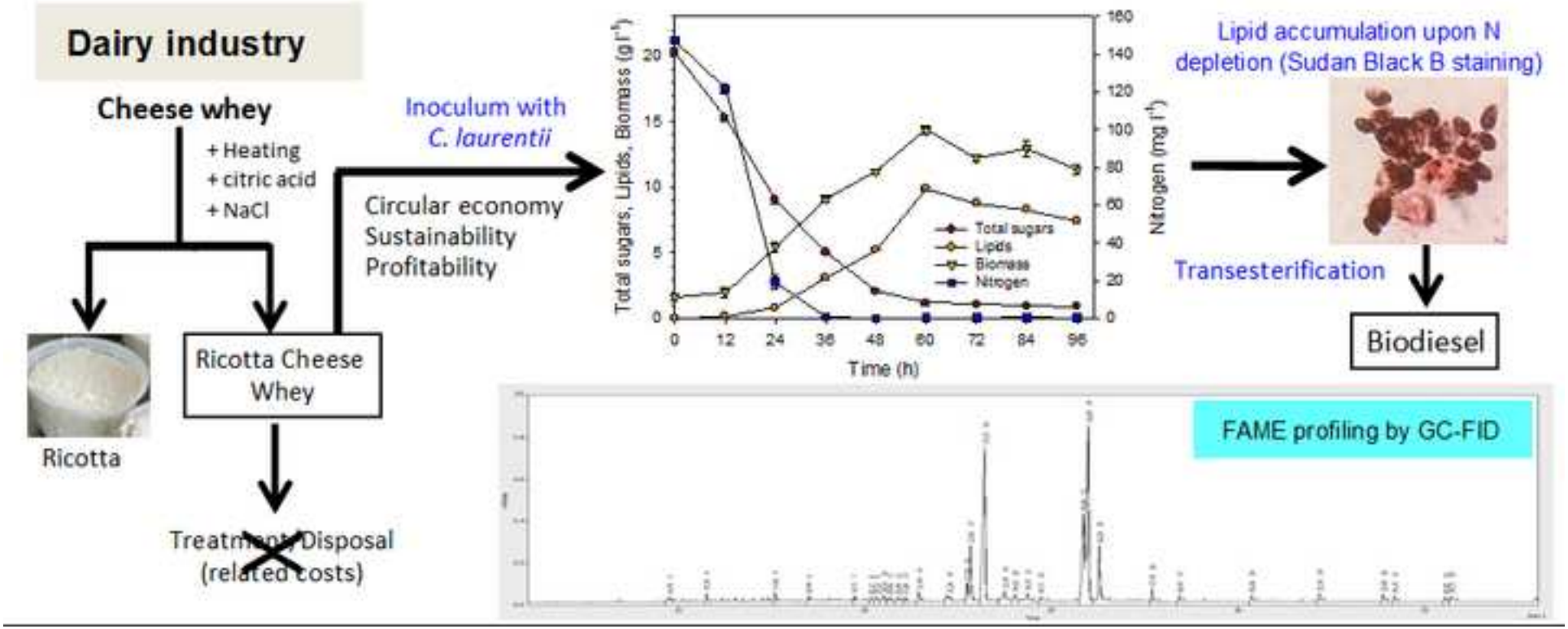
Remark #3: P 6, line 145: most performing oleaginous?
Answer: "most performing oleaginous" has been changed to "best"

Remark#4: P7, line 147: "mL" not "ml"
Answer: Changed here and elsewhere as requested

Remark #5: P14, line 335-336: unclear
Answer: The sentence in question has been changed to "In particular, when nitrogen depletion takes place, the residual carbon in the medium is readily converted to storage lipids."

Remark #6: There is no data to support the conclusion in the manuscript that "Last but not least, the non-seasonal availability of RCW makes this byproduct a valuable feedstock ensuring a continuous supply".
Answer: Although we are sure that dairy plants where cheese whey is routinely converted into ricotta ensure a continuous supply of RCW, we have not statistical figures supporting this statement. For this reason, we have deleted that sentence as suggested by the Reviewer.

*Graphical Abstract



Highlights

- Ricotta cheese whey (RCW) used as a lipid-production medium
- Oleaginous yeasts (OY) screened for lipid-producing ability on RCW
- *C. curvatus* and *C. laurentii* produced substantial amounts of lipids on RCW
- Dominant fatty acids were oleic, linoleic and palmitic acids in *C. laurentii*
- *C. laurentii* lipid production was faster in STR than shaken flask

Formatted: Left: 2.54 cm, Right: 1.63 cm, Top: 2.54 cm, Bottom: 2.54 cm, Width: 21 cm, Height: 29.7 cm

A sustainable use of Ricotta Cheese Whey for microbial biodiesel production

Carota, Eleonora; Crognale, Silvia; D'Annibale, Alessandro*; Gallo, Anna Maria ~~Gallo~~; Stazi, Silvia Rita; Petruccioli, Maurizio

Department for Innovation in Biological, Agro-food and Forest Systems (DIBAF), University of Tuscia, Via S. Camillo De Lellis, 01100 Viterbo, Italy

(*) Correspondence should be addressed to:
D'Annibale, Alessandro, PhD
Department for Innovation in Biological, Agro-food and Forest Systems (DIBAF)
University of Tuscia, Via S. Camillo De Lellis, 01100 Viterbo, Italy
Phone: ++390761357368
E-mail: dannib@unitus.it

Abstract

The increasing demand of plant oils for biodiesel production has highlighted the need for alternative strategies based either on non-food crops or agro-industrial wastes that do not compete with food and feed production. In this context, the combined use of wastewater and oleaginous microorganisms could be a valuable production option. Ricotta cheese whey (RCW), one of the major byproducts of the dairy industry, is produced in very high and steadily increasing amounts and, due to its high organic load, its disposal is cost-prohibitive.

In the present study, in order to assess the adequacy of RCW as a growth medium for lipid production, 18 strains of oleaginous yeasts were investigated in shaken flask for their growth and lipid-producing capabilities on this substrate. Among them, *Cryptococcus curvatus* NRRL Y-1511 and *Cryptococcus laurentii* UCD 68-201~~,~~ adequately grew therein producing substantial amounts of lipids (6.8 and 5.1 g L^{-1}, respectively). A high similarity between the percent fatty acid methyl esters (FAME) composition of lipids from the former and the latter strain was found with a predominance of oleic acid (52.8 vs. 48.7%) and of total saturated fatty acids (37.9 vs. 40.8%). The subsequent scale transfer of the *C. laurentii* UCD 68-201 lipid production process on RCW to a 3-L~~l~~ STR led to significantly improved biomass and total lipid ~~accumulation and productivity~~productions (14.4 and 9.9 g L^{-1} ~~and 4.0 g l^{-1} d^{-1}~~, respectively) with the biodiesel yield amounting to 32.6%. Although the *C. laurentii* FAME profile was modified upon process transfer, it resembled that of the Jatropha oil, a well established feedstock for biodiesel production. In conclusion, *C. laurentii* UCD 68-201, for which there is very limited amount of available information, turned out to be a very promising candidate for biodiesel production and wide margins of process improvement might be envisaged.

Keywords:

Ricotta Cheese Whey, *Cryptococcus laurentii*, oleaginous yeasts, biodiesel, single cell oil

1. Introduction

Ricotta cheese whey (RCW) is a by-product of dairy industry derived from ricotta cheese production. During this process, the whey is heated at 80-90 _ °C~~C~~ and generally added with organic acids and salts to induce the denaturation and consequent precipitation of whey proteins. The curd thus obtained is allowed to cool and filtered to separate the solid part (ricotta) from the liquid waste which is referred to as RCW (Lavarda, 1972).

Although the large majority of RCW is produced in the Mediterranean basin (about 1 Mton per year only in Italy) (Sansonetti et al., 2009), the manufacturing of ricotta cheese is also widespread in USA, where this product is often referred to as ricottone. Therefore, the technical hurdles related to either upgrading or disposal of this dairy byproduct are not only a regional issue. The typical composition of RCW is 4.8-5.0% lactose, 1.0-1.3% salts, 0.15-0.22% proteins, 0.20-0.25% organic acids and 0.20% fats with a COD ranging between 50.000 to 80.000 mg L^{-1} (Sansonetti et al., 2009). However, despite the presence of residual nutrients, the aforementioned presence of additives impairs its nutritional value and, consequently, its profitable use as a feed for livestock, causing additional costs for the dairy industry due to the need of its disposal. Moreover, RCW might cause a considerable environmental impact in case of an inappropriate disposal procedure.

Despite the appreciable sugar content in RCW which makes it a putative candidate as a growth medium in microbial production processes, only few studies have been conducted for this purpose. These studies include bio-ethanol production by *Kluyveromyces marxianus* (Sansonetti et al., 2009; Zoppellari and Bardi, 2013) and lactic acid production by mixed and pure bacterial cultures (Secchi et al., 2012). Most of research has focused instead on cheese whey, which, however, differs substantially from RCW, due to its lower concentration of salts and organic acids and higher content of whey proteins (Ykema et al., 1988; Takakuwa and Saito, 2010).

Microbial biodiesel production is among the most promising upgrading options of low cost feedstocks characterized by a high content in carbohydrates associated with low n~~N~~itrogen content. In this respect, RCW seems to meet ~~meets~~ these nutritional requirements. Biodiesel is a mixture of fatty acids alkyl monoesters derived from renewable resources such as higher plant oils, or, to a lesser extent, animal fats and waste cooking oil (Srivastava and Prasad, 2000) produced from transesterification reactions of triacylglycerides (TAG). Compared to conventional diesel, it has several advantages, being non-toxic, renewable and biodegradable. However, the rising costs of plant oils, from which the large majority of biodiesel is derived (around 95% of total world production), and the increasing demand for biofuels have given rise to concerns about land-use practices, increase of food price and oil production strategies (Atabani et al., 2012). Therefore, the use of microbial oils might represent a promising alternative to mitigate the problems associated with the ‘‘food vs. fuel’’ issue. Several microorganisms, belonging to yeasts, molds and microalgae, have the reported ability to accumulate intracellular lipids up to 70% (w/w) of their dry weight; the microbial fatty acid composition is often similar to that of vegetable oils used for biodiesel (Cristophe et al., 2012). Among them, oleaginous yeasts (OY) seem to be more suitable for an industrial production, being easy to cultivate, fast-growing and receptive to scale-up (Li et al., 2008).

Lipid accumulation in OY occurs through two different mechanisms depending on the nature of growth medium. “Ex-novo” synthesis is observed on hydrophobic substrates, while “de novo” synthesis occurs on sugar-based media in concomitance with a shortage of a key element, usually nitrogen.

In this study, the adequacy of RCW as a growth medium for the production of microbial oil was assessed. In fact, apparently the chemical composition of RCW characterized by high C/N ratios might be compatible with microbial “de novo synthesis” of lipids (Pirozzi et al., 2013). The exploitation of RCW in this direction would be in line with sustainability since it would enable a

partial replacement of edible oils as feedstock for biodiesel, giving back land use to food crops. Furthermore, in view of a possible development of a circular economy (Cicatiello et al., 2016), RCW upgrading would be beneficial for both dairy industries, due to a reduction of production costs, and for the environment since the spent medium derived from fermentation, would have a negligible organic load.

To this aim, a screening was initially performed with several OY belonging to well known lipid-producing species, some of which previously isolated from dairy products (Corbo et al., 2001), to assess their respective abilities to grow and produce lipids on that dairy byproduct and to determine productivities. The fatty acid methyl ester compositions derived from transesterification of lipids produced by some selected strains were analyzed and compared with well established feedstocks for biodiesel production. The most promising strain was then tested in a stirred tank reactor in view of a preliminary assessment of the process scale transfer feasibility.

2. Materials and methods

2.1 Microbial strains and maintenance

The strains under study were obtained from various culture collections or were isolated from environmental matrices and were chosen on the basis of their reported lipid accumulation ability on synthetic media. *Candida rugosa* NRRL Y-95, *Cryptococcus curvatus* NRRL Y-1511, *Lipomyces starkeii* NRRL 11557, *Rhodosporidium torouloides* NRRL Y-1091, *Rhodosporidium torouloides* NRRL Y-17902, *Trichosporon fermentans* NRRL Y-1492, *Yarrowia lipolytica* NRRL YB-423, *Y. lipolytica* NRRL Y-1095 and *Y. lipolytica* NRRL Y-7208 were provided by the ARS Culture Collection (NRRL, Peoria, IL). *Cryptococcus albidus* UCD 68-150, *C. albidus* UCD 68-174, *Cryptococcus laurentii* UCD 68-201, *Rhodotorula glutinis* UCD 68-255 and *Rhodotorula minuta* UCD 68-280 were obtained from the UCD Collection (Davis, California), while *Rhodotorula glutinis* DBVPG 3853 from DBVPG Collection (Perugia, Italy). *Pichia*

guilliermondii 1067 and *Pichia anomala* AN/4 were a kind gift of Prof. Cardinali (University of Perugia; Italy). *Pichia membranifaciens* 6C1 was isolated from olive brine (Crognale et al., 2012) and identified on the basis of its ITS sequence (GenBank Accession number JN900498).

During the study, the strains were maintained on potato dextrose agar (PDA) slants at 4°C and sub-cultured every month.

2.2 Growth medium

RCW was collected from a cheese processing plant (Formaggi Boccea S.r.l., Rome, Italy) and stored at -20 °C until used. RCW had the following characteristics (g L~~l~~$^{-1}$): dry weight, 48.2±4.10; Chemical Oxygen Demand (COD), 43.5±3.8; Total Organic Carbon (TOC), 16.3±1.4; lactose, 40.2±0.8; galactose, 1.6±0.2, total n~~N~~itrogen, 0.053±0.04; protein, 0.008±0.001; C/N, 307; ash, 4.5±0.4. Initial pH was 5.8. After thawing, the RCW was centrifuged (8000 x g, 15 min), two-fold diluted with deionized water, added with $(NH_4)_2SO_4$ so as to reach a C/N ratio of 55 and finally its pH was adjusted to 5.5 with 0.1 M NaOH.

