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Bioconversion of agro-industrial waste into microbial oils by filamentous fungi

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ABSTRACT

Microbial oils are regarded as a sustainable alternative to vegetable oils for biodiesel manufacturing. However, in order to develop a cost-effective process, high-lipid producer microorganisms should be combined with low-cost renewable growth substrates. For this reason, the objective of the present study was to assess comparatively the oil-producing performance of 9 oleaginous fungi belonging to the *Aspergillus*, *Mucor*, *Mortierella* and *Cunninghamella* genera on three relevant and widespread waste, such as glycerol, orange peel extract (OPE) and ricotta cheese whey (RCW). This screening was performed at the shaken flask level and, among the strains under study, *Mortierella isabellina* NRRL 1757 turned out to be the most efficient and versatile and its lipid profile was found to be highly compatible with biodiesel production. Process transfer of *M. isabellina* lipid production to the lab-scale Stirred Tank Reactor on all the three waste-based media, was shown to be feasible, achieving a lipid productivity of 0.46, 1.24 and 0.91 g/(L d) on glycerol, OPE and RCW, respectively. Noteworthy, the fatty acid analysis of the oils produced, confirmed their suitability for biodiesel manufacturing, exhibiting a high similarity to palm and Jatropha oils commonly used as feedstock for this production.

1. Introduction

Microbial oils are considered a potential and more sustainable alternative source than that derived from higher plants and are currently exploited in a variety of applications including biofuels and nutraceuticals manufacturing and in oleochemical industry (Ratledge, 2013). Among the so called oleaginous microorganisms, namely those able to accumulate intracellular lipids over 20% of their dry weight (Rossi et al., 2010), filamentous fungi represent an interesting option mainly due to their great versatility and efficiency in metabolizing complex substrates (Ferreira et al., 2013).

Oleaginous filamentous fungi include species belonging predominantly to Zygomycota and, in particular, to genera such as *Mucor*, *Mortierella* and *Cunninghamella* (Ferreira et al., 2013) and, to a lesser extent, to Ascomycota, mainly belonging to the *Aspergillus* genus (Hui et al., 2010; Venkata Subhash and Venkata Mohan, 2011).

Furthermore, the Zygomycota are valuable sources of industrially interesting lipids for the food industry, such as linoleic and γ-linolenic acids (ω-6) or eicosapentanoic acid and docosahexaenoic acid (long-chain ω-3). In addition to these high-value added compounds, they also produce more common fatty acids, such as palmitic, palmitoleic, oleic and stearic acids that could be used as renewable and sustainable feedstock for biodiesel or other oleochemical production (Bellou et al., 2012).

However, a disadvantage related to the use of filamentous fungi in submerged cultures with respect to other microorganisms, derives from their assumption of a variety of morphological forms depending on culture conditions (e.g., inoculum size and medium composition) that might affect negatively process performance. Usually, the pellet growth mode is preferred for industrial applications because it poses less rheological and technical problems (Gibbs et al., 2000); however, the management of fungal morphology during a given bioprocess is not an easy task.

To date, the oil-producing ability of several oleaginous filamentous fungi, such as *Mortierella isabellina*, *Thamnidium elegans, Cunninghamella elegans* and *Mortierella ramanniana*, has been investigated on a wide array of wastes. These waste have included starchy hydrolysate (Zhu et al., 2003), lignocellulosic hydrolyzates (Peng and Chen, 2008; Economou et al., 2010; Zhang and Hu, 2014), and agro-industrial waste, such as whey (Demir et al., 2013) and potato-related byproducts (Lunin et al., 2013). In addition, industrial waste, such as raw glycerol (Bellou et al., 2012; Fakas et al., 2009; Papanikolaou et al., 2008; Silva et al., 2012) and corn thin

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stillage derived from corn ethanol production (Mitra et al., 2012) have been used.

Although a variety of waste have been examined for their abilities of supporting oil production by filamentous fungi, there is scant information regarding the use of orange peel waste (OPW) and cheese whey byproducts for this purpose. OPW is a widespread waste derived from juice manufacturing, produced in huge amounts at a worldwide level, and the large majority of this waste remains unused apart from some applications involving the recovery of essential oils or its use as an animal feed (Balu et al., 2012). Although this byproduct is known to contain high concentration of easily assimilable sugars, such as glucose, sucrose and fructose, orange peels have been poorly studied as growth substrate in the context of microbial oil production (Gema et al., 2002; Park et al., 2014). The same applies to cheese whey and related byproducts for which only two studies have been performed with the aim of producing oils from filamentous fungi (Demir et al., 2013; Vamvakaki et al., 2010). Ricotta cheese whey (RCW) is the liquid waste derived from ricotta cheese, the manufacturing of which involves heating of cheese whey to promote thermal protein precipitation. In addition to the Mediterranean area, where the RCW annual production amounts to several Mtons (about 1 Mton per year only in Italy), it is also produced in other countries of Central Europe and in Brazil (Leal et al., 2016). Compared to simple cheese whey, which can be reused and exploited for protein extraction or animal feeding, RCW is just considered as a waste, posing environmental and disposal problems, due to its high content in protein-precipitating additives (i.e, NaCl and organic acids) and very low protein concentration.

An additional gap of information regards the current lack of studies dealing with a comparative assessment of the oil-producing process performance with different waste.

For these reasons, the main objective of this study was to compare the oil-production performance of several strains of filamentous fungi on three different waste. To this aim, 9 strains belonging to the *Aspergillus*, *Cunninghamella*, *Mortierella* and *Mucor* genera were grown on either orange peel extract (OPE), derived from hot aqueous extraction of OPW, RCW or glycerol.

Although, as already mentioned, several studies are available for glycerol, a by-product of several oleochemical processes involving the transesterification of vegetable oils and animal fats (e.g., biodiesel production) (Tchakouteu et al., 2015), a surplus of this waste is expected to occur in the brief term considering a projected increase of biodiesel annual production to 11×10^{10} L by the year 2020 (OECD/FAO, 2011).

The biomass-producing and lipid-accumulating abilities of the strains under study on these waste were first assessed in shaken flasks and the fatty acid profiles of the most versatile ones determined. Based on biomass and lipids yields and rates obtained, the most efficient strain was further investigated in stirred-tank reactor in order to evaluate the technical feasibility of the process transfer to the reactor scale.