2.3 Culture conditions

2.3.1 Shaken flask experiments

The microorganisms mentioned above were firstly screened in shaken flasks to select the ~~most performing oleaginous~~best strain in terms of biomass and lipid production. Regardless of the strain, each inoculum was obtained by suspending 72-h-old PDA slants with sterile physiological solution. Inocula were added to 250-mL~~l~~ Erlenmeyer flasks containing 50 mL~~l~~ of RCW-based medium so as to yield an initial value of optical density of 600 (OD_{600}) equal to 0.2. After inoculation, flasks were incubated at 30 °C in an orbital shaker (185 rpm) for 5 days. Samples were collected on a daily basis. All experiments were performed in triplicate. ~~Biomass ($Y_{X/S}$) and product ($Y_{P/S}$) yields were determined by relating yeast biomass and lipid production, respectively,~~

~~to the extent of total sugars consumed at the time of maximal lipid concentration. Specific product yield ($Y_{P/X}$) was calculated by relating lipid to biomass production.~~

2.3.2 Bioreactor experiments

Bioreactor experiments were performed in a 3-L~~l~~ jacketed bench-top stirred tank reactor (STR) (Applikon, Schiedam, NL) filled with 2 L~~l~~ of medium. The bioreactor was endowed with a top stirrer bearing two six-blade Rushton-type turbines (diameter 4.5 cm, blade width 1.4 cm, blade length 1.4 cm) and three baffles (width 1.4 cm). Air was introduced through a perforated pipe sparger located under the bottom turbine. The top plate was equipped with the following probes: dissolved oxygen and pH sensors (Applikon) and a PT 100 temperature sensor. Standard bioprocess conditions were as follows: impeller speed, 600 rpm (impeller tip speed = 141 cm s^{-1}); aeration rate, 1.5 vvm; temperature, 30 °C; initial dissolved oxygen concentration, 100% of saturation. Silicon Antifoam 289 (0.5 mL~~l~~ L~~l~~$^{-1}$) (Sigma Chemical Co., St Louis, MO, USA) was added before inoculation and an additional 1 mL~~l~~ L~~l~~$^{-1}$ was added when needed. The fermentation parameters (temperature, pH and dissolved oxygen) were monitored in bioreactors by an ADI 1030 (Applikon) adaptive/PID digital controller.

Pre-inoculum was grown at 30 °C in shaken flasks on Potato Dextrose Broth medium for 24 h, under orbital shaking (185 rpm) and added to the reactor so as to yield an initial value of OD_{600} equal to 0.2. Reactor experiments were performed in duplicate and culture samples collected every 12 h. Besides yields ($Y_{P/S}$, $Y_{P/X}$, $Y_{X/S}$)~~, described in subsection 2.3.1, kinetic parameters~~, such as specific growth rate (μ), lipid and biomass production rates (r_P and r_X, respectively) and N and total sugars consumption rates (r_N and r_S, respectively) were calculated, as described in subsection 2.4.~~.~~

2.4 Determination of yields and rates

The specific growth rate (μ) was calculated according to the following equation

$$\mu = \frac{1}{X} \cdot \frac{\delta X}{\delta t} \qquad (1)$$

where X is the biomass concentration (g L~~l~~$^{-1}$) at time t (h).

The biomass yield ($Y_{X/S}$) and product yield ($Y_{P/S}$) were calculated according to Equations (2) and (3), respectively:

$$Y_{X/S} = \frac{\Delta X}{\Delta S} \qquad (2)$$

$$Y_{P/S} = \frac{\Delta P}{\Delta S} \qquad (3)$$

where ΔX is the amount of biomass and ΔP is the product (either as total lipids or biodiesel calculated as the amounts of total FAME), respectively, and ΔS is the amount of substrate consumed.

~~where ΔX and ΔP are the amounts of biomass and lipids produced, respectively, and ΔS is the amount of substrate consumed.~~

The volumetric growth and production rates (r_X and r_P, respectively) were calculated according to Equations (4) and (5) by relating the amounts of biomass and ~~lipids~~biodiesel, respectively, to the time required to attain the lipid production peak (Δt):

$$r_X = \frac{\Delta X}{\Delta t} \qquad (4)$$

$$r_P = \frac{\Delta P}{\Delta t} \qquad (5)$$

Formatted: Not Highlight

substrate (r_S) and nitrogen (r_N) consumption rates were calculated by Equation (6) by relating the amounts of total sugars and nitrogen consumed, respectively, to the time required to attain the lipid production peak (Δt):

$$r_{[S,N]} = \frac{\Delta[S,N]}{\Delta t} \quad (6)$$

2.5 Analytical methods

Cell biomass was collected from 5 mL~~l~~-samples in pre-weighed Falcon tubes. The suspension was centrifuged at 8000 x g for 10 minutes and washed 3 times with distilled water. Dry cell weight was determined gravimetrically after lyophilisation for 48 h.

Total sugars content was determined by using the phenol-sulphuric acid method (Dubois et al., 1956). The concentrations of lactose and galactose were determined by ion-moderated partitioning chromatography in a Varian HPLC system equipped with an Aminex HPX 87-P column (Biorad Laboratories, Milan, IT). Samples were eluted with Milli-Q water (flow rate 0.6 mL~~l~~ min^{-1}) at 65 °C and the elution profile was monitored by an IR4 refractive index detector (Varian, Sunnyvale, CA). Determination of total lipids ~~content~~ was performed according to the method of Izard and Limberger (2003). COD and TOC were determined according to Standard Methods (APHA, 2005). Ashes were determined gravimetrically after 12-h ignition at 550 °C in a muffle furnace.

Nitrogen was determined by a modified Kjeldahl method (Domini et al., 2009). Digestions were carried out in a batch microwave digestion system (MarsXpress, CEM, Matthews, NC, USA) at 500 W power, 200 °C for 10 minutes by adding a mixture of 37% HCl (Carlo Erba Reagenti, Milan, Italy) and 30% H_2O_2 (Merck KGaA, Darmstadt, Germany) to 1 mL~~l~~ sample. Afterwards, nitrogen present in the form of ammonium was determined spectrophotometrically at 650 nm using the nitroprusside method described by Anderson and Ingram (1993).

Yeast cells were stained with Sudan Black B followed by counterstaining with Safranin to detect the presence of intracellular storage lipids as described by Ravikumar and collaborators (2012). Characterisation of lipid profiles was performed by a direct transesterification on lyophilised cells (Schutter and Dick, 2000) to obtain fatty acid methyl esters (FAMEs), which were analysed by a Master GC gas chromatograph (DANI Instrument SpA, Cologno Monzese, Italy) equipped with a Rxi-5MS (Restek, Germany) capillary column (0.25 mm id x 30 m length). FAMEs were eluted by using the following program: isothermal at 89 °C for 2 min; temperature gradient from 89 to 280 °C at a 6 °C $/$min^{-1}; hold at 280 °C for 5 min. The temperatures of the injector and flame ionization detector were set at 280 and 300 °C, respectively. Each FAME was identified by comparing its retention time with that of authentic standards contained in the FAME Mix C8-C24 (Sigma Aldrich, 18918-1AMP, USA). For quantification purposes, an internal standard (i.e., methyl nonadecanoate) was added to each sample prior to the transesterification. Biodiesel yield was calculated by relating the amounts of FAME to those of cellular lipids in dry biomass.

Formatted: Superscript

3. Results

3.1 Screening of yeast strains

Table 1 comparatively reports biomass and lipid productions and related yields calculated at the time (t) of maximal lipid accumulation. Regardless of the strain under study, the duration of the production process was brief with best lipid accumulation being observed between 48 and 96 h.

Among the tested strains, *C. curvatus* NRRL Y-1511 and *C. laurentii* UCD 68-201 proved to efficiently grow on RCW-based medium, achieving biomass productions of 10.77 and 7.28 g L^{-1}, respectively. The ability of the former and the latter strain to use RCW as a growth substrate was confirmed by marked reduction in COD (86.7 and 77.9%, respectively) (data not shown). *C. curvatus* was able to produce 6.83 g L^{-1} of intracellular lipids which amounted to 63% of biomass

dry weight, while *C. laurentii* achieved a slightly lower production level (5.06 g L~~l~~$^{-1}$) but with a higher yield with respect to biomass, amounting to 70%.

The other strains ~~tested~~ showed greater difficulties in adapting to this substrate but some of them, despite a low growth, were able to accumulate high percentages of total lipids with respect to biomass. In particular, ~~lipid accumulation referred to biomass (~~$Y_{P/X}$~~)~~ values in *L. starkeii* and *R. toruloides* NRRL Y-17902 amounted to 0.63 and 0.79 although their biomass productions were 0.79 and 0.64 g L~~l~~$^{-1}$, respectively (Table 1). Conversely, although *R. glutinis* UCD 68-255, *T. fermentans* and *P. membranifaciens* exhibited substantial growth in the RCW-based medium their relative $Y_{P/X}$ values were very low (i.e., 0.09, 0.20 and 0.13, respectively).

Thus, on the basis of the performance observed in shaken cultures, the determination of the lipid profiles was limited to some selected strains. Figure 1A, reporting the percent concentrations of each identified fatty acid with respect to total FAME~~lipids~~, shows that in all the strains considered, the main fatty acids were palmitic (C16:0), oleic (C18:1 Δ9), linoleic (C18:2 Δ9,12) and stearic (C18:0) acids, while the concentrations of polyunsaturated fatty acids (PUFAs), such as linolenic (C 18:3 Δ 9,12,15), eicosadienoic (C20:2 Δ11,14) and arachidonic (20:4 Δ5,8,11,14) acids, were either negligible or even absent. The FAME profiles of *C. curvatus*, *C. laurentii* and *L. starkeii* were rather similar while that of *R. toruloides* greatly differed from the others due to its higher content in total saturated (52.6%) and polyunsaturated (15%) fatty acids, as ~~it can be inferred from~~ shown in Figure 1A.

Although the biodiesel yields of *C. curvatus* and *C. laurentii* (35.77±3.51% and 27.1±4.80%, respectively) were lower than those of *R. toruloides* and *L. starkeii* (88.90±11.2% and 54.32±3.99%, respectively) (Figure 1B), ~~Taking into account~~ their volumetric lipid productions, ~~the~~ lipid accumulation capacities~~y~~ and ~~the~~ specific fatty acid profiles, ~~*C. curvatus* and *C. laurentii*~~ were ~~found to be the most~~deemed to be suitable ~~strains in view of~~for subsequent transfer of the process to the reactor scale. ~~Noteworthy, for both strains, the lipid production peak was attained~~

~~after 24 h from the depletion of N from the growth medium.~~ Although *C. curvatus* exhibited better production properties than *C. laurentii*, the latter was selected since a scant amount of information is currently available regarding its use for biodiesel production. A typical fermentation of *C. laurentii* in RCW medium in shaken flask is reported in Figure S1 and shows that maximal lipid accumulation took place after 24 h from the depletion of N from the growth medium. ~~-~~ Taking into account the ~~-~~lipid production peak (5.06 g L^{-1}) and the relative biodiesel yield (27.1%), the volumetric production of FAME derived from lipid transesterification was estimated to amount to 1.37 g L^{-1} (Figure 1B).

Formatted: Font: (Tipo di carattere testo asiati, Superscript

Formatted: Font: (Tipo di carattere testo asiati, Superscript

3.2 Reactor experiments with C. laurentii

To assess the feasibility of upscaling, the production process with *C. laurentii* was performed in a stirred tank reactor, as shown in Fig. 2.

In STR, nitrogen starvation occurred after only 36 h thus resulting in an anticipated peak of lipid production (9.93 g L^{-1} ~~)~~ at 60 h~~) from the inoculation~~. The lipid production peak coincided with the occurrence of ~~both~~ maximal biomass production (14.37 g ~~l~~L^{-1})~~ and lipid specific yield ($Y_{P/X}$ equal to 0.69) (Table 2)~~. ~~Moreover, the conversion efficiency of the substrate into product was significantly higher in STR than in shaken flask, with the respective $Y_{P/S}$ being 0.53 and 0.36. At that time, the average lipid productivity (r_P) amounted to 3.97 g l^{-1} d^{-1}, as shown in Table 2.~~ The decrease in total sugars concentration proceeded at an almost linear rate in the early 48 h to dramatically decline thereafter (Fig. 2A). As a consequence, the oxygen percent saturation, after a significant drop in the same time interval, tended to rise again to reach values which approached initial ones. Conversely, the pH did not significantly change throughout the process as shown in Fig. 2B. Noteworthy, at the lipid production peak, the soluble COD was reduced by 83.4% reaching values as low as 3600 mg L^{-1} (data not shown). Table 2 shows that at the time of maximal lipid accumulation, biodiesel yield amounted to 32.64±3.24%; on this basis, the maximal

volumetric amounts of total FAME derived from transesterification were estimated to be 3.24 g L^{-1}. As a result of this calculation, $Y_{P/X}$ and $Y_{P/S}$ were 0.23±0.01 and 0.17±0.01, respectively.

Table 2, reporting also the percent FAME composition of *C. laurentii* lipids obtained in STR, shows that the main fatty acids were oleic acid, linoleic and palmitic acids (47.2, 23.7 and 18.5%, respectively). Noteworthy, a significant change in FAME composition was observed in bioreactor as compared to shaken cultures, with a significant decrease in total saturated fatty acids (27.9 vs. 38.2%) and a 2.8-fold increase in linoleic acid (23.768 vs. 8.3%).