2. Materials and methods

2.1. Strains, maintenance and inoculum preparation

Aspergillus tubingensis NRRL 4700, *Cunninghamella echinulata* NRRL 3655, *Mortierella isabellina* NRRL 1757, *Mucor circinelloides* NRRL 3631, *Mucor miehei* NRRL 3169 and *Mucor racemosus* UCD 71-20 were obtained from the ARS (NRRL, Peoria and Davis, California) and UCD (Davis, California) Culture Collections. *A. tubingensis* E1 (Accession number KF482450) was isolated from wood chips (Barontini et al., 2014), while *Mucor moelleri* LAS_E (Accession number KY678773) and *Chaetomium* sp. LAS_G (Accession number KY688070) were isolated from As- and Hg-polluted soils collected from the Pestarena decommissioned gold mine site (Crognale et al., 2017). Identification was performed on the basis of ITS sequence and, for *Chaetomium* sp. LAS_G, on the basis of 18S rDNA, too. During the study, the strains were maintained on potato dextrose agar (PDA) slants at 4 °C and sub-cultured every month.

Regardless of the strain, pre-inocula for shaken flask and bioreactor experiments were prepared by growing 10^6 spores/mL obtained from 2 week-old plates, in 250-mL flasks containing 50 mL of PDB medium, at 30 °C for 3 days under orbital shaking (185 rpm).

2.2. Growth media

The glycerol-based medium had the following composition (g/L): technical-grade glycerol (90%, Carlo Erba Reagents, Val-de-Reuil, France), 20; $(NH_4)_2SO_4$, 0.5; yeast extract (containing 10% total nitrogen), 1.0; NaH_2PO_4, 3.0 and KH_2PO_4, 0.7. The medium's pH was adjusted to pH 5.5 prior to sterilization in autoclave (121 °C, 20 min). After sterilization, 0.1% of filter-sterilized 1000x stock mineral salt solution was added; this solution (pH 5.5) contained (g/L): $ZnSO_4$, 4.40; $MnCl_2{\cdot}4H_2O$, 1.10; $CuSO_4$, 0.31; $CaCl_2$, 1.47 and $FeSO_4$,1.0.

Orange peel waste, from which OPE was obtained, was kindly supplied by Agrumi-Gel srl (Barcellona P.G., ME, Italy) in powder form with a moisture content of 6.9 ± 0.3%. OPE was prepared by crushing air-dried orange peels with a blender and extracting them in distilled water at 100 °C for 1 h. The aqueous extract thus obtained, after centrifugation and filtration through Whatman filter n. 41, had a dry weight of 25.43 ± 0.54 g/L composed of 39.85 ± 0.15% total carbon and 1.32 ± 0.02% total nitrogen determined by elemental analysis. A small amount of $(NH_4)_2SO_4$ was added after autoclave sterilization to adjust the C/N ratio to 25. This adjustment was done to enable a quick adaptation of the tested strains to the new substrate after inoculation thus ensuring a rapid initial growth. Substrate composition, determined by HPLC and enzymatic kits (Megazyme International Ireland Ltd, Wicklow, Ireland), was as follows (g/L): glucose, 5.68 ± 0.17; sucrose, 5.98 ± 0.21; fructose, 5.04 ± 0.17; mannose, 1.08 ± 0.22 and pectin, 1.78 ± 0.14.

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): dry weight, 48.2 ± 4.10; 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, it was centrifuged ($8000 \times g$, 15 min), diluted 1:2 with distilled water, supplemented with $(NH_4)_2SO_4$ to reach a C/N ratio of 25, and finally its pH was brought to 5.5 with 0.1 M NaOH.

2.3. Culture conditions

2.3.1. Shaken flask experiments

Shaken culture experiments were performed in triplicate in 250-mL Erlenmeyer flasks containing 45 mL of growth medium inoculated with 5 mL of pre-inoculum. Cultures were incubated in an orbital shaker (185 rpm) at 30 °C for 5 days. Samples were collected on a daily basis for analysis. Yields ($Y_{P/S}$, $Y_{P/X}$, $Y_{X/S}$) were calculated as described in Section 2.4.

2.3.2. Bioreactor experiments

Bioreactor experiments were performed in a 3-L jacketed bench-top STR (Applikon, Schiedam, NL) filled with 2 L of medium. Mechanical agitation was ensured by 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). Aeration was provided through a perforated pipe sparger located under the bottom turbine, while monitoring of the fermentation processes was

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ensured by the following probes: dissolved oxygen and pH sensors (Applikon) and a PT 100 temperature sensor.

For all the fermentations tested, the following conditions were used: impeller speed, 450 rpm (impeller tip speed = 141 cm/s); aeration rate, 1.5 vvm; temperature, 30 °C; initial dissolved oxygen concentration, 100% of saturation. Silicon Antifoam 289 (0.5 mL/L) (Sigma Chemical Co., St Louis, MO, USA) was added before inoculation and an additional 1 mL/L was added when needed. The fermentation parameters (temperature, pH and dissolved oxygen) were monitored by an ADI 1030 (Applikon) adaptive/PID digital controller.

Bioreactor experiments were done in duplicate and culture samples collected every 12 h. Yields ($Y_{P/S}$, $Y_{P/X}$, $Y_{X/S}$), specific growth rate (μ), lipid and biomass production rates (rP and rX, respectively) and N and total sugars consumption rates (rN and rS, respectively) were calculated, as described in Section 2.4.

2.4. Determination of yields and rates

The Eq. (1) was used to calculate the specific growth rate (μ):

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

where X represents the concentration of the fungal biomass (g/L) at the time t (h).

The Eqs. (2) and (3) were used to calculate the biomass and the product yield ($Y_{X/S}$ and $Y_{P/S}$, respectively):

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

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

where ΔX and ΔP are the biomass and total lipids amounts, respectively and ΔS is the amount of substrate consumed. The volumetric growth and production rates (rX and rP, respectively) were calculated according to Eqs. (4) and (5) by referring the biomass and biodiesel amounts, respectively, to the time needed to achieve the lipid production peak (Δt):

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

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

Substrate (rS) consumption rate was calculated by Eq. (6) by relating the amounts of total sugars consumed to the time needed to achieve the lipid production peak (Δt):

$$r_S = \frac{\Delta S}{\Delta t} \quad (6)$$

2.5. Analytical methods

2.5.1. Analysis on cell biomass

Biomass was recovered from 5 mL samples after centrifugation at 8000 × *g* for 10 min followed by 3 washing steps with distilled water. Dry cell weight was determined gravimetrically after freeze-drying for 72 h. Determination of total lipids along the fermentation time was performed according to the method of Izard and Limberger (2003) on fresh biomass pellet derived from 2.0 mL of cell suspension, after centrifugation.

Quantification and characterisation of fungal fatty acids was performed directly on lyophilised cells by a modification of an alkaline methanolysis method described by Schutter and Dick (2000). In particular, the lyophilised fungal biomass (15.0 ± 0.1 mg dry weight) was added with a 0.2 M KOH solution in methanol (15 mL) and 100 µL of methyl-nonadecanoate (0.23 mg/mL in hexane) as the internal standard in a 50-mL teflon-lined, screw-cap centrifuge glass tube. After mixing, the reaction mixture was incubated at 37 °C for 1 h and vortexed periodically (every 10 min). At the end of the incubation, samples were added with 6.6 mL of 1.0 M acetic acid solution in Milli-Q water to neutralize the pH. Finally, to enable the partitioning to an organic phase of the fatty acid methyl esters (FAMEs) thus obtained, the mixture was added with hexane (20 mL), mixed repeatedly by gentle inversion and centrifuged (500 × *g*; 10 min). After removal of the upper organic phase, a fresh aliquot of hexane (20 mL) was added, and mixed and centrifuged again under the same conditions. The pooled organic phases were transferred to a clean vacuum flask and hexane removed by rotary evaporation at 30 °C in a R-210 Rotavapor (Büchi Labortechnik, Flawil, CH). The dried residue was then dissolved again in 1.0 mL hexane and transferred to a GC vial prior to chromatographic analysis. FAMEs were analyzed 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 × 30 m length) and a flame ionization detector. The operating conditions were as follows: initial oven temperature at 89 °C for 2 min, followed by a temperature gradient from 89 to 280 °C at 6 °C/min and by a final elution step at 280 °C for 5 min. The injector and detector temperatures 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) and quantified by using the aforementioned internal standard. Empirical equations for the calculation of saponification (SV) and iodine value (IV) developed by (Kalayasiri et al., 1996) and of cetane number (CN) (Krisnangkura, 1986) were used to predict these values and, thus, to evaluate the suitability of microbial lipids for biodiesel production. To this aim, the Standard EN14214 was used as the reference. These predicted values were compared with those estimated by the model of Gopinath et al. (2009) which showed very good accuracy in predicting these properties in several commonly used oils in biodiesel production. Predicted SV and IV values were used to calculate the higher heating value (HHV) by using the empirical model of Demirbas (1998).

2.5.2. Analysis on the supernatant

Supernatants were recovered after centrifugation at 8000 × *g* for 10 min.

Total sugars content was determined by using the phenol-sulphuric acid method (Dubois et al., 1956). Ashes were determined gravimetrically after 12-h ignition at 550 °C in a muffle furnace.

Total 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 min by adding a mixture of 37% HCl (Carlo Erba Reagenti, Milan, Italy) and 30% H_2O_2 (Merck KGaA, Darmstadt, Germany) to 1.0 mL 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).

During fermentation, substrate consumption was assessed by means of enzymatic kits. Specifically, glucose, fructose and sucrose were analyzed using the SUCROSE, D-FRUCTOSE and D-GLUCOSE kits (Megazyme International Ireland Ltd, Wicklow, Ireland), following the procedure described by the manufacturer.

Maltose and glycerol were determined by means of Maltose/Sucrose/D-Glucose K-GCROLGK Assay Kits (Megazyme International Ireland Ltd, Wicklow, Ireland), respectively, as described by the manufacturer.

Pectin was indirectly quantified by galacturonic acid assay (Taylor and Buchanan-Smith, 1992) using pectin as the standard for calibration curve in a range between 10 and 200 µg/mL. Although orange peels are composed of 15–25% (w/w) of pectin (Grohmann et al., 1995), under the mild extraction conditions adopted in this

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Table 1
Q6 Volumetric biomass (X) and lipid (P) productions and yield parameters ($Y_{X/S}$, $Y_{P/X}$, $Y_{P/S}$) calculated at the time of maximal lipid accumulation (t) obtained with each of the 9 filamentous fungi strains grown on the glycerol-based medium.

Strain	X (g/L)	P (g/L)	t (h)	$Y_{X/S}$ (g/g)	$Y_{P/X}$ (g/g)	$Y_{P/S}$ (g/g)
A. tubingensis E1	10.82 ± 0.12^{de}	2.00 ± 0.17^{d}	96	0.54 ± 0.05^{bc}	0.19 ± 0.02^{c}	0.10 ± 0.02^{e}
A. tubingensis NRRL 4700	9.64 ± 0.83^{cd}	1.39 ± 0.12^{c}	96	0.54 ± 0.09^{bcd}	0.14 ± 0.03^{d}	0.08 ± 0.01^{a}
C. echinulata NRRL 3655	11.66 ± 0.14^{e}	2.12 ± 0.18^{de}	96	0.58 ± 0.06^{cd}	0.18 ± 0.02^{d}	0.11 ± 0.02^{f}
Chaetomium sp. LAS_G	3.45 ± 0.13^{a}	0.28 ± 0.02^{a}	96	0.36 ± 0.04^{a}	0.08 ± 0.01^{b}	0.03 ± 0.00^{c}
M. isabellina NRRL 1757	7.24 ± 0.37^{b}	1.99 ± 0.07^{d}	72	0.36 ± 0.03^{a}	0.28 ± 0.02^{f}	0.10 ± 0.01^{f}
M. circinelloides NRRL 3631	12.07 ± 0.24^{e}	0.43 ± 0.03^{ab}	72	0.60 ± 0.06^{d}	0.04 ± 0.00^{a}	0.02 ± 0.00^{b}
M. miehei NRRL 3169	6.46 ± 0.88^{b}	0.65 ± 0.01^{b}	96	0.36 ± 0.05^{a}	0.10 ± 0.02^{b}	0.04 ± 0.00^{d}
Mucor moelleri LAS_E	8.77 ± 0.61^{c}	2.10 ± 0.20^{de}	96	0.44 ± 0.07^{ab}	0.24 ± 0.04^{e}	0.11 ± 0.01^{f}
M. racemosus UCD 71-20	10.16 ± 0.65^{d}	2.33 ± 0.21^{e}	96	0.51 ± 0.08^{bcd}	0.23 ± 0.04^{e}	0.12 ± 0.00^{g}

$Y_{X/S}$ = biomass yield; $Y_{P/X}$ = specific lipid yield; $Y_{P/S}$ = lipid yield referred to consumed substrate, calculated by relating total lipids, determined according to Izard and Limberger (2003), to the amounts of total glycerol consumed. Data are the mean± SD of three replicates. Multiple pair-wise comparisons were performed by the Tukey test ($P \leq 0.05$). Same superscript letters indicate the lack of statistically significant difference ($P \leq 0.05$) within column means.

study, a very small content of this component was found in the final OPE.