4. Discussion

The objective of this study was to investigate the feasibility of a second-generation biodiesel production by oleaginous yeasts grown on a dairy wastewater byproduct (i.e., RCW), for which, as opposed to cheese whey, there is a limited amount of information as a feedstock (Pirozzi et al., 2013; Castanha et al., 2014). Although both initial C/N ratio and absolute N amounts in the RCW-based medium might be putatively conducive to substantial yeast growth (Beopoulos et al., 2011), only 4 out the 18 strains under study were able to produce a biomass higher than 3 g L^{-1}. The strains that met this requirement were *R. glutinis* UCD 68-255, *T. fermentans* NRRL Y-1492 and two species belonging to the genus *Cryptococcus* (i.e., *C. curvatus* and *C. laurentii*). The *Cryptococcus* and *Trichosporon* genera encompass several species with lactose-assimilating ability. Thus, the failure of the remaining tested strains to substantially grow on RCW might be due to their weak or absent lactose-assimilating ability. In this respect, it may be noted here that lactose-assimilating capacity in yeasts is not very widespread (Frengova et al., 2004) and, in for some species, such as *L. starkeii* and *Kluyveromyces marxianus*, this feature has been shown to be strain-dependent (Slodki and Wickerham, 1966; Naumov, 2006). However, irrespective of their growth abilities on RCW, the large majority of the strains under study met the requirement of

Formatted: Superscript

Formatted: Line spacing: Double

oleaginicity accumulating intracellular lipids to larger than 20% of their cellular dry mass (Ratledge, 2002) with the only exceptions of *P. membranifaciens* 6C1, *C. rugosa* NRRL Y-95 and *R. glutinis* UCD 68-225.

C. curvatus NRLL Y-1511 was the best performing strain in the screening oin the RCW-based medium in terms of production, productivity and lipid accumulation capacity. However, this species is widely recognized as a good lipid producer and this attitude has been already tested on several low-cost substrates, including biodiesel-derived glycerol (Thiru et al., 2011; Tchakouteu et al., 2015), beet molasses (Takakuwa and Saito, 2010), olive mill wastewater (Yousuf et al., 2010), hydrolysates of sweet sorghum bagasse and wheat straw (Liang et al., 2012; Yu et al., 2014) and this attitude has been already tested on several substrates (Thiru et al., 2011, Yu et al., 2011, Liang et al., 2012) and even in a similar growth medium such as cheese whey (Seo et al., 2014). OnIn theat last substratestudy, which is highly related to that used in the present study, in particular, a maximum lipid productivity of 4.68 g L^{-1} d^{-1} was obtained. Conversely, the lipid-producing capacity of *C. laurentii* has not been investigated on whey-based media with a sole exception where another strain of this species was tested on cheese whey supplemented with sugarcane molasses (Castanha et al., 2014). In addition, this species deserves particular attention due to its remarkable ability to use a variety of carbon sources and to its tolerance to potential inhibitors of yeast growth (Sitepu et al., 2014). In the present study, lipid volumetric production and productivity of *C. laurentii* (5.06 g L^{-1} and 1.7 g L^{-1} d^{-1}) were significantly higher than those reported (2.96 g L^{-1} and 0.102 g L^{-1} d^{-1}) in the aforementioned study of Castanha and collaborators (2014) and these parameters were further improved at the reactor scale.

In fact, the process transfer from shaken flask to the STR was successful leading to remarkably improved biomass and lipid productions; on a volumetric basis, the amounts of FAME derived from STR were found to be 2.4-fold higher than in shaken cultures. yields and comparable specific yield. Moreover, the attainment of the lipid peak was anticipated with respect to shaken

flask thus resulting in higher productivity. This anticipation observed in the STR was due to a better mass transfer of both O_2 and nutrients thus leading to an earlier occurrence of nitrogen starvation in bioreactor, an event known to trigger lipid accumulation in oleaginous yeasts. In particular, when nitrogen ~~is limiting for biomass production~~depletion takes place, the residual carbon in the medium~~source~~ is readily converted to storage lipids ~~with the lipid yield increasing with increased C/N ratios~~ (Ratledge, 2002; Papanikolau and Aggelis, 2011). Noteworthy, the ~~performance~~ productivity observed in the present study was~~ere~~ higher than that~~ose~~ reported for *C. laurentii* DMKU-AmC14 on a glycerol-based medium ~~where lipid productivity and $Y_{P/X}$ amounted to 0.18 g l^{-1} d^{-1} and 0.28, respectively~~ (Polburee et al., 2015). Although a marked reduction in organic load was observed in the spent medium in concomitance with the lipid production peak, ~~a marked reduction in organic load was observed in the spent medium as compared to initial RCW,~~ residual COD values were well above the regulatory standards for effluent discharge into receiving water bodies. However, treatment costs of this spent medium in a wastewater treatment plant ~~the marked reductions in organic load~~ would ~~result undoubtedly i~~be significantly lower than those for RCW since, ~~a decrease in wastewater treatment costs which,~~ in the absence of other critical parameters (e.g., toxic pollutants, chromophoric substances etc.), ~~such as in the case of RCW,~~they mostly depend on residual COD rather than on the volumes conferred.

The determination of the lipid profile of *C. laurentii* indicated the predominance of 16- and 18-carbon chain saturated and monosaturated fatty acids in agreement with Castanha et al. (2014) and this was associated with a low content in PUFA. Noteworthy, the FAME profile of lipids obtained in the bioreactor significantly differed from that obtained in shaken flask with a concomitant increase in linoleic acid and decrease in total saturated fatty acids. In this respect, dissolved oxygen levels in the medium~~, which are influenced reportedly by both aeration and agitation,~~ can markedly modify the fatty acid profile produced by oleaginous microorganisms (Laoteng et al., 2011) and a decrease in unsaturated fatty acids concomitant to the reduction of

available oxygen was observed in *Saccharomyces cerevisiae* (Bardi et al., 1999) and *Apiotrichum curvatum* (Davies et al., 1990) cultures. This is not surprising since oxygen acts as the terminal electron acceptor in reactions catalyzed by fatty acid desaturases which play a key role in the synthesis of MUFA and PUFA (Lee et al., 2016); moreover, an oxygen-induced increase in the transcripts levels of desaturase genes was observed in *Mucor rouxii* cultures (Ruenwai et al., 2010). Although an increase in PUFA has been shown to be detrimental to the oxidative stability of the biodiesel (Jakeria et al., 2014), in the present study, the reactor-induced change led to a FAME composition which substantially resembled that of the Jatropha oil (Thiru et al., 2011), the use of which is well established in biodiesel production. It has been reported that oil sources containing high amounts of oleic acid and long chain saturated fatty acids would be ideal candidates for biodiesel purposes, because this composition positively impacts on biodiesel performance parameters, such as cetane number, kinematic viscosity, melting point, oxidative stability and heat of combustion (Knothe, 2005). In this respect, the FAME composition of *C. laurentii* appears to meet these requirements.

4. Conclusions

On the one hand, the screening performed with a variety of strains belonging to well known lipid-accumulating species confirmed that whey-related substrates, such as RCW, are often not adequate to support yeast growth since the large majority of carbon in this byproduct is found as lactose, the assimilation of which is not widespread among yeasts. Thus, a profitable use of this dairy byproduct as a feedstock for microbial production processes involving yeasts would require its pretreatment either by acid hydrolysis and subsequent pH correction or enzymatic hydrolysis with β-galactosidase which is commercially available at low costs. This might be beneficial to lipid production processes involving *L. starkeii* and *R. toruloides*, which despite a limited growth on RCW, maintained a significant lipid accumulation capacity and yielded FAME profiles which

were compatible with good biodiesel properties; studies are underway to assess this hypothesis. On the other hand, the successful transfer of the lipid production process of *C. laurentii* grown on RCW from shaken flask to STR offers wide and promising perspectives of further improvements. ~~Last but not least, the non-seasonal availability of RCW makes this byproduct a valuable feedstock ensuring a continuous supply.~~

Formatted: Line spacing: Double

References

Anderson, J.M., Ingram, J.S.I., 1993. Tropical Soil Biology and Fertility: A Handbook of Method. Second Edition CAB International, Wallingford, UK.

APHA, Standard Methods for the Examination of Water and Wastewater, 2005. 21th edition American Public Health Association, AWWA, WEF, Baltimore, USA.

Atabani, A.E., Silitonga, A.S., Badruddin, I.A., Mahlia, T.M.I., Masjuki, H.H., Mekhilef, S., 2012. A comprehensive review on biodiesel as an alternative energy resource and its characteristics. Renew. Sustain. Energy Rev. 16, 2070–2093. http://dx.doi.org/10.1016/j.rser.2012.01.003

Bardi, L., Cocito, C., Marzona, M., 1999. *Saccharomyces cerevisiae* cell fatty acid composition and release during fermentation without aeration and in absence of exogenous lipids. Int. J. Food Microbiol. 47, 133-140. http://dx.doi.org/10.1016/S0168-1605(98)00203-7

Beopoulos, A., Nicaud, J.M., Gaillardin, C., 2011. An overview of lipid metabolism in yeasts and its impact on biotechnological processes. Appl. Microbiol. Biotechnol. 90, 1193–1206. http://dx.doi.org/10.1007/s00253-011-3212-8.

Castanha, R.F., Mariano, A.P., de Morais, L.A.S., Scramin, S., Monteiro, R.T.R., 2014. Optimization of lipids production by *Cryptococcus laurentii* 11 using cheese whey with molasses. Brazilian J. Microbiol. 45, 379–387. http://dx.doi.org/10.1590/S1517-83822014000200003.

Formatted: English (United States)

Christophe, G., Kumar, V., Nouaille, R., Gaudet, G., Fontanille, P., Pandey, A., Soccol, C.R., Larroche, C., 2012. Recent developments in microbial oils production: A possible alternative to vegetable oils for biodiesel without competition with human food? Brazilian Arch. Biol. Technol. 55, 29–46. http://dx.doi.org/10.1590/S1516-89132012000100004.

Cicatiello C., Franco S., Pancino B., Blasi E., 2016. The value of food waste: An exploratory study on retailing. Journal of. Retailing Retail, and Consum.er Serv.ices 30, 96-104. http://dx.doi.org/10.1016/j.jretconser.2016.01.004.

Corbo, M.R., Lanciotti, R., Albenzio, M., Sinigaglia, M., 2001. Occurrence and characterization of yeasts isolated from milks and dairy products of Apulia region. Int. J. Food Microbiol. 69, 147-152. http://dx.doi.org/10.1016/S0168-1605(01)00585-2.

Crognale, S., Pesciaroli, L., Petruccioli, M., D'Annibale, A., 2012. Phenoloxidase-producing halotolerant fungi from olive brine wastewater. Process Biochem. 47, 1433-1437. http://dx.doi.org/10.1016/j.procbio.2012.05.014.

Davies, R.J., Holdsworth, J.E., Reader, S.L., 1990. The effect of low oxygen uptake rate on the fatty acid profile of the oleaginous yeast *Apiotrichum curvatum*. Appl. Microbiol. Biotechnol. 33, 569–573. http://dx.doi.org/10.1007/BF00172553.

Domini, C., Vidal, L., Cravotto, G., Canals, A., 2009. A simultaneous, direct microwave/ultrasound-assisted digestion procedure for the determination of total Kjeldahl nitrogen. Ultrason. Sonochem. 16, 564–569. http://dx.doi.org/10.1016/j.ultsonch.2008.12.006.

Dubois, M., Gilles, K., Hamilton, J., Rebers, P., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350–356. http://dx.doi.org/10.1021/ac60111a017.

Frengova G, Simova E, Beshkova D. 2004. Use of whey ultrafiltrate as a substrate for production of carotenoids by the yeast *Rhodotorula rubra*. Appl. Biochem. Biotechnol 112.: 133-141. http://dx.doi.org/10.1385/ABAB:112:3:133.

Formatted: Justified, Space Before: 0 pt, After: 0 pt
Formatted: English (United States)
Formatted: English (United States)
Formatted: English (United States)
Formatted: No underline, Font color: Auto, English (United States)
Formatted: English (United States)
Formatted: No underline, Font color: Auto, English (United States)
Formatted: No underline, Font color: Auto, Italian (Italy)
Formatted: No underline, Font color: Auto, Italian (Italy)
Formatted: No underline, Font color: Auto, Italian (Italy)
Formatted: No underline, Font color: Auto, Italian (Italy)
Formatted: No underline, Font color: Auto
Formatted: No underline, Font color: Auto, Italian (Italy)
Formatted: Italian (Italy)
Formatted: No underline, Font color: Auto
Formatted: No underline, Font color: Auto
Formatted: No underline, Font color: Auto, English (United States)
Formatted: English (United States)
Formatted: No underline, Font color: Auto, English (United States)
Formatted: English (United States)
Formatted: No underline, Font color: Auto, English (United States)
Formatted: No underline, Font color: Auto
Formatted: No underline, Font color: Auto

Izard, J., Limberger, R.J., 2003. Rapid screening method for quantitation of bacterial cell lipids from whole cells. J. Microbiol. Methods 55, 411–418. http://dx.doi.org/10.1016/s0167-7012(03)00193-3.

Jakeria, M.R., Fazal, M.A., Haseeb, A.S.M.A., 2014. Influence of different factors on the stability of biodiesel: A review. Renew. Sustain. Energy Rev. 30, 154–163. http://dx.doi.org/10.1016/j.rser.2013.09.024

Knothe, G., 2005. Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. Fuel Process. Technol. 86, 1059–1070. http://dx.doi.org/10.1016/j.fuproc.2004.11.002.

Laoteng, K., Čertík, M., Cheevadhanark, S., 2011. Mechanisms controlling lipid accumulation and polyunsaturated fatty acid synthesis in oleaginous fungi. Chemical Papers 65, 97-103. http://dx.doi.org/10.2478/s11696-010-0097-4.

Lavarda, J. 1972. Preparation of ricotta cheese curd. United States Patent 3,704,136 A. 1972 Nov 28.

Lee, J.M., Lee, H., Kang, S., Park, W. J., 2016. Fatty aAcid dDesaturases, pPolyunsaturated fFatty aAcid rRegulation, and bBiotechnological aAdvances. Nutrients 8, 23. http://dx.doi.org/10.3390/nu8010023.

Li, Q., Du, W., Liu, D., 2008. Perspectives of microbial oils for biodiesel production. Appl. Microbiol. Biotechnol. 80, 749–756. http://dx.doi.org/10.1007/s00253-008-1625-9 .

Liang, Y., Tang, T., Siddaramu, T., Choudhary, R., Umagiliyage, A.L., 2012. Lipid production from sweet sorghum bagasse through yeast fermentation. Renew. Energ 40:130-136. http://dx.doi.org/10.1016/j.renene.2011.09.035.