2.6. Statistical analysis

The experiments in flasks were carried out with three independent replicates, while experiments in bioreactor in duplicate. The data were analyzed by one-way or two-way analysis of variance (ANOVA test) and the means compared by a post-hoc Tukey's test using SigmaStat Software package version 3.5 (Jandel Scientific, Germany). In order to assess similarity relationships between the FAME profiles of microbial oils obtained in the present study and those of commonly used plant oils, principal component analysis (PCA) was performed on arcsin of the square-root transformed data. To this aim, soft-scaled were subjected to PCA by using the SIMCA software package v. 13 (Umetrics, Umea, Sweden). The possible presence of either moderate or strong outliers in observations was checked by the squared prediction errors of residuals and Hotelling (T^2) of t-scores, respectively (MacGregor and Kourti, 1995).

3. Results and discussion

3.1. Lipid production by fungal strains on glycerol

Table 1 shows several parameters including volumetric biomass (X) and lipid (P) productions at the time of maximum lipid accumulation (t), together with biomass and lipids yields referred to substrate consumed ($Y_{X/S}$, $Y_{P/S}$, respectively).

All the strains under study grew profusely on the glycerol-based medium, with the only exception of *Chaetomium* sp. LAS_G, which showed a low ability to metabolize this polyol. The highest biomass levels were produced by *M. circinelloides* and *C. echinulata*, which also showed high conversion yields ($Y_{X/S}$ equal to 0.60 and 0.58 g/g, respectively). However, fungal growth on this substrate was not generally conducive to high lipid accumulation with the exceptions of *M. isabellina*, *M. moelleri* and *M. racemosus* (1.99 ± 0.07, 2.10 ± 0.20 and 2.33 ± 0.21 g/L), the respective $Y_{P/X}$ values of which were higher than 20% thus satisfying the requirement of oleaginicity under these growth conditions (Rossi et al., 2010). The remaining strains did not meet this requirement on glycerol.

To the best of our knowledge, two screening studies have been conducted with Zygomycetes on glycerol as the growth substrate (Fakas et al., 2009; Bellou et al., 2012). In particular, although all the strains tested by Bellou et al. (2012) exhibited higher $Y_{P/X}$ values than those obtained in the present study, the volumetric lipid productions and time requirements to reach the lipid peak were invariably lower and longer, respectively, as compared to the current study. In the present study, *C. echinulata* NRLL 3655 gave similar performance to those of a strain of the same species, grown on glycerol (Fakas et al., 2009). Moreover, *M. isabellina* NRRL 1757 (present study) exhibited a markedly lower lipid accumulation capacity than that of other strains, grown on glycerol-based media, and belonging to the same species (Papanikolaou et al., 2008; Fakas et al., 2009). Noteworthy, product yields per glycerol consumed ranged from 0.02 to 0.12 g/g with the highest values being observed with *M. racemosus* and *M. moelleri*. In this respect, it has to be taken into account that around 30 mol of acetyl-CoA are needed for the synthesis of 1 mol of triacylglycerol (Ratledge and Wynn, 2002) and that 1 mol of acetyl-CoA derives from the conversion of 1 mol of glycerol; on this basis, it has been calculated that the maximum theoretical $Y_{P/S}$ on glycerol amounts to 0.30 g/g assuming that all the acetyl-CoA pool is channeled towards lipid synthesis (Ratledge and Wynn, 2002; Papanikolaou and Aggelis, 2009). In the case of glycerol utilization by oleaginous molds, however, $Y_{P/S}$ around 0.10 g/g were observed in agreement with the present study (Papanikolaou et al., 2008). These low yields have been ascribed either to a poor regulation of the enzymes (glycerol kinase and glycerol-3-phosphate dehydrogenase) involved in the primary steps of glycerol metabolism (Papanikolaou and Aggelis, 2009) or to an inefficient uptake system. In other studies, a growth inhibition was observed as the initial content of glycerol in the production medium exceeded species-dependent concentration threshold and this effect was ascribed to an increase in the medium's osmolarity (Meesters et al., 1996; Rakicka et al., 2015; Yang et al., 2014).

3.2. Lipid production by fungal strains on OPE

All the results obtained by growing the strains under study on OPE are summarized in Table 2. This extract, as already mentioned, was a balanced mixture of easily assimilable monosaccharides, such as glucose, fructose and sucrose, and it was obtained from a D-limonene-free orange peel waste (Santi et al., 2015). Among the strains tested, *A. tubingensis* E1, *A. tubingensis* NRRL 4700, *M. miehei* and *M. isabellina* showed a remarkable growth on OPE. The last strain exhibited the highest lipid production (3.10 ± 0.16 g/L at 72 h) and the highest conversion yield $Y_{P/S}$ (0.17 ± 0.00 g/g) and these results were not far from those attained by *M. isabellina* ATHUM2935 grown on a glucose-rich medium (60 g/L) which yielded a volumetric lipid production and $Y_{P/S}$ equal to 3.9 g/L and 0.11 g/g (Chatzifragkou et al., 2010). *M. miehei* NRRL 3169, which had shown scarce lipid-producing and accumulating capacities on the glycerol-based medium, was the second most-performing strain on OPE attaining volumetric lipid production and conversion yield amounting to 2.23 g/L and 0.12 g/g, respectively. Although the lipid yield of *C. echinulata* NRRL 3655 on OPE was not dissimilar from those attained by another strain of the same species (i.e., ATHUM4411) on glucose- and fructose-rich media (0.26 vs. 0.30 and 0.21, respectively) (Chatzifragkou et al., 2010), its low growth on OPE led to scant volumetric lipid production (1.08 g/L) as shown in Table 2.

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Table 2
Volumetric biomass (X) and lipid (P) productions and yield parameters ($Y_{X/S}$, $Y_{P/X}$, $Y_{P/S}$) calculated at the time of maximal lipid accumulation (t) obtained for each of the 9 filamentous fungi strains grown on OPE medium.

Strain	X (g/L)	P (g/L)	t (h)	YX/S (g/g)	$Y_{P/X}$ (g/g)	$Y_{P/S}$ (g/g)
A. tubingensis E1	11.23 ± 0.13^{f}	1.82 ± 0.08^{c}	96	0.57 ± 0.01^{d}	0.16 ± 0.01^{a}	0.09 ± 0.00^{c}
A. tubingensis NRRL 4700	11.41 ± 0.25^{f}	1.80 ± 0.16^{c}	48	0.56 ± 0.02^{d}	0.16 ± 0.01^{a}	0.09 ± 0.00^{c}
C. echinulata NRRL 3655	4.08 ± 0.03^{b}	1.08 ± 0.04^{b}	48	0.27 ± 0.00^{b}	0.26 ± 0.01^{bc}	0.07 ± 0.00^{b}
Chaetomium sp. LAS_G	5.03 ± 0.04^{c}	1.19 ± 0.27^{b}	72	0.26 ± 0.00^{b}	0.24 ± 0.06^{bc}	0.06 ± 0.01^{b}
M. isabellina NRRL 1757	11.21 ± 0.35^{f}	3.10 ± 0.16^{e}	72	0.58 ± 0.04^{d}	0.28 ± 0.02^{c}	0.16 ± 0.01^{f}
M. circinelloides NRRL 3631	8.63 ± 0.04^{e}	1.81 ± 0.15^{c}	72	0.47 ± 0.00^{c}	0.21 ± 0.02^{ab}	0.10 ± 0.01^{de}
M. miehei NRRL 3169	11.05 ± 0.01^{f}	2.23 ± 0.02^{d}	72	0.58 ± 0.00^{d}	0.20 ± 0.00^{ab}	0.12 ± 0.00^{e}
Mucor moelleri LAS_E	5.45 ± 0.14^{d}	1.01 ± 0.08^{b}	96	0.28 ± 0.01^{b}	0.18 ± 0.00^{a}	0.05 ± 0.00^{ab}
M. racemosus UCD 71-20	2.59 ± 0.23^{a}	0.54 ± 0.02^{a}	96	0.16 ± 0.03^{a}	0.21 ± 0.02^{ab}	0.03 ± 0.00^{a}

$Y_{X/S}$ = 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. Data are the mean± SD of three replicates. Multiple pair-wise comparisons were performed by the Tukey test ($P \leq 0.05$). Same superscript letters indicate lack of statistically significant difference within column means.

Table 3
Volumetric biomass (X) and lipid (P) productions and yield parameters (YX/S, YP/X, YP/S) calculated at the time of maximal lipid accumulation (t) obtained for each of the 9 filamentous fungi strains grown on RCW medium.

Strain	X (g/L)	P (g/L)	t (h)	$Y_{X/S}$ (g/g)	$Y_{P/X}$ (g/g)	$Y_{P/S}$ (g/g)
A. tubingensis E1	10.01 ± 0.93^{d}	1.86 ± 0.03^{b}	96	0.58 ± 0.09^{c}	0.19 ± 0.02^{bc}	0.11 ± 0.01^{cd}
A. tubingensis NRRL 4700	5.85 ± 0.58^{b}	0.75 ± 0.0^{a}	72	0.30 ± 0.05^{ab}	0.13 ± 0.02^{a}	0.04 ± 0.00^{a}
C. echinulata NRRL 3655	6.49 ± 0.66^{bc}	1.09 ± 0.07^{a}	72	0.35 ± 0.06^{ab}	0.17 ± 0.03^{b}	0.06 ± 0.01^{abc}
Chaetomium sp. LAS_G	3.95 ± 0.31^{a}	0.98 ± 0.02^{a}	96	0.26 ± 0.03^{a}	0.25 ± 0.02^{d}	0.06 ± 0.00^{abc}
M. isabellina NRRL 1757	11.89 ± 0.71^{e}	4.49 ± 0.05^{d}	72	0.59 ± 0.06^{c}	0.38 ± 0.03^{f}	0.22 ± 0.01^{d}
M. circinelloides NRRL 3631	7.61 ± 0.17^{cd}	1.60 ± 0.16^{b}	72	0.52 ± 0.04^{abc}	0.21 ± 0.03^{c}	0.11 ± 0.02^{bcd}
M. miehei NRRL 3169	7.64 ± 0.16^{cd}	0.77 ± 0.02^{a}	72	0.51 ± 0.05^{abc}	0.10 ± 0.00^{a}	0.05 ± 0.01^{ab}
Mucor moelleri LAS_E	6.30 ± 0.71^{bc}	0.85 ± 0.09^{a}	72	0.36 ± 0.04^{ab}	0.13 ± 0.03^{a}	0.05 ± 0.01^{ab}
M. racemosus UCD 71-20	8.68 ± 0.66^{d}	2.63 ± 0.07^{c}	72	0.56 ± 0.08^{bc}	0.30 ± 0.03^{e}	0.17 ± 0.02^{d}

$Y_{X/S}$ = 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. Data are the mean± SD of three replicates. Multiple pair-wise comparisons were performed by the Tukey test ($P \leq 0.05$). Same letters in apex near the values indicate lack of statistically significant difference within column means.

3.3. Lipid production by fungal strains on RCW

The main carbon source in the RCW-based medium was lactose (20 g/L) the presence of which was associated with a small amount of galactose (0.4 g/L). Lactose is generally deemed to be an unsuitable substrate for supporting the growth of yeasts (Carota et al., 2017; González Siso, 1996) and filamentous fungi (Somashekar et al., 2002; Dyal and Narine, 2005; Ahmed et al., 2006). However, all the fungi tested exhibited a substantial growth capacity on RCW, as shown in Table 3. Even *C. echinulata* NRRL3655, another strain of which had shown a negligible growth on a lactose-containing medium (Papanikolaou et al., 2007), produced significant biomass levels (6.49 g/L) associated with moderate lipid-accumulating capacity (0.17 g/g). This discrepancy suggests the presence of an inter-strain variability within the *C. echinulata* species. *M. racemosus* grew well and exhibited significant lipid accumulation capacity. However, among the tested strains, *M. isabellina* NRRL 1757 performed best on the RCW-based medium, with its volumetric biomass and lipids productions amounting to 11.89 ± 0.71 and 4.49 ± 0.50 g/L, respectively, after 72 h of fermentation; its lipid-accumulating capacity was very similar to that of another strain (0.38 vs. 0.36) that had been grown on lactose as the sole carbon source (Papanikolaou et al., 2007). On a comparative basis with the other waste-based media, and irrespective of the strain, this was the best $Y_{P/X}$ value obtained in the present study.

3.4. Comparison of lipid profiles

The GC-FID characterization of lipid profiles was only limited to those strains that had shown a good versatility in using the waste under study. In addition to *M. isabellina* NRRL1757 and *M. racemosus* UCD 71-20 that satisfied this criterion, investigations were also extended to *A. tubingensis* E1, due to both its profuse growth in all the waste-based media and to its constant $Y_{P/X}$ value which was only slightly lower than that required by oleagenicity (0.19 vs. 0.20 g/g). Moreover, with the exceptions of two studies carried out under solid-state conditions (Cheirsilp and Kitcha, 2015) for the last species there is no information regarding its fatty acid profiles. Fig. 1 shows the percent compositions of fatty methyl esters (FAMEs) and biodiesel yields obtained after transesterification of total lipids produced