Naumov, G.I., 2006. Genetics of lactose utilization polymorphism in the yeast *Kluyveromyces marxianus*. Doklady Biol.ogical Sci.ences 409, 317-319. http://dx.doi.org/10.1134/S0012496606040144.

Formatted: No underline, Font color: Auto, English (United States)

Formatted: No underline, Font color: Auto

Formatted: English (United States)

Formatted: No underline, Font color: Auto, English (United States)

Formatted: No underline, Font color: Auto, English (United States)

Papanikolaou, S., Aggelis, G., 2011. Lipids of oleaginous yeasts. Part I: Biochemistry of single cell oil production. Eur. J. Lipid Sci. Technol. 113, 1031-1051. http://dx.doi.org/10.1002/ejlt.201100014.

Pirozzi, D., Ausiello, A., Zuccaro, G., Sannino, F., Yousuf, A., 2013. Culture of oleaginous yeasts in dairy industry wastewaters to obtain lipids suitable for the production of II-generation Biodiesel. In: Proceedings of World Academy of Science, Engineering and Technology (No. 76, p. 57). World Academy of Science, Engineering and Technology (WASET).

Polburee, P., Yongmanitchai, W., Lertwattanasakul, N., Ohashi, T., Fujiyama, K., Limtong, S., 2015. Characterization of oleaginous yeasts accumulating high levels of lipid when cultivated in glycerol and their potential for lipid production from biodiesel-derived crude glycerol. Fungal Biol. 119, 1194-1204. http://dx.doi.org/10.1016/j.funbio.2015.09.002.

Ravikumar, K., Dakshayini, J., Girisha, S.T., 2012. Biodiesel production from oleaginous fungi. Int. J. Life Sci. 6, 43-49. http://dx.doi.org/10.3126/ijls.v6i1.5721

Ratledge, C. (2002). Regulation of lipid accumulation in oleaginous micro-organisms. Biochem. Soc. Trans. 30(6), 1047-1050. http://dx.doi.org/10.1042/bst0301047.

Ruenwai, R., Cheevadhanarak, S., Rachdawong, S., Tanticharoen,M., Laoteng, K., 2010. Oxygen-induced expression of Δ^6-, Δ^9- and Δ^{12}-desaturase genes modulates fatty acid composition in *Mucor rouxii*. Appl. Microbiol. Biotechnol. 86, 327–334. http://dx.doi.org/10.1007/s00253-009-2338-4.

Sansonetti, S., Curcio, S., Calabrò, V., Iorio, G., 2009. Bio-ethanol production by fermentation of ricotta cheese whey as an effective alternative non-vegetable source. Biomass and Bioenergy 33, 1687–1692. http://dx.doi.org/10.1016/j.biombioe.2009.09.002.

Formatted: No underline, Font color: Auto

Formatted: No underline, Font color: Auto

Formatted: No underline, Font color: Auto

Formatted: No underline, Font color: Auto

Formatted: Left, Space Before: 0 pt, After: 0 pt

Formatted: No underline, Font color: Auto

Formatted: No underline, Font color: Auto

Schutter, M.E., Dick, R.P., 2000. Comparison of fFatty aAcid mMethyl eEster (FAME) mMethods for cCharacterizing mMicrobial cCommunities. Soil Sci. Soc. Am. J. 64, 1659–1668. http://dx.doi.org/10.2136/sssaj2000.6451659x.

Secchi, N., Giunta, D., Pretti, L., García, M.R., Roggio, T., Mannazzu, I., Catzeddu, P., 2012. Bioconversion of ovine scotta into lactic acid with pure and mixed cultures of lactic acid bacteria. J. Ind. Microbiol. Biotechnol. 39, 175–181. http://dx.doi.org/10.1007/s10295-011-1013-9.

Seo, Y.H., Lee, I., Jeon, S.H., Han, J.I., 2014. Efficient conversion from cheese whey to lipid using *Cryptococcus curvatus*. Biochem. Eng. J. 90, 149–153. http://dx.doi.org/10.1016/j.bej.2014.05.018

Sitepu, I., Selby, T., Lin, T., Zhu, S., Boundy-Mills, K., 2014. Carbon source utilization and inhibitor tolerance of 45 oleaginous yeast species. J. Ind. Microbiol. Biotechnol. 41, 1061-1070. http://dx.doi.org/10.1007/s10295-014-1447-y.

Slodki, M.E., Wickerham, L.J. 1966. Extracellular polysaccharides and classification of the genus Lipomyces. Microbiology, 42(3), 381-385. http://dx.doi.org/10.1099/00221287-42-3-381.

Srivastava, A., Prasad, R., 2000. Triglycerides-based diesel fuels. Renew. Sustain. Eenerg.y Rev. 4, 111–133. http://dx.doi.org/10.1016/S1364-0321(99)00013-1

Tchakouteu, S.S., Kalantzi, O., Gardeli, C., Koutinas, A.A., Aggelis, G., Papanikolaou, S., 2015. Lipid production by yeasts growing on biodiesel-derived crude glycerol: strain selection and impact of substrate concentration on the fermentation efficiency. J. Appl. Microbiol. 118, 911-927. http://dx.doi.org/10.1111/jam.12736

~~Zoppellari, F., Bardi, L., 2013. Production of bioethanol from effluents of the dairy industry by *Kluyveromyces marxianus*. New Biotechnol. 30, 607–613. http://dx.doi.org/10.1016/j.nbt.2012.11.017~~

Formatted: No underline, Font color: Auto, English (United States)

Formatted: No underline, Font color: Auto

Formatted: Font: Not Italic, No underline, Font color: Auto

Formatted: Font: Not Italic, No underline, Font color: Auto

Formatted: No underline, Font color: Auto, Italian (Italy)

Formatted: No underline, Font color: Auto

Formatted: Italian (Italy)

Formatted: No underline, Font color: Auto, Italian (Italy)

Formatted: Justified

Takakuwa, N., Saito, K., 2010. Conversion of beet molasses and cheese whey into fatty acid methyl esters by the yeast *Cryptococcus curvatus*. J. Oleo Sci. 59, 255-260. http://dx.doi.org/10.5650/jos.59.255.

Thiru, M., Sankh, S., Rangaswamy, V., 2011. Process for biodiesel production from *Cryptococcus curvatus*. Bioresour. Technol. 102, 10436-10440. http://dx.doi.org/10.1016/j.biortech.2011.08.102.

Ykema, A., Verbree, E.C., Kater, M.M., Smit, H., 1988. Optimization of lipid production in the oleaginous yeast *Apiotricum curvatum* in whey permeate. Appl. Microbiol. Biotechnol. 29:211-218. http://dx.doi.org/10.1007/BF00251704.

Yousuf, A., Sannino, F., Addorisio, V., Pirozzi, D., 2010. Microbial conversion of olive oil mill wastewaters into lipids suitable for biodiesel production. J. Agric. Food Chem. 58, 8630-8635. http://dx.doi.org/10.1021/jf101282t

Yu, X., Zeng, J., Zheng, Y., Chen, S., 2014. Effect of lignocellulose degradation products on microbial biomass and lipid production by the oleaginous yeast *Cryptococcus curvatus*. Process Biochem. 49, 457-465. http://dx.doi.org/10.1016/j.procbio.2013.10.016

Zoppellari, F., Bardi, L., 2013. Production of bioethanol from effluents of the dairy industry by *Kluyveromyces marxianus*. New Biotechnol. 30, 607–613. http://dx.doi.org/10.1016/j.nbt.2012.11.017

~~Yu, X., Zheng, Y., Dorgan, K.M., Chen, S. 2011. Oil production by oleaginous yeasts using the hydrolysate from pretreatment of wheat straw with dilute sulfuric acid. Bioresour Technol 102:6134-6140. http://dx.doi.org/10.1016/j.biortech.2011.02.081.~~

Formatted: No underline, Font color: Auto, English (United Kingdom)

Formatted: No underline, Font color: Auto, English (United Kingdom)

Formatted: No underline, Font color: Auto, English (United Kingdom)

Formatted: English (United Kingdom)

Formatted: No underline, Font color: Auto, English (United Kingdom)

Formatted: English (United Kingdom)

Formatted: No underline, Font color: Auto, English (United States)

Formatted: No underline, Font color: Auto

Formatted: Font: Italic, No underline, Font color: Auto

Formatted: No underline, Font color: Auto, Swedish (Sweden)

Formatted: No underline, Font color: Auto

Formatted: Swedish (Sweden)

Formatted: No underline, Font color: Auto, Swedish (Sweden)

Formatted: Justified

Figure legends

Figure 1. Percent compositions of fatty methyl esters (FAMEs) derived from lipids produced by *C. curvatus* NRRL Y-1511, *C. laurentii* UCD 68-201, *L. starkeii* NRRL 11557 and *R. toruloides* Y-17~~0~~9021~~1~~ grown in shaken flask on the RCW-based medium (A) and biodiesel yields (%) and FAME amounts obtained from lipid transesterification and referred to unit volume of culture broth (B). Data are referred to the time of maximal lipid accumulation and are the means of six chromatographic runs (2 technical replicates for each independent culture carried out in triplicate). Abbreviations: 16:1, palmitoleic acid; 16:0, palmitic acid; 18:2, linoleic acid; 18:1 oleic acid; 18:0, stearic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Figure 2. Time courses of lipid production and residual nitrogen and total sugar concentrations (Plot A) and biomass production, pH and dissolved oxygen (Plot B) in *C. laurentii* UCD 68-201 cultures grown in a 3-L~~l~~ stirred tank reactor (impeller speed, 600 rpm; aeration rate, 1.5 vvm). Data are the means ± standard deviations of duplicate reactor experiments. Inset in plot A contains a micrograph of 72-h-old cells stained according to Ravikumar et al. (2012) to detect the presence of storage lipid bodies.

Figure S1. Time courses of lipid and biomass productions and concentrations of residual nitrogen and total sugars in *C. laurentii* UCD 68-201 cultures grown in shaken flask. Data are the means ± standard deviations of triplicate cultures.

Table 1. Biomass (X) and lipid (P) productions, yield parameters ($Y_{X/S}$, $Y_{P/X}$, $Y_{P/S}$) and time of maximal lipid accumulation (t) obtained for each of the 18 yeast strains grown on RCW-based medium.

	X (g L^{-1})	P (g L^{-1})	$Y_{X/S}$	$Y_{P/X}$§	$Y_{P/S}$§	t (h)
C. rugosa NRRL Y-95	1.74±0.01	0.30±0.01	0.13±0.01	0.17±0.01	0.02±0.00	72
C. albidus UCD 68-150	2.00±0.07	0.72±0.10	0.15±0.01	0.36±0.06	0.06±0.01	72
C. albidus UCD 68-174	0.71±0.13	0.35±0.02	0.06±0.01	0.49±0.04	0.03±0.00	48
C. curvatus NRRL Y-1511	10.77±0.21	6.83±0.14	0.5084±0.04	0.63±0.02	0.3753±0.02	72
C. laurentii UCD 68-201	7.28±0.10	5.06±0.28	0.52±0.02	0.70±0.05	0.36±0.03	72
L. starkeii NRRL 11557	0.79±0.10	0.50±0.07	0.08±0.01	0.63±0.14	0.05±0.01	96
P. anomala AN/4	1.50±0.06	0.34±0.01	0.10±0.01	0.23±0.01	0.02±0.00	72
P. guilliermondii 1067	0.74±0.00	0.35±0.02	0.05±0.00	0.48±0.03	0.02±0.00	72
P. membranifaciens 6C1	2.71±0.20	0.34±0.05	0.31±0.03	0.13±0.03	0.04±0.01	72
R. glutinis DBVPG 3853	0.95±0.05	0.31±0.04	0.07±0.01	0.32±0.06	0.02±0.00	72
R. glutinis UCD 68-255	3.03±0.15	0.27±0.01	0.21±0.01	0.09±0.01	0.02±0.00	72
R. minuta UCD 68-280	0.66±0.03	0.37±0.03	0.06±0.00	0.56±0.07	0.03±0.00	72
R. toruloides NRRL 1091	0.86±0.07	0.41±0.03	0.06±0.01	0.48±0.07	0.03±0.00	72
R. torouloides NRRL Y-17902	0.64±0.03	0.51±0.01	0.05±0.00	0.79±0.05	0.04±0.00	96
T. fermentans NRRL Y-1492	3.50±0.22	0.69±0.06	0.32±0.03	0.20±0.03	0.06±0.01	48
Y. lipolytica NRRL YB-423	1.15±0.19	0.38±0.04	0.08±0.01	0.33±0.09	0.03±0.00	72
Y. lipolytica NRRL Y-1095	1.56±0.08	0.39±0.02	0.18±0.01	0.25±0.03	0.05±0.00	72
Y. lipolytica NRRL Y-7208	1.35±0.15	0.38±0.01	0.18±0.02	0.28±0.02	0.05±0.01	72

$Y_{X/S}$=biomass yield; $Y_{P/X}$= specific lipid yield; $Y_{P/S}$= lipid yield referred to consumed sugars; § Calculated by relating total lipids, determined according to Izard and Limberger (2003), to the amounts of total sugars consumed.

Table 2. Performance indicators of ~~lipid~~ biodiesel production process in STR by *C. laurentii* including yields (Biodiesel yield, $Y_{P/S}$, $Y_{P/X}$, $Y_{X/S}$) and biodiesel~~lipid~~ and biomass production rates (r_P and r_X, respectively), N and total sugars consumption rates (r_N, r_S) specific growth rate (μ) and percent fatty acid composition. All values have been calculated at the time of maximal lipid production.

	Dimension unit	Value
~~$Y_{P/S}$~~	-	~~0.53±0.03~~
	(%)	32.64±3.24
	(g g^{-1})	0.2369±0.016
~~$Y_{X/S}$~~	-	~~0.76±0.02~~
	(g g^{-1})	0.17±0.01
	(g g^{-1})	0.65±0.02
	(g L $^{-1}$ d^{-1})	13.3097±0.0928
	(g L $^{-1}$ h^{-1})	0.31±0.00
	(mg L $^{-1}$ h^{-1})	2.49±0.00
	(g L $^{-1}$ h^{-1})	0.24±0.00
	(h^{-1})	0.02±0.00
	(%)	0.39±0.05
	(%)%	18.53±1.24
	(%)%	23.47±1.01
	(%)%	47.16±0.97
	(%)%	5.45±0.28
A)	(%)%	3.93±0.73
MUFA)	(%)%	0.30±0.07
PUFA)	(%)%	0.77±0.14
	(%)%	27.91±2.25
	(%)%	47.85±1.09
	(%)%	24.24±1.15

§Data are the means ± standard deviations of duplicate reactor experiments; ǂ Calculated on the basis of the biodiesel yield; †Data are the means ± standard

Formatted: No underline, Superscript
Formatted: No underline, Not Superscript/ Subscript
Formatted: No underline, Not Superscript/ Subscript
Formatted: No underline, Not Superscript/ Subscript
Formatted: No underline, Not Superscript/ Subscript
Formatted: No underline, Not Superscript/ Subscript
Formatted: Line spacing: single

deviations of 4 chromatographic runs (2 technical replicates for each reactor experiment); ‡ Predominant fatty acids are listed as a function of increasing retention time.

A sustainable use of Ricotta Cheese Whey for microbial biodiesel production

Carota, Eleonora; Crognale, Silvia; D'Annibale, Alessandro*; Gallo, Anna Maria; Stazi, Silvia Rita; Petruccioli, Maurizio

Department for Innovation in Biological, Agro-food and Forest Systems (DIBAF), University of Tuscia, Via S. Camillo De Lellis, 01100 Viterbo, Italy

(*) Correspondence should be addressed to:
D'Annibale, Alessandro, PhD
Department for Innovation in Biological, Agro-food and Forest Systems (DIBAF)
University of Tuscia, Via S. Camillo De Lellis, 01100 Viterbo, Italy
Phone: ++390761357368
E-mail: dannib@unitus.it

Abstract

The increasing demand of plant oils for biodiesel production has highlighted the need for alternative strategies based either on non-food crops or agro-industrial wastes that do not compete with food and feed production. In this context, the combined use of wastewater and oleaginous microorganisms could be a valuable production option. Ricotta cheese whey (RCW), one of the major byproducts of the dairy industry, is produced in very high and steadily increasing amounts and, due to its high organic load, its disposal is cost-prohibitive.

In the present study, in order to assess the adequacy of RCW as a growth medium for lipid production, 18 strains of oleaginous yeasts were investigated in shaken flask for their growth and lipid-producing capabilities on this substrate. Among them, *Cryptococcus curvatus* NRRL Y-1511 and *Cryptococcus laurentii* UCD 68-201 adequately grew therein producing substantial amounts of lipids (6.8 and 5.1 g L^{-1}, respectively). A high similarity between the percent fatty acid methyl esters (FAME) composition of lipids from the former and the latter strain was found with a predominance of oleic acid (52.8 vs. 48.7%) and of total saturated fatty acids (37.9 vs. 40.8%). The subsequent scale transfer of the *C. laurentii* UCD 68-201 lipid production process on RCW to a 3-L STR led to significantly improved biomass and total lipid productions (14.4 and 9.9 g L^{-1}, respectively) with the biodiesel yield amounting to 32.6%. Although the *C. laurentii* FAME profile was modified upon process transfer, it resembled that of the Jatropha oil, a well established feedstock for biodiesel production. In conclusion, *C. laurentii* UCD 68-201, for which there is very limited amount of available information, turned out to be a very promising candidate for biodiesel production and wide margins of process improvement might be envisaged.

Keywords:

Ricotta Cheese Whey, *Cryptococcus laurentii*, oleaginous yeasts, biodiesel, single cell oil

1. Introduction

Ricotta cheese whey (RCW) is a by-product of dairy industry derived from ricotta cheese production. During this process, the whey is heated at 80-90 °C and generally added with organic acids and salts to induce the denaturation and consequent precipitation of whey proteins. The curd thus obtained is allowed to cool and filtered to separate the solid part (ricotta) from the liquid waste which is referred to as RCW (Lavarda, 1972).

Although the large majority of RCW is produced in the Mediterranean basin (about 1 Mton per year only in Italy) (Sansonetti et al., 2009), the manufacturing of ricotta cheese is also widespread in USA, where this product is often referred to as ricottone. Therefore, the technical hurdles related to either upgrading or disposal of this dairy byproduct are not only a regional issue. The typical composition of RCW is 4.8-5.0% lactose, 1.0-1.3% salts, 0.15-0.22% proteins, 0.20-0.25% organic acids and 0.20% fats with a COD ranging between 50.000 to 80.000 mg L^{-1} (Sansonetti et al., 2009). However, despite the presence of residual nutrients, the aforementioned presence of additives impairs its nutritional value and, consequently, its profitable use as a feed for livestock, causing additional costs for the dairy industry due to the need of its disposal. Moreover, RCW might cause a considerable environmental impact in case of an inappropriate disposal procedure.

Despite the appreciable sugar content in RCW which makes it a putative candidate as a growth medium in microbial production processes, only few studies have been conducted for this purpose. These studies include bio-ethanol production by *Kluyveromyces marxianus* (Sansonetti et al., 2009; Zoppellari and Bardi, 2013) and lactic acid production by mixed and pure bacterial cultures (Secchi et al., 2012). Most of research has focused instead on cheese whey, which, however, differs substantially from RCW, due to its lower concentration of salts and organic acids and higher content of whey proteins (Ykema et al., 1988; Takakuwa and Saito, 2010).

Microbial biodiesel production is among the most promising upgrading options of low cost feedstocks characterized by a high content in carbohydrates associated with low nitrogen content. In this respect, RCW seems to meet these nutritional requirements. Biodiesel is a mixture of fatty acids alkyl monoesters derived from renewable resources such as higher plant oils, or, to a lesser extent, animal fats and waste cooking oil (Srivastava and Prasad, 2000) produced from transesterification reactions of triacylglycerides (TAG). Compared to conventional diesel, it has several advantages, being non-toxic, renewable and biodegradable. However, the rising costs of plant oils, from which the large majority of biodiesel is derived (around 95% of total world production), and the increasing demand for biofuels have given rise to concerns about land-use practices, increase of food price and oil production strategies (Atabani et al., 2012). Therefore, the use of microbial oils might represent a promising alternative to mitigate the problems associated with the ‘‘food vs. fuel’’ issue. Several microorganisms, belonging to yeasts, molds and microalgae, have the reported ability to accumulate intracellular lipids up to 70% (w/w) of their dry weight; the microbial fatty acid composition is often similar to that of vegetable oils used for biodiesel (Cristophe et al., 2012). Among them, oleaginous yeasts (OY) seem to be more suitable for an industrial production, being easy to cultivate, fast-growing and receptive to scale-up (Li et al., 2008).

Lipid accumulation in OY occurs through two different mechanisms depending on the nature of growth medium. “Ex-novo” synthesis is observed on hydrophobic substrates, while “de novo” synthesis occurs on sugar-based media in concomitance with a shortage of a key element, usually nitrogen.

In this study, the adequacy of RCW as a growth medium for the production of microbial oil was assessed. In fact, apparently the chemical composition of RCW characterized by high C/N ratios might be compatible with microbial “de novo synthesis” of lipids (Pirozzi et al., 2013). The exploitation of RCW in this direction would be in line with sustainability since it would enable a

partial replacement of edible oils as feedstock for biodiesel, giving back land use to food crops. Furthermore, in view of a possible development of a circular economy (Cicatiello et al., 2016), RCW upgrading would be beneficial for both dairy industries, due to a reduction of production costs, and for the environment since the spent medium derived from fermentation, would have a negligible organic load.

To this aim, a screening was initially performed with several OY belonging to well known lipid-producing species, some of which previously isolated from dairy products (Corbo et al., 2001), to assess their respective abilities to grow and produce lipids on that dairy byproduct and to determine productivities. The fatty acid methyl ester compositions derived from transesterification of lipids produced by some selected strains were analyzed and compared with well established feedstocks for biodiesel production. The most promising strain was then tested in a stirred tank reactor in view of a preliminary assessment of the process scale transfer feasibility.

2. Materials and methods

2.1 Microbial strains and maintenance

The strains under study were obtained from various culture collections or were isolated from environmental matrices and were chosen on the basis of their reported lipid accumulation ability on synthetic media. *Candida rugosa* NRRL Y-95, *Cryptococcus curvatus* NRRL Y-1511, *Lipomyces starkeii* NRRL 11557, *Rhodosporidium torouloides* NRRL Y-1091, *Rhodosporidium torouloides* NRRL Y-17902, *Trichosporon fermentans* NRRL Y-1492, *Yarrowia lipolytica* NRRL YB-423, *Y. lipolytica* NRRL Y-1095 and *Y. lipolytica* NRRL Y-7208 were provided by the ARS Culture Collection (NRRL, Peoria, IL). *Cryptococcus albidus* UCD 68-150, *C. albidus* UCD 68-174, *Cryptococcus laurentii* UCD 68-201, *Rhodotorula glutinis* UCD 68-255 and *Rhodotorula minuta* UCD 68-280 were obtained from the UCD Collection (Davis, California), while *Rhodotorula glutinis* DBVPG 3853 from DBVPG Collection (Perugia, Italy). *Pichia*

guilliermondii 1067 and *Pichia anomala* AN/4 were a kind gift of Prof. Cardinali (University of Perugia; Italy). *Pichia membranifaciens* 6C1 was isolated from olive brine (Crognale et al., 2012) and identified on the basis of its ITS sequence (GenBank Accession number JN900498).

During the study, the strains were maintained on potato dextrose agar (PDA) slants at 4°C and sub-cultured every month.

2.2 Growth medium

RCW was collected from a cheese processing plant (Formaggi Boccea S.r.l., Rome, Italy) and stored at -20 °C until used. RCW had the following characteristics (g L^{-1}): dry weight, 48.2±4.10; Chemical Oxygen Demand (COD), 43.5±3.8; Total Organic Carbon (TOC), 16.3±1.4; lactose, 40.2±0.8; galactose, 1.6±0.2, total nitrogen, 0.053±0.04; protein, 0.008±0.001; C/N, 307; ash, 4.5±0.4. Initial pH was 5.8. After thawing, the RCW was centrifuged (8000 x g, 15 min), two-fold diluted with deionized water, added with $(NH_4)_2SO_4$ so as to reach a C/N ratio of 55 and finally its pH was adjusted to 5.5 with 0.1 M NaOH.

2.3 Culture conditions

2.3.1 Shaken flask experiments

The microorganisms mentioned above were firstly screened in shaken flasks to select the best strain in terms of biomass and lipid production. Regardless of the strain, each inoculum was obtained by suspending 72-h-old PDA slants with sterile physiological solution. Inocula were added to 250-mL Erlenmeyer flasks containing 50 mL of RCW-based medium so as to yield an initial value of optical density of 600 (OD_{600}) equal to 0.2. After inoculation, flasks were incubated at 30 °C in an orbital shaker (185 rpm) for 5 days. Samples were collected on a daily basis. All experiments were performed in triplicate.

147 *2.3.2 Bioreactor experiments*

148 Bioreactor experiments were performed in a 3-L jacketed bench-top stirred tank reactor (STR)
149 (Applikon, Schiedam, NL) filled with 2 L of medium. The bioreactor was endowed with a top
150 stirrer bearing two six-blade Rushton-type turbines (diameter 4.5 cm, blade width 1.4 cm, blade
151 length 1.4 cm) and three baffles (width 1.4 cm). Air was introduced through a perforated pipe
152 sparger located under the bottom turbine. The top plate was equipped with the following probes:
153 dissolved oxygen and pH sensors (Applikon) and a PT 100 temperature sensor. Standard
154 bioprocess conditions were as follows: impeller speed, 600 rpm (impeller tip speed = 141 cm s^{-1});
155 aeration rate, 1.5 vvm; temperature, 30 °C; initial dissolved oxygen concentration, 100% of
156 saturation. Silicon Antifoam 289 (0.5 mL L^{-1}) (Sigma Chemical Co., St Louis, MO, USA) was
157 added before inoculation and an additional 1 mL L^{-1} was added when needed. The fermentation
158 parameters (temperature, pH and dissolved oxygen) were monitored in bioreactors by an ADI 1030
159 (Applikon) adaptive/PID digital controller.
160 Pre-inoculum was grown at 30 °C in shaken flasks on Potato Dextrose Broth medium for 24 h,
161 under orbital shaking (185 rpm) and added to the reactor so as to yield an initial value of OD_{600}
162 equal to 0.2. Reactor experiments were performed in duplicate and culture samples collected every
163 12 h. Besides yields ($Y_{P/S}$, $Y_{P/X}$, $Y_{X/S}$), kinetic parameters, such as specific growth rate (μ), lipid
164 and biomass production rates (r_P and r_X, respectively) and N and total sugars consumption rates (r_N
165 and r_S, respectively) were calculated, as described in subsection 2.4.
166

167 *2.4 Determination of yields and rates*

168 The specific growth rate (μ) was calculated according to the following equation

169 $$\mu = \frac{1}{X} \cdot \frac{\delta X}{\delta t} \qquad (1)$$

170 where X is the biomass concentration (g L^{-1}) at time t (h).

The biomass yield ($Y_{X/S}$) and product yield ($Y_{P/S}$) were calculated according to Equations (2) and (3), respectively:

$$Y_{X/S} = \frac{\Delta X}{\Delta S} \quad (2)$$

$$Y_{P/S} = \frac{\Delta P}{\Delta S} \quad (3)$$

where ΔX is the amount of biomass and ΔP is the product (either as total lipids or biodiesel calculated as the amounts of total FAME), respectively, and ΔS is the amount of substrate consumed.