Irrespective of the strain under study, fungal lipids were mainly composed of long chain fatty acids with 16 and 18 carbon atoms and the five predominant ones were oleic acid ($C18:1^{\Delta 9}$), palmitic acid (C16:0), linoleic acid ($C18:2^{\Delta 9,12}$), stearic acid (C18:0) and palmitoleic acid ($C16:1^{\Delta 9}$), as usually found in oleaginous fungi (Rossi et al., 2010). The amounts of poly-unsaturated fatty acids (PUFA) in *A. tubingensis* E1 lipids were largely affected by the waste-based medium; in this strain, in fact, the relative abundance of $C18:2^{\Delta 9,12}$ passed from 32% on glycerol, to 11% on OPE and 44% on RCW, at the expense principally of oleic acid, which dropped from 53% on OPE to 15% on RCW. In this case, such a variable lipid profile associated with a high percentage of PUFA might not be deemed a good starting basis for biodiesel manufacturing, being saturated fatty acids (SFA) and mono-unsaturated fatty acids (MUFA) preferred for this purpose (Patel et al., 2017). Noteworthy, when *A. tubingensis* E1 was grown on glycerol, it produced 50 mg/L γ-linolenic acid ($C18:3^{\Delta 9,12,15}$) thus representing about 2.5% of total lipids. This fatty acid has found several applications in food industry, such as for instance, as an additive for infant formula, and in the pharmaceutical sector in the treatment of several diseases (e.g., eczema, rheumatoid arthritis and premenstrual tension). However, its production levels by the strain under study were far from those of other fungi (Béligon et al., 2016).

As far as *M. isabellina* was concerned, its lipid profiles were less affected by the growth medium than those of *A. tubingensis*, with the oleic acid content ranging from 35% to 50% and a degree of polyunsaturation that never exceeded 28%. Thus, the FAME profiles

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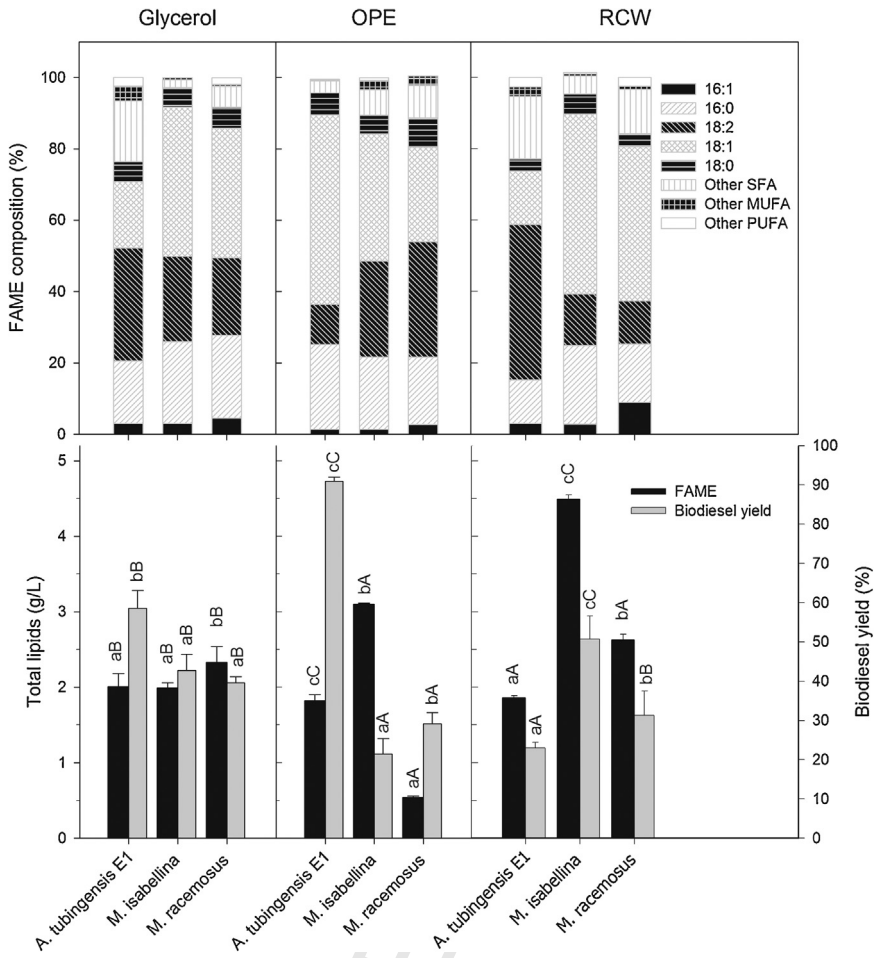

Fig. 1. Percent compositions of fatty methyl esters (FAMEs) derived from lipids produced by 3 selected strains grown in shaken flask on glycerol (A), OPE (B) and RCW (C). Corresponding biodiesel yields after transesterification (%) and total lipids determined by colorimetric assay are reported (D: glycerol; E: OPE; F: RCW). 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. Multiple pair-wise comparisons were performed by the Tukey test ($P \leq 0.05$) and same letters above the bars indicate lack of statistically significant difference ($P \leq 0.05$). Lowercase letters are referred to the comparison of values (FAME or biodiesel yield) obtained on each substrate individually; uppercase letter are referred to the comparison of values obtained by the same strain on different substrates.

of *M. isabellina*, especially those obtained on glycerol, are suitable for the production of biodiesel. *M. racemosus* exhibited similar lipid profiles to those of *M. isabellina* and, thus, it might be considered a good candidate for biodiesel production.

Among the tested strains, *M. isabellina* was the best one in terms of versatility, lipid production and FAME profiles. Consequently, this strain was selected to assess the transferability of the lipid production process to the reactor scale. To this aim, the bioprocesses with the selected strain were conducted in a lab stirred-tank bioreactor (STR) on all the three waste-based media.

3.5. *Transfer of* M. isabellina *lipid production process to STR*

In general, process transfer to bioreactor was less problematic than it might be expected. In fact, the set impeller speed (450 rpm) was able to prevent from both the formation of large cell aggregates and fouling phenomena which, instead, were observed with *M. isabellina* in a previous study thus leading to severe problems related to culture homogeneity and sampling difficulties (Papanikolaou et al., 2007). However, with the exception of the OPE-based medium, STR experiments led to significantly lower biomass production as shown in Table 4. In fact, biomass levels at the time of maximal lipid accumulation were reduced by 36.5 and 38.6% on glycerol and RCW in STR, as compared to shaken cultures; on these media, this effect was not counteracted by the increased values in $Y_{P/X}$ (i.e., 0.35 and 0.49, respectively). Thus, these results appear to confirm the reported growth inhibition phenomena exerted by high agitation rates towards the closely-related species *M. alpina* (Koike et al., 2001) and *M. isabellina* (Chatzifragkou et al., 2010; Koike et al., 2001; Papanikolaou et al., 2007). However, at least for *M. isabellina*, the problem was solved by adopting a stirring regime as low as 220 rpm thus leading to a biomass production on an YPG medium around 18 g/L with a $Y_{P/X}$ of 0.72 (Chatzifragkou et al., 2010).