The volumetric growth and production rates (r_X and r_P, respectively) were calculated according to Equations (4) and (5) by relating the amounts of biomass and biodiesel, respectively, to the time required to attain the lipid production peak (Δt):

$$r_X = \frac{\Delta X}{\Delta t} \quad (4)$$

$$r_P = \frac{\Delta P}{\Delta t} \quad (5)$$

substrate (r_S) and nitrogen (r_N) consumption rates were calculated by Equation (6) by relating the amounts of total sugars and nitrogen consumed, respectively, to the time required to attain the lipid production peak (Δt):

$$r_{[S,N]} = \frac{\Delta[S,N]}{\Delta t} \quad (6)$$

2.5 Analytical methods

189 Cell biomass was collected from 5 mL-samples in pre-weighed Falcon tubes. The suspension was
190 centrifuged at 8000 x g for 10 minutes and washed 3 times with distilled water. Dry cell weight
191 was determined gravimetrically after lyophilisation for 48 h.
192 Total sugars content was determined by using the phenol-sulphuric acid method (Dubois et al.,
193 1956). The concentrations of lactose and galactose were determined by ion-moderated partitioning
194 chromatography in a Varian HPLC system equipped with an Aminex HPX 87-P column (Biorad
195 Laboratories, Milan, IT). Samples were eluted with Milli-Q water (flow rate 0.6 mL min^{-1}) at 65
196 °C and the elution profile was monitored by an IR4 refractive index detector (Varian, Sunnyvale,
197 CA). Determination of total lipids was performed according to the method of Izard and Limberger
198 (2003). COD and TOC were determined according to Standard Methods (APHA, 2005). Ashes
199 were determined gravimetrically after 12-h ignition at 550 °C in a muffle furnace.
200 Nitrogen was determined by a modified Kjeldahl method (Domini et al., 2009). Digestions were
201 carried out in a batch microwave digestion system (MarsXpress, CEM, Matthews, NC, USA) at
202 500 W power, 200 °C for 10 minutes by adding a mixture of 37% HCl (Carlo Erba Reagenti,
203 Milan, Italy) and 30% H_2O_2 (Merck KGaA, Darmstadt, Germany) to 1 mL sample. Afterwards,
204 nitrogen present in the form of ammonium was determined spectrophotometrically at 650 nm using
205 the nitroprusside method described by Anderson and Ingram (1993).
206 Yeast cells were stained with Sudan Black B followed by counterstaining with Safranin to detect
207 the presence of intracellular storage lipids as described by Ravikumar and collaborators (2012).
208 Characterisation of lipid profiles was performed by a direct transesterification on lyophilised cells
209 (Schutter and Dick, 2000) to obtain fatty acid methyl esters (FAMEs), which were analysed by a
210 Master GC gas chromatograph (DANI Instrument SpA, Cologno Monzese, Italy) equipped with a
211 Rxi-5MS (Restek, Germany) capillary column (0.25 mm id x 30 m length). FAMEs were eluted by
212 using the following program: isothermal at 89 °C for 2 min; temperature gradient from 89 to 280
213 °C at a 6 °C min^{-1}; hold at 280 °C for 5 min. The temperatures of the injector and flame ionization

detector were set at 280 and 300 °C, respectively. Each FAME was identified by comparing its retention time with that of authentic standards contained in the FAME Mix C8-C24 (Sigma Aldrich, 18918-1AMP, USA). For quantification purposes, an internal standard (i.e., methyl nonadecanoate) was added to each sample prior to the transesterification.

3. Results

3.1 Screening of yeast strains

Table 1 comparatively reports biomass and lipid productions and related yields calculated at the time (t) of maximal lipid accumulation. Regardless of the strain under study, the duration of the production process was brief with best lipid accumulation being observed between 48 and 96 h.

Among the tested strains, *C. curvatus* NRRL Y-1511 and *C. laurentii* UCD 68-201 proved to efficiently grow on RCW-based medium, achieving biomass productions of 10.77 and 7.28 g L^{-1}, respectively. The ability of the former and the latter strain to use RCW as a growth substrate was confirmed by marked reduction in COD (86.7 and 77.9%, respectively) (data not shown). *C. curvatus* was able to produce 6.83 g L^{-1} of intracellular lipids which amounted to 63% of biomass dry weight, while *C. laurentii* achieved a slightly lower production level (5.06 g L^{-1}) but with a higher yield with respect to biomass, amounting to 70%.

The other strains showed greater difficulties in adapting to this substrate but some of them, despite a low growth, were able to accumulate high percentages of total lipids with respect to biomass. In particular, $Y_{P/X}$ values in *L. starkeii* and *R. toruloides* NRRL Y-17902 amounted to 0.63 and 0.79 although their biomass productions were 0.79 and 0.64 g L^{-1}, respectively (Table 1). Conversely, although *R. glutinis* UCD 68-255, *T. fermentans* and *P. membranifaciens* exhibited substantial growth in the RCW-based medium their relative $Y_{P/X}$ values were very low (i.e., 0.09, 0.20 and 0.13, respectively).

Thus, on the basis of the performance observed in shaken cultures, the determination of the lipid profiles was limited to some selected strains. Figure 1A, reporting the percent concentrations of each identified fatty acid with respect to total FAME, shows that in all the strains considered, the main fatty acids were palmitic (C16:0), oleic (C18:1 Δ9), linoleic (C18:2 Δ9,12) and stearic (C18:0) acids, while the concentrations of polyunsaturated fatty acids (PUFAs), such as linolenic (C 18:3 Δ 9,12,15), eicosadienoic (C20:2 Δ11,14) and arachidonic (20:4 Δ5,8,11,14) acids, were either negligible or even absent. The FAME profiles of *C. curvatus*, *C. laurentii* and *L. starkeii* were rather similar while that of *R. toruloides* greatly differed from the others due to its higher content in total saturated (52.6%) and polyunsaturated (15%) fatty acids, as shown in Figure 1A.

Although the biodiesel yields of *C. curvatus* and *C. laurentii* (35.77±3.51% and 27.1±4.80%, respectively) were lower than those of *R. toruloides* and *L. starkeii* (88.90±11.2% and 54.32±3.99%, respectively) (Figure 1B), their volumetric lipid productions, lipid accumulation capacities and specific fatty acid profiles, were deemed to be suitable for subsequent transfer of the process to the reactor scale. Although *C. curvatus* exhibited better production properties than *C. laurentii*, the latter was selected since a scant amount of information is currently available regarding its use for biodiesel production. A typical fermentation of *C. laurentii* in RCW medium in shaken flask is reported in Figure S1 and shows that maximal lipid accumulation took place after 24 h from the depletion of N from the growth medium. Taking into account the lipid production peak (5.06 g L^{-1}) and the relative biodiesel yield (27.1%), the volumetric production of FAME derived from lipid transesterification was estimated to amount to 1.37 g L^{-1} (Figure 1B).

3.2 Reactor experiments with C. laurentii

To assess the feasibility of upscaling, the production process with *C. laurentii* was performed in a stirred tank reactor, as shown in Fig. 2.

In STR, nitrogen starvation occurred after only 36 h, thus resulting in an anticipated peak of lipid production (9.93 g L^{-1} at 60 h). The lipid production peak coincided with the occurrence of maximal biomass production (14.37 g L^{-1}). The decrease in total sugars concentration proceeded at an almost linear rate in the early 48 h to dramatically decline thereafter (Fig. 2A). As a consequence, the oxygen percent saturation, after a significant drop in the same time interval, tended to rise again to reach values which approached initial ones. Conversely, the pH did not significantly change throughout the process as shown in Fig. 2B. Noteworthy, at the lipid production peak, the soluble COD was reduced by 83.4% reaching values as low as 3600 mg L^{-1} (data not shown). Table 2 shows that at the time of maximal lipid accumulation, biodiesel yield amounted to 32.64±3.24%; on this basis, the maximal volumetric amounts of total FAME derived from transesterification were estimated to be 3.24 g L^{-1}. As a result of this calculation, $Y_{P/X}$ and $Y_{P/S}$ were 0.23±0.01 and 0.17±0.01, respectively.

Table 2, reporting also the percent FAME composition of *C. laurentii* lipids obtained in STR, shows that the main fatty acids were oleic acid, linoleic and palmitic acids (47.2, 23.7 and 18.5%, respectively). Noteworthy, a significant change in FAME composition was observed in bioreactor as compared to shaken cultures, with a significant decrease in total saturated fatty acids (27.9 vs. 38.2%) and a 2.8-fold increase in linoleic acid (23.7 vs. 8.3%).

4. Discussion

The objective of this study was to investigate the feasibility of a second-generation biodiesel production by oleaginous yeasts grown on a dairy wastewater byproduct (i.e., RCW), for which, as opposed to cheese whey, there is a limited amount of information as a feedstock (Pirozzi et al., 2013; Castanha et al., 2014). Although both initial C/N ratio and absolute N amounts in the RCW-based medium might be putatively conducive to substantial yeast growth (Beopoulos et al., 2011), only 4 out the 18 strains under study were able to produce a biomass higher than 3 g L^{-1}. The

288 strains that met this requirement were *R. glutinis* UCD 68-255, *T. fermentans* NRRL Y-1492 and
289 two species belonging to the genus *Cryptococcus* (i.e., *C. curvatus* and *C. laurentii*). The
290 *Cryptococcus* and *Trichosporon* genera encompass several species with lactose-assimilating
291 ability. Thus, the failure of the remaining tested strains to substantially grow on RCW might be
292 due to their weak or absent lactose-assimilating ability. In this respect, it may be noted here that
293 lactose-assimilating capacity in yeasts is not very widespread (Frengova et al., 2004) and, for some
294 species, such as *L. starkeii* and *Kluyveromyces marxianus*, this feature has been shown to be strain-
295 dependent (Slodki and Wickerham, 1966; Naumov, 2006). However, irrespective of their growth
296 abilities on RCW, the large majority of the strains under study met the requirement of oleaginicity
297 accumulating intracellular lipids to larger than 20% of their cellular dry mass (Ratledge, 2002)
298 with the only exceptions of *P. membranifaciens* 6C1, *C. rugosa* NRRL Y-95 and *R. glutinis* UCD
299 68-225.

300 *C. curvatus* NRLL Y-1511 was the best performing strain in the screening on the RCW-based
301 medium in terms of production, productivity and lipid accumulation capacity. However, this
302 species is widely recognized as a good lipid producer and this attitude has been already tested on
303 several low-cost substrates, including biodiesel-derived glycerol (Thiru et al., 2011; Tchakouteu et
304 al., 2015), beet molasses (Takakuwa and Saito, 2010), olive mill wastewater (Yousuf et al., 2010),
305 hydrolysates of sweet sorghum bagasse and wheat straw (Liang et al., 2012; Yu et al., 2014) and
306 cheese whey (Seo et al., 2014). On the last substrate, which is highly related to that used in the
307 present study, in particular, a maximum lipid productivity of 4.68 g L^{-1} d^{-1} was obtained.
308 Conversely, the lipid-producing capacity of *C. laurentii* has not been investigated on whey-based
309 media with a sole exception where another strain of this species was tested on cheese whey
310 supplemented with sugarcane molasses (Castanha et al., 2014). In addition, this species deserves
311 particular attention due to its remarkable ability to use a variety of carbon sources and to its
312 tolerance to potential inhibitors of yeast growth (Sitepu et al., 2014). In the present study, lipid

volumetric production and productivity of *C. laurentii* (5.06 g L^{-1} and 1.7 g L^{-1} d^{-1}) were significantly higher than those reported (2.96 g L^{-1} and 0.102 g L^{-1} d^{-1}) in the aforementioned study of Castanha and collaborators (2014) and these parameters were further improved at the reactor scale.

In fact, the process transfer from shaken flask to the STR was successful leading to remarkably improved biomass and lipid productions; on a volumetric basis, the amounts of FAME derived from STR were found to be 2.4-fold higher than in shaken cultures. Moreover, the attainment of the lipid peak was anticipated with respect to shaken flask thus resulting in higher productivity. This anticipation observed in the STR was due to a better mass transfer of both O_2 and nutrients thus leading to an earlier occurrence of nitrogen starvation in bioreactor, an event known to trigger lipid accumulation in oleaginous yeasts. In particular, when nitrogen depletion takes place, the residual carbon in the medium is readily converted to storage lipids (Ratledge, 2002; Papanikolau and Aggelis, 2011). Noteworthy, the productivity observed in the present study was higher than that reported for *C. laurentii* DMKU-AmC14 on a glycerol-based medium (Polburee et al., 2015). Although a marked reduction in organic load was observed in the spent medium in concomitance with the lipid production peak, residual COD values were well above the regulatory standards for effluent discharge into receiving water bodies. However, treatment costs of this spent medium in a wastewater treatment plant would be significantly lower than those for RCW since, in the absence of other critical parameters (e.g., toxic pollutants, chromophoric substances etc.), they mostly depend on residual COD rather than on the volumes conferred.

The determination of the lipid profile of *C. laurentii* indicated the predominance of 16- and 18-carbon chain saturated and monosaturated fatty acids in agreement with Castanha et al. (2014) and this was associated with a low content in PUFA. Noteworthy, the FAME profile of lipids obtained in the bioreactor significantly differed from that obtained in shaken flask with a concomitant increase in linoleic acid and decrease in total saturated fatty acids. In this respect,

dissolved oxygen levels in the medium can markedly modify the fatty acid profile produced by oleaginous microorganisms (Laoteng et al., 2011) and a decrease in unsaturated fatty acids concomitant to the reduction of available oxygen was observed in *Saccharomyces cerevisiae* (Bardi et al., 1999) and *Apiotrichum curvatum* (Davies et al., 1990) cultures. This is not surprising since oxygen acts as the terminal electron acceptor in reactions catalyzed by fatty acid desaturases which play a key role in the synthesis of MUFA and PUFA (Lee et al., 2016); moreover, an oxygen-induced increase in the transcripts levels of desaturase genes was observed in *Mucor rouxii* cultures (Ruenwai et al., 2010). Although an increase in PUFA has been shown to be detrimental to the oxidative stability of the biodiesel (Jakeria et al., 2014), in the present study, the reactor-induced change led to a FAME composition which substantially resembled that of the Jatropha oil (Thiru et al., 2011), the use of which is well established in biodiesel production. It has been reported that oil sources containing high amounts of oleic acid and long chain saturated fatty acids would be ideal candidates for biodiesel purposes, because this composition positively impacts on biodiesel performance parameters, such as cetane number, kinematic viscosity, melting point, oxidative stability and heat of combustion (Knothe, 2005). In this respect, the FAME composition of *C. laurentii* appears to meet these requirements.