For sake of exemplification, a typical fermentation kinetics of *M. isabellina* grown on one of the tested waste-based media, namely on OPE, is shown in Fig. 2. Unlike the other waste, where the carbon source was dominated by a single component, on this growth medium, a sequential utilization of carbohydrates was observed. In fact, glucose was rapidly depleted in the early 24 h as opposed to fructose and sucrose the quantitative consumptions of which from the medium took place after 36 and 72 h, respectively. Noteworthy, dissolved oxygen was not ever limiting since its percent saturation was always higher than 60% even at the end of the exponential growth phase which ended after 24 h from the inoculation.

These results are in line with other studies with different strains of *M. isabellina* showing that sucrose was metabolized less efficiently than other sugars (Chatzifragkou et al., 2010; Zhang and

Table 4
Comparison among performance indicators of biodiesel production process in STR by *M. isabellina* on glycerol, OPE and RCW, including: yields (biodiesel yield, $Y_{P/S}$, $Y_{P/X}$, $Y_{X/S}$), specific growth rate (μ), lipids and biomass production rates (rP and rX, respectively), total sugars or glycerol consumption rates (rS) and percent fatty acid composition and, finally, predicted saponification value (SV), iodine value (IV), high heating value (HHV) and cetane number (CN). All values are referred to the time of the lipid production peak.

Parameter	Dimension unit	Glycerol	OPE	RCW
Volumetric productions				
Biomass‡	g/L	4.6 ± 0.4^{a}	11.1 ± 0.8^{c}	7.3 ± 0.2^{b}
Lipids*†	g/L	1.6 ± 0.1^{a}	3.72 ± 0.22^{b}	3.62 ± 0.06^{b}
t	(h)	84	72	96
Yields §				
Biodiesel yield	(%)	47.3 ± 5.0^{b}	50.8 ± 6.2^{b}	20.0 ± 2.00^{a}
$Y_{P/X}$‡	(g/g)	0.35 ± 0.04^{a}	0.34 ± 0.04^{a}	0.49 ± 0.05^{b}
$Y_{P/S}$‡	(g/g)	0.11 ± 0.01^{a}	0.20 ± 0.02^{b}	0.26 ± 0.02^{b}
$Y_{X/S}$‡	(g/g)	0.30 ± 0.02^{a}	0.58 ± 0.06^{c}	0.49 ± 0.02^{b}
Rates §				
rP‡	g/(L h)	0.02 ± 00^{a}	0.05 ± 00^{b}	0.04 ± 0.00^{b}
rS‡	g/(L h)	0.15 ± 0.01^{a}	0.24 ± 0.01^{b}	0.14 ± 0.01^{a}
rX‡	g/(L h)	0.04 ± 0.00^{a}	0.14 ± 0.01^{b}	0.07 ± 0.00^{a}
μ‡	1/h	0.012 ± 0.001^{a}	0.014 ± 0.001^{a}	0.010 ± 0.001^{a}
Lipid profile†‡				
Palmitoleic acid	(%)	2.4 ± 0.5^{a}	2.0 ± 0.1^{a}	2.7 ± 0.4^{a}
Palmitic acid	(%)	23.3 ± 3.1^{b}	24.13 ± 1.13^{b}	20.33 ± 1.49^{a}
Linoleic acid	(%)	15.2 ± 3.4^{a}	13.9 ± 1.5^{a}	27.8 ± 1.9^{b}
Oleic acid	(%)	51.9 ± 2.7^{b}	49.8 ± 4.3^{b}	41.2 ± 1.3^{a}
Stearic acid	(%)	5.0 ± 0.4^{a}	4.6 ± 0.4^{a}	4.0 ± 0.2^{a}
Other SFA	(%)	1.5 ± 0.1^{a}	4.3 ± 0.4^{c}	2.8 ± 0.5^{b}
Other MUFA	(%)	0.50 ± 0.1^{a}	0.80 ± 0.19^{a}	1.02 ± 0.16^{a}
Other PUFA	(%)	0.10 ± 0.0^{a}	0.44 ± 0.1^{c}	0.23 ± 0.1^{b}
SV#	(mg KOH/g oil)	203.72(203.82)	203.22(206.25)	204.90(204.11)
IV#	(g I2/100 g oil)	77.32 (76.38)	73.52 (73.99)	91.62 (87.30)
HHV#	(MJ/kg)	39.92 (39.92)	40.00 (39.86)	39.65 (39.75)
CN#	–	55.69 (55.88)	56.62 (56.12)	52.32 (53.40)

‡Data are the means ± SD of duplicate reactor experiments; *calculated on the basis of lipids determined by colorimetric method (Izard and Limberger, 2003); †data are the means ± standard deviations of 4 determinations (2 technical replicates for each reactor experiment); ‡predominant fatty acids are listed as a function of increasing retention time; # the values of these parameters were predicted by the empirical models of either Kalayasiri et al. (1996) or Gopinath et al. (2009); predicted values by the latter model are shown between round brackets. Multiple pair-wise comparisons among yields, rates and single fatty acids were performed by the Tukey test ($P \leq 0.05$). Same superscript letters indicate lack of statistically significant difference within row means.

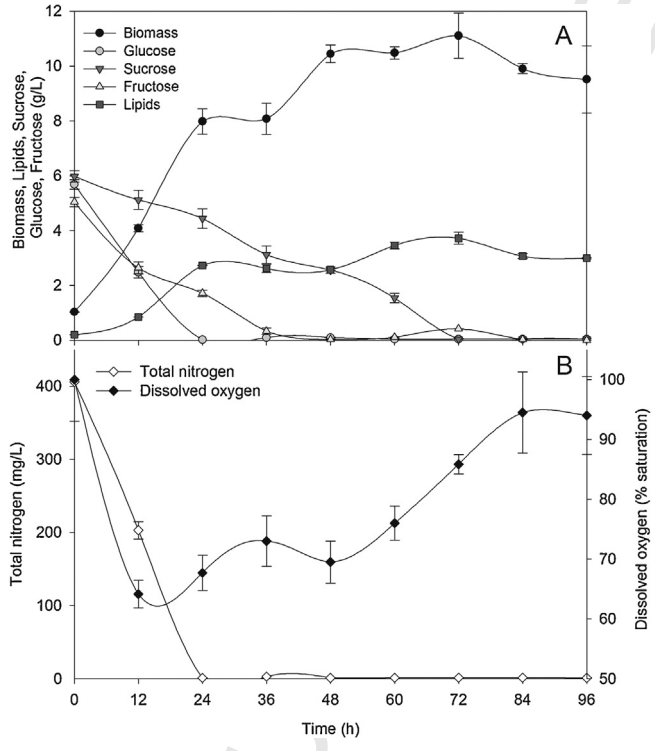

Fig. 2. Batch fermentation in stirred tank reactor of *M. isabellina* grown on OPE: (A) Time courses of lipid and biomass productions and glucose, sucrose and fructose consumption profiles; (B) nitrogen consumption and trend of dissolved oxygen saturation Data are the means ± standard deviations of duplicate reactor experiments.