4. Conclusions

On the one hand, the screening performed with a variety of strains belonging to well known lipid-accumulating species confirmed that whey-related substrates, such as RCW, are often not adequate to support yeast growth since the large majority of carbon in this byproduct is found as lactose, the assimilation of which is not widespread among yeasts. Thus, a profitable use of this dairy byproduct as a feedstock for microbial production processes involving yeasts would require its pretreatment either by acid hydrolysis and subsequent pH correction or enzymatic hydrolysis with β-galactosidase which is commercially available at low costs. This might be beneficial to lipid

363 production processes involving *L. starkeii* and *R. toruloides*, which despite a limited growth on
364 RCW, maintained a significant lipid accumulation capacity and yielded FAME profiles which
365 were compatible with good biodiesel properties; studies are underway to assess this hypothesis. On
366 the other hand, the successful transfer of the lipid production process of *C. laurentii* grown on
367 RCW from shaken flask to STR offers wide and promising perspectives of further improvements.
368
369 **References**

370 Anderson, J.M., Ingram, J.S.I., 1993. Tropical Soil Biology and Fertility: A Handbook of Method.
371 Second Edition CAB International, Wallingford, UK.
372 APHA, Standard Methods for the Examination of Water and Wastewater, 2005. 21th edition
373 American Public Health Association, AWWA, WEF, Baltimore, USA.
374 Atabani, A.E., Silitonga, A.S., Badruddin, I.A., Mahlia, T.M.I., Masjuki, H.H., Mekhilef, S., 2012.
375 A comprehensive review on biodiesel as an alternative energy resource and its characteristics.
376 Renew. Sustain. Energy Rev. 16, 2070–2093. http://dx.doi.org/10.1016/j.rser.2012.01.003
377 Bardi, L., Cocito, C., Marzona, M., 1999. *Saccharomyces cerevisiae* cell fatty acid composition
378 and release during fermentation without aeration and in absence of exogenous lipids. Int. J.
379 Food Microbiol. 47, 133-140. http://dx.doi.org/10.1016/S0168-1605(98)00203-7
380 Beopoulos, A., Nicaud, J.M., Gaillardin, C., 2011. An overview of lipid metabolism in yeasts and
381 its impact on biotechnological processes. Appl. Microbiol. Biotechnol. 90, 1193–1206.
382 http://dx.doi.org/10.1007/s00253-011-3212-8
383 Castanha, R.F., Mariano, A.P., de Morais, L.A.S., Scramin, S., Monteiro, R.T.R., 2014.
384 Optimization of lipids production by *Cryptococcus laurentii* 11 using cheese whey with
385 molasses. Brazilian J. Microbiol. 45, 379–387. http://dx.doi.org/10.1590/S1517-
386 83822014000200003

387 Christophe, G., Kumar, V., Nouaille, R., Gaudet, G., Fontanille, P., Pandey, A., Soccol, C.R.,
388 Larroche, C., 2012. Recent developments in microbial oils production: A possible alternative
389 to vegetable oils for biodiesel without competition with human food? Brazilian Arch. Biol.
390 Technol. 55, 29–46. http://dx.doi.org/10.1590/S1516-89132012000100004
391 Cicatiello C., Franco S., Pancino B., Blasi E., 2016. The value of food waste: An exploratory study
392 on retailing. J. Retail. Cons. Serv. 30, 96-104.
393 http://dx.doi.org/10.1016/j.jretconser.2016.01.004
394 Corbo, M.R., Lanciotti, R., Albenzio, M., Sinigaglia, M., 2001. Occurrence and characterization of
395 yeasts isolated from milks and dairy products of Apulia region. Int. J. Food Microbiol. 69,
396 147-152. http://dx.doi.org/10.1016/S0168-1605(01)00585-2
397 Crognale, S., Pesciaroli, L., Petruccioli, M., D'Annibale, A., 2012. Phenoloxidase-producing
398 halotolerant fungi from olive brine wastewater. Process Biochem. 47, 1433-1437.
399 http://dx.doi.org/10.1016/j.procbio.2012.05.014
400 Davies, R.J., Holdsworth, J.E., Reader, S.L., 1990. The effect of low oxygen uptake rate on the
401 fatty acid profile of the oleaginous yeast *Apiotrichum curvatum*. Appl. Microbiol. Biotechnol.
402 33, 569–573. http://dx.doi.org/10.1007/BF00172553
403 Domini, C., Vidal, L., Cravotto, G., Canals, A., 2009. A simultaneous, direct
404 microwave/ultrasound-assisted digestion procedure for the determination of total Kjeldahl
405 nitrogen. Ultrason. Sonochem. 16, 564–569. http://dx.doi.org/10.1016/j.ultsonch.2008.12.006
406 Dubois, M., Gilles, K., Hamilton, J., Rebers, P., Smith, F., 1956. Colorimetric method for
407 determination of sugars and related substances. Anal. Chem. 28, 350–356.
408 http://dx.doi.org/10.1021/ac60111a017
409 Frengova G, Simova E, Beshkova D. 2004. Use of whey ultrafiltrate as a substrate for production
410 of carotenoids by the yeast *Rhodotorula rubra*. Appl. Biochem. Biotechnol. 112, 133-141.
411 http://dx.doi.org/10.1385/ABAB:112:3:133

412 Izard, J., Limberger, R.J., 2003. Rapid screening method for quantitation of bacterial cell lipids
413 from whole cells. J. Microbiol. Methods 55, 411–418. http://dx.doi.org/10.1016/s0167-
414 7012(03)00193-3
415 Jakeria, M.R., Fazal, M.A., Haseeb, A.S.M.A., 2014. Influence of different factors on the stability
416 of biodiesel: A review. Renew. Sustain. Energy Rev. 30, 154–163.
417 http://dx.doi.org/10.1016/j.rser.2013.09.024
418 Knothe, G., 2005. Dependence of biodiesel fuel properties on the structure of fatty acid alkyl
419 esters. Fuel Process. Technol. 86, 1059–1070. http://dx.doi.org/10.1016/j.fuproc.2004.11.002.
420 Laoteng, K., Čertík, M., Cheevadhanark, S., 2011. Mechanisms controlling lipid accumulation and
421 polyunsaturated fatty acid synthesis in oleaginous fungi. Chemical Papers 65, 97-103.
422 http://dx.doi.org/10.2478/s11696-010-0097-4
423 Lavarda, J. 1972. Preparation of ricotta cheese curd. United States Patent 3,704,136 A. 1972 Nov
424 28.
425 Lee, J.M., Lee, H., Kang, S., Park, W. J., 2016. Fatty acid desaturases, polyunsaturated fatty acid
426 regulation, and biotechnological advances. Nutrients 8, 23.
427 http://dx.doi.org/10.3390/nu8010023
428 Li, Q., Du, W., Liu, D., 2008. Perspectives of microbial oils for biodiesel production. Appl.
429 Microbiol. Biotechnol. 80, 749–756. http://dx.doi.org/10.1007/s00253-008-1625-9
430 Liang, Y., Tang, T., Siddaramu, T., Choudhary, R., Umagiliyage, A.L., 2012. Lipid production
431 from sweet sorghum bagasse through yeast fermentation. Renew. Energ. 40, 130-136.
432 http://dx.doi.org/10.1016/j.renene.2011.09.035
433 Naumov, G.I., 2006. Genetics of lactose utilization polymorphism in the yeast *Kluyveromyces*
434 *marxianus*. Doklady Biol. Sci. 409, 317-319. http://dx.doi.org/10.1134/S0012496606040144

435 Papanikolaou, S., Aggelis, G., 2011. Lipids of oleaginous yeasts. Part I: Biochemistry of single
436 cell oil production. Eur. J. Lipid Sci. Technol. 113, 1031-1051.
437 http://dx.doi.org/10.1002/ejlt.201100014.
438 Pirozzi, D., Ausiello, A., Zuccaro, G., Sannino, F., Yousuf, A., 2013. Culture of oleaginous yeasts
439 in dairy industry wastewaters to obtain lipids suitable for the production of II-generation
440 Biodiesel. In: Proceedings of World Academy of Science, Engineering and Technology (No.
441 76, p. 57). World Academy of Science, Engineering and Technology (WASET).
442 Polburee, P., Yongmanitchai, W., Lertwattanasakul, N., Ohashi, T., Fujiyama, K., Limtong, S.,
443 2015. Characterization of oleaginous yeasts accumulating high levels of lipid when cultivated
444 in glycerol and their potential for lipid production from biodiesel-derived crude glycerol.
445 Fungal Biol. 119, 1194-1204. http://dx.doi.org/10.1016/j.funbio.2015.09.002.
446 Ravikumar, K., Dakshayini, J., Girisha, S.T., 2012. Biodiesel production from oleaginous fungi.
447 Int. J. Life Sci. 6, 43-49. http://dx.doi.org/10.3126/ijls.v6i1.5721
448 Ratledge, C. (2002). Regulation of lipid accumulation in oleaginous micro-organisms. Biochem.
449 Soc. Trans, 30, 1047-1050. http://dx.doi.org/10.1042/bst0301047.
450 Ruenwai, R., Cheevadhanarak, S., Rachdawong, S., Tanticharoen,M., Laoteng, K., 2010. Oxygen-
451 induced expression of Δ^6-, Δ^9- and Δ^{12}-desaturase genes modulates fatty acid composition in
452 *Mucor rouxii*. Appl. Microbiol. Biotechnol. 86, 327–334. http://dx.doi.org/10.1007/s00253-
453 009-2338-4
454 Sansonetti, S., Curcio, S., Calabrò, V., Iorio, G., 2009. Bio-ethanol production by fermentation of
455 ricotta cheese whey as an effective alternative non-vegetable source. Biom. Bioenerg. 33,
456 1687–1692. http://dx.doi.org/10.1016/j.biombioe.2009.09.002
457 Schutter, M.E., Dick, R.P., 2000. Comparison of Fatty Acid Methyl Ester (FAME) methods for
458 characterizing microbial communities. Soil Sci. Soc. Am. J. 64, 1659–1668.
459 http://dx.doi.org/10.2136/sssaj2000.6451659x

460 Secchi, N., Giunta, D., Pretti, L., García, M.R., Roggio, T., Mannazzu, I., Catzeddu, P., 2012.
461 Bioconversion of ovine scotta into lactic acid with pure and mixed cultures of lactic acid
462 bacteria. J. Ind. Microbiol. Biotechnol. 39, 175–181. http://dx.doi.org/10.1007/s10295-011-
463 1013-9.
464 Seo, Y.H., Lee, I., Jeon, S.H., Han, J.I., 2014. Efficient conversion from cheese whey to lipid using
465 *Cryptococcus curvatus*. Biochem. Eng. J. 90, 149–153.
466 http://dx.doi.org/10.1016/j.bej.2014.05.018
467 Sitepu, I., Selby, T., Lin, T., Zhu, S., Boundy-Mills, K., 2014. Carbon source utilization and
468 inhibitor tolerance of 45 oleaginous yeast species. J. Ind. Microbiol. Biotechnol. 41, 1061-
469 1070. http://dx.doi.org/10.1007/s10295-014-1447-y
470 Slodki, M.E., Wickerham, L.J. 1966. Extracellular polysaccharides and classification of the genus
471 Lipomyces. Microbiology 42, 381-385. http://dx.doi.org/10.1099/00221287-42-3-381
472 Srivastava, A., Prasad, R., 2000. Triglycerides-based diesel fuels. Renew. Sustain. Energ. Rev. 4,
473 111–133. http://dx.doi.org/10.1016/S1364-0321(99)00013-1
474 Tchakouteu, S.S., Kalantzi, O., Gardeli, C., Koutinas, A.A., Aggelis, G., Papanikolaou, S., 2015.
475 Lipid production by yeasts growing on biodiesel-derived crude glycerol: strain selection and
476 impact of substrate concentration on the fermentation efficiency. J. Appl. Microbiol. 118,
477 911-927. http://dx.doi.org/10.1111/jam.12736
478 Takakuwa, N., Saito, K., 2010. Conversion of beet molasses and cheese whey into fatty acid
479 methyl esters by the yeast *Cryptococcus curvatus*. J. Oleo Sci. 59, 255-260.
480 http://dx.doi.org/10.5650/jos.59.255
481 Thiru, M., Sankh, S., Rangaswamy, V., 2011. Process for biodiesel production from *Cryptococcus*
482 *curvatus*. Bioresour. Technol. 102, 10436-10440.
483 http://dx.doi.org/10.1016/j.biortech.2011.08.102.

484 Ykema, A., Verbree, E.C., Kater, M.M., Smit, H., 1988. Optimization of lipid production in the
485 oleaginous yeast *Apiotricum curvatum* in whey permeate. Appl. Microbiol. Biotechnol. 29,
486 211-218. http://dx.doi.org/10.1007/BF00251704
487 Yousuf, A., Sannino, F., Addorisio, V., Pirozzi, D., 2010. Microbial conversion of olive oil mill
488 wastewaters into lipids suitable for biodiesel production. J Agric Food Chem 58, 8630-8635.
489 http://dx.doi.org/10.1021/jf101282t
490 Yu, X., Zeng, J., Zheng, Y., Chen, S., 2014. Effect of lignocellulose degradation products on
491 microbial biomass and lipid production by the oleaginous yeast *Cryptococcus curvatus*.
492 Process Biochem. 49, 457-465. http://dx.doi.org/10.1016/j.procbio.2013.10.016
493 Zoppellari, F., Bardi, L., 2013. Production of bioethanol from effluents of the dairy industry by
494 *Kluyveromyces marxianus*. New Biotechnol. 30, 607–613.
495 http://dx.doi.org/10.1016/j.nbt.2012.11.017
496
497

Figure legends

Figure 1. Percent compositions of fatty methyl esters (FAMEs) derived from lipids produced by *C. curvatus* NRRL Y-1511, *C. laurentii* UCD 68-201, *L. starkeii* NRRL 11557 and *R. toruloides* Y-17902 grown in shaken flask on the RCW-based medium (A) and biodiesel yields (%) and FAME amounts obtained from lipid transesterification and referred to unit volume of culture broth (B). Data are referred to the time of maximal lipid accumulation and are the means of six chromatographic runs (2 technical replicates for each independent culture carried out in triplicate). Abbreviations: 16:1, palmitoleic acid; 16:0, palmitic acid; 18:2, linoleic acid; 18:1 oleic acid; 18:0, stearic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Figure 2. Time courses of lipid production and residual nitrogen and total sugar concentrations (Plot A) and biomass production, pH and dissolved oxygen (Plot B) in *C. laurentii* UCD 68-201 cultures grown in a 3-L stirred tank reactor (impeller speed, 600 rpm; aeration rate, 1.5 vvm). Data are the means ± standard deviations of duplicate reactor experiments. Inset in plot A contains a micrograph of 72-h-old cells stained according to Ravikumar et al. (2012) to detect the presence of storage lipid bodies.

Figure S1. Time courses of lipid and biomass productions and concentrations of residual nitrogen and total sugars in *C. laurentii* UCD 68-201 cultures grown in shaken flask. Data are the means ± standard deviations of triplicate cultures.

Table 1
Click here to download Table: Carota_Table 1.docx

519 **Table 1.** Biomass (X) and lipid (P) productions, yield parameters ($Y_{X/S}$, $Y_{P/X}$, $Y_{P/S}$) and time of maximal
520 lipid accumulation (t) obtained for each of the 18 yeast strains grown on RCW-based medium.

	X (g L^{-1})	P (g L^{-1})	$Y_{X/S}$	$Y_{P/X}$§	$Y_{P/S}$§	t (h)
C. rugosa NRRL Y-95	1.74±0.01	0.30±0.01	0.13±0.01	0.17±0.01	0.02±0.00	72
C. albidus UCD 68-150	2.00±0.07	0.72±0.10	0.15±0.01	0.36±0.06	0.06±0.01	72
C. albidus UCD 68-174	0.71±0.13	0.35±0.02	0.06±0.01	0.49±0.04	0.03±0.00	48
C. curvatus NRRL Y-1511	10.77±0.21	6.83±0.14	0.50±0.04	0.63±0.02	0.37±0.02	72
C. laurentii UCD 68-201	7.28±0.10	5.06±0.28	0.52±0.02	0.70±0.05	0.36±0.03	72
L. starkeii NRRL 11557	0.79±0.10	0.50±0.07	0.08±0.01	0.63±0.14	0.05±0.01	96
P. anomala AN/4	1.50±0.06	0.34±0.01	0.10±0.01	0.23±0.01	0.02±0.00	72
P. guilliermondii 1067	0.74±0.00	0.35±0.02	0.05±0.00	0.48±0.03	0.02±0.00	72
P. membranifaciens 6C1	2.71±0.20	0.34±0.05	0.31±0.03	0.13±0.03	0.04±0.01	72
R. glutinis DBVPG 3853	0.95±0.05	0.31±0.04	0.07±0.01	0.32±0.06	0.02±0.00	72
R. glutinis UCD 68-255	3.03±0.15	0.27±0.01	0.21±0.01	0.09±0.01	0.02±0.00	72
R. minuta UCD 68-280	0.66±0.03	0.37±0.03	0.06±0.00	0.56±0.07	0.03±0.00	72
R. toruloides NRRL 1091	0.86±0.07	0.41±0.03	0.06±0.01	0.48±0.07	0.03±0.00	72
R. torouloides NRRL Y-17902	0.64±0.03	0.51±0.01	0.05±0.00	0.79±0.05	0.04±0.00	96
T. fermentans NRRL Y-1492	3.50±0.22	0.69±0.06	0.32±0.03	0.20±0.03	0.06±0.01	48
Y. lipolytica NRRL YB-423	1.15±0.19	0.38±0.04	0.08±0.01	0.33±0.09	0.03±0.00	72
Y. lipolytica NRRL Y-1095	1.56±0.08	0.39±0.02	0.18±0.01	0.25±0.03	0.05±0.00	72
Y. lipolytica NRRL Y-7208	1.35±0.15	0.38±0.01	0.18±0.02	0.28±0.02	0.05±0.01	72

521 $Y_{X/S}$=biomass yield; $Y_{P/X}$= specific lipid yield; $Y_{P/S}$= lipid yield referred to consumed sugars; §
522 Calculated by relating total lipids, determined according to Izard and Limberger (2003), to the
523 amounts of total sugars consumed.

Table 2. Performance indicators of biodiesel production process in STR by *C. laurentii* including yields (Biodiesel yield, $Y_{P/S}$, $Y_{P/X}$, $Y_{X/S}$) and biodiesel and biomass production rates (r_P and r_X, respectively), N and total sugars consumption rates (r_N, r_S) specific growth rate (μ) and percent fatty acid composition. All values have been calculated at the time of maximal lipid production.

Parameter	Dimension unit	Value
Yields §		
Biodiesel yield	(%)	32.64±3.24
$Y_{P/X}$ǂ	(g g^{-1})	0.23±0.01
$Y_{P/S}$ǂ	(g g^{-1})	0.17±0.01
$Y_{X/S}$	(g g^{-1})	0.65±0.02
Rates §		
r_Pǂ	(g L^{-1} d^{-1})	1.30±0.09
r_S	(g L^{-1} h^{-1})	0.31±0.00
r_N	(mg L^{-1} h^{-1})	2.49±0.00
r_X	(g L^{-1} h^{-1})	0.24±0.00
μ	(h^{-1})	0.02±0.00
Lipid profile †‡		
Palmitoleic acid	(%)	0.39±0.05
Palmitic acid	(%)	18.53±1.24
Linoleic acid	(%)	23.47±1.01
Oleic acid	(%)	47.16±0.97
Stearic acid	(%)	5.45±0.28
Other Saturated fatty acids (SFA)	(%)	3.93±0.73
Other Monounsaturated fatty acids (MUFA)	(%)	0.30±0.07
Other polyunsaturated fatty acids (PUFA)	(%)	0.77±0.14
Total SFA	(%)	27.91±2.25
Total MUFA	(%)	47.85±1.09
Total PUFA	(%)	24.24±1.15

§Data are the means ± standard deviations of duplicate reactor experiments; ǂCalculated on the basis of the biodiesel yield; †Data are the means ± standard deviations of 4 chromatographic runs (2 technical replicates for each reactor experiment); ‡Predominant fatty acids are listed as a function of increasing retention time.

Figure 1

Click here to download high resolution image

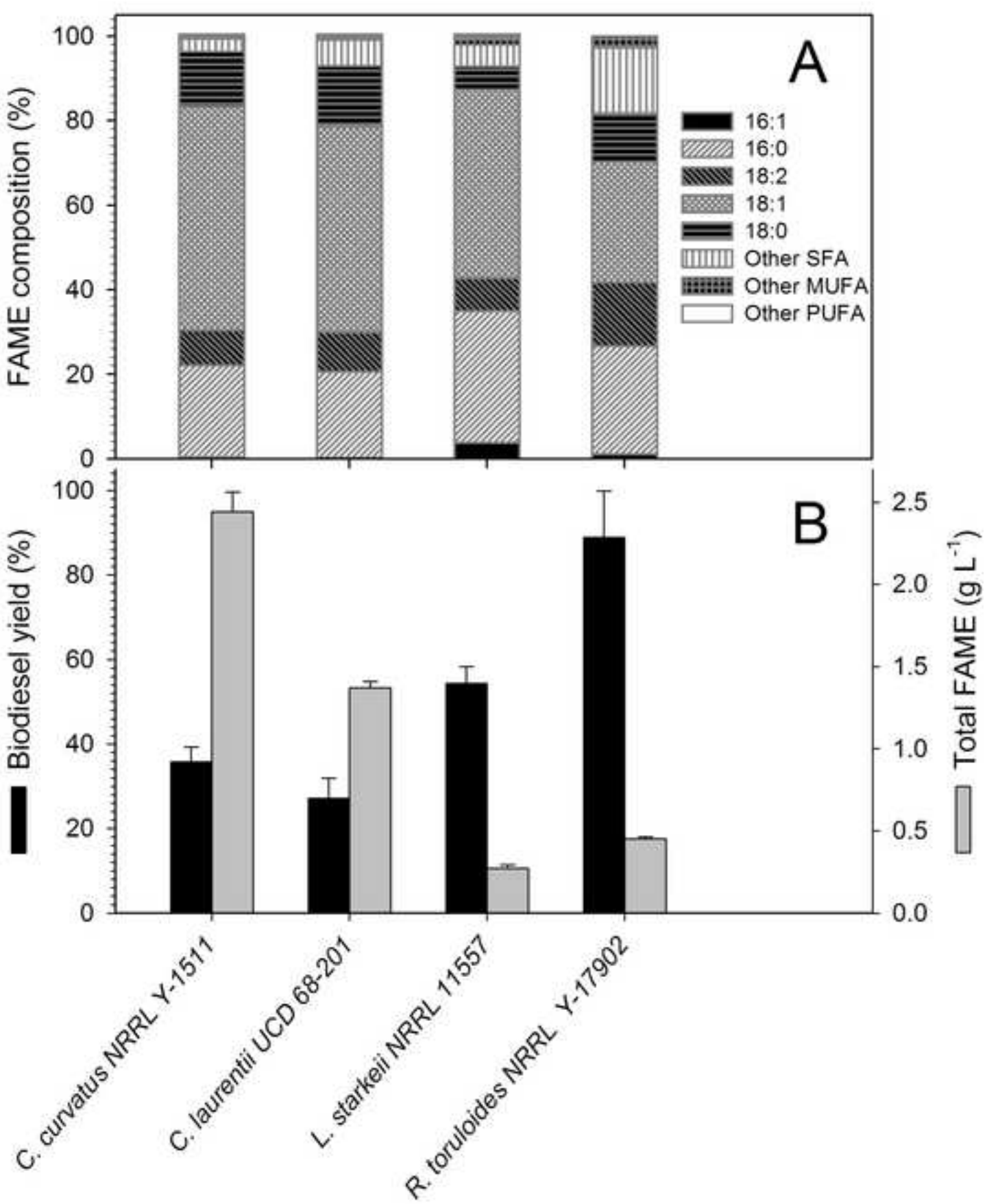


Figure 2

Click here to download high resolution image

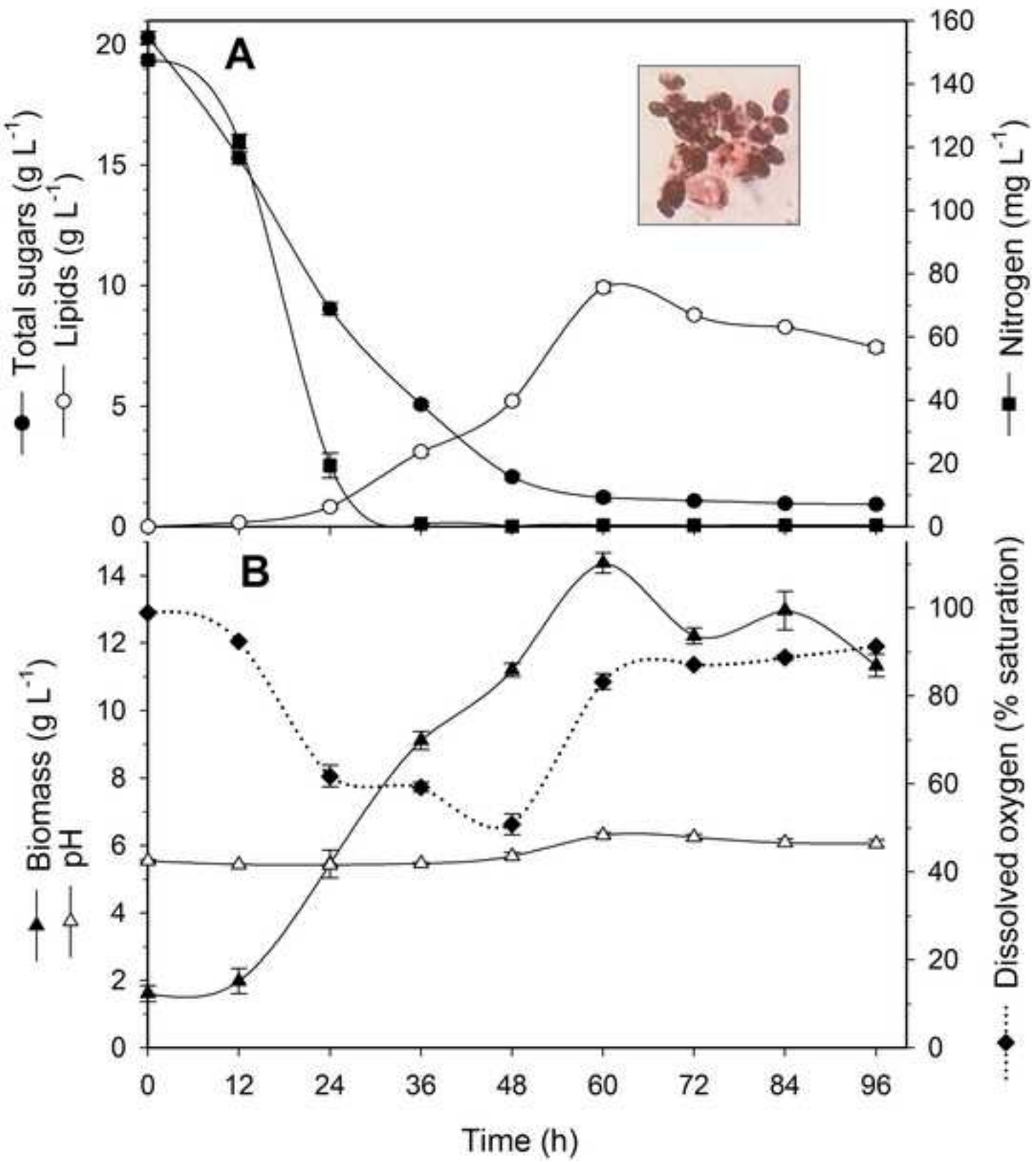


Figure S1

Click here to download Supplementary material for on-line publication only: FigureS1.TIF