Hu, 2014). From a certain point of view, this phenomenon, if properly managed, could enable the maintenance of a sort of reserve sugars to be used to support the lipid accumulation phase. Pectin, instead, was not depleted at all from the medium although this species had been shown to grow efficiently on a medium containing pectin (Zhang and Hu, 2012).

Table 4 reports comparatively growth and process performance and FAME compositions of lipids obtained from *M. isabellina* NRRL 1757 cultures grown in STR on the three waste-based media. With the only exception of linoleic acid and, as a consequence, of oleic acid, the FAME profiles of lipids obtained in the tree waste-based media were rather similar. Although biomass production in STR was generally lower than that observed in shaken cultures, it was offset by a higher lipid accumulation capacity ($Y_{P/X}$) that amounted to 35, 34 and 50% on glycerol, OPE and RCW, respectively. On the basis of the FAME profiles, important biodiesel properties, such as IV, SV, CN and HHV were calculated by two alternative models (Kalayasiri et al., 1996; Gopinath et al., 2009) and the predicted values compared each other. Irrespective of the parameter considered, the two models yielded very similar predicted values as shown in Table 4. In fact, with the only exception of predicted IVs of *M. isabellina* lipids grown on RCW, the relative differences between values predicted by the two models were invariably lower than 1.5%. The predicted SVs and HHVs were within the ranges (180–240 mg KOH/g oil and 39–41 MJ/kg, respectively) of plant oils generally used in biodiesel manufacturing (Gopinath et al., 2009). Predicted IVs and CN were largely lower and higher than the thresholds (<130 g I_2/100 g oil and 51, respectively) set by European biodiesel standards (EN14214), as shown in Table 4.

In order to assess the possible similarity of the FAME profiles of lipids obtained from *M. isabellina* grown on the three waste-based media with those of oils from higher plants commonly used

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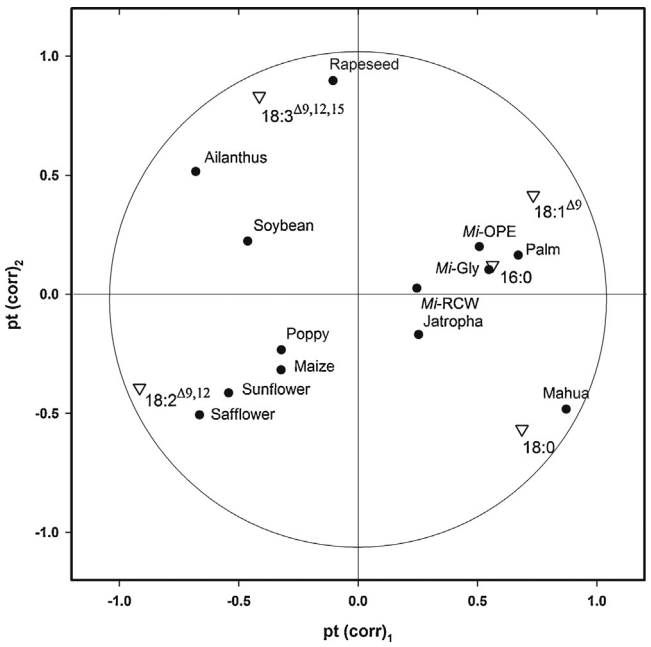

Fig. 3. Correlation biplot of lipid profiles of *M. isabellina* grown on glycerol, orange peel extract and ricotta cheese whey (Mi-Gly, Mi-OPE and Mi-RCW, respectively) and plant oils of various origins. Empty triangles represent loadings of palmitic acid (16:0), stearic acid (18:0), oleic acid ($18:1^{\Delta 9}$), linoleic acid ($18:2^{\Delta 9,12}$) and linolenic acid ($18:3^{\Delta 9,12,15}$). Percent variance explained by the first and second component amounted to 47.7% and 27.1%, respectively. The fatty acid compositions of reference plant oils have been derived from Gopinath et al. (2009).

in biodiesel production (Gopinath et al., 2009), a PCA analysis was performed. Fig. 3 shows a correlation biplot with the first and second component explaining 46.7 and 27.1% of total variance, respectively. The main variables driving separation along the first component were linoleic and oleic acids while linolenic acid mostly affected scores segregation along the second one. Irrespective of the production medium, *M. isabellina* lipids were all located in the upper right quadrant; in particular, those obtained by growing the fungus on OPE and glycerol showed a high similarity to palm oil while those obtained on RCW were more resemblant to those of Jatropha oil as shown in Fig. 3.

4. Conclusions

The feasibility of biodiesel production depends necessarily on the reduction of production costs that, in turn, are related to the microbial performance, used growth substrate, and to the fermentation and downstream processing complexity (Dourou et al., 2018). The first two items are those on which it is possible to intervene in a more significant way and represent, at the same time, the major bottleneck for second generation biodiesel production (Luque et al., 2016; Dourou et al., 2018). Recent estimates have established that the cost of the substrate can affect the economics of biodiesel production as much as 75% of the overall cost of the process; consequently, the sector is affected by the worldwide increased commercial demand for edible vegetable oils as feedstock for biodiesel industry, with consequent increase in these oils' prizes (Atabani et al., 2012; Luque et al., 2016). Moreover, the agricultural production of some feedstock, in particular palm oil that is widely used for biodiesel production, are highly controversial (Balat, 2011). Thus, the assessment of growth and lipid production potential, of oleaginous fungi on low-cost substrates still maintains a great relevance also in light of a widely reported inter-strain variability and of the lower amount of information regarding Zygomycetes as compared to oleaginous yeasts. With this in mind, several fungal strains were comparatively examined on technical glycerol and two widespread agro-industrial waste and, on the basis of their fatty acid profiles, the adequacy of their lipids for biodiesel manufacturing was evaluated. Among the strains tested, *M. isabellina* NRRL 1757 turned out to be the most versatile and performing and, for this strain, the process transfer to the lab-reactor scale was shown to be feasible, albeit with a biomass reduction which was evident with two out the three tested media. *M. isabellina* NRRL 1757 FAME profiles of lipids obtained in STR resembled to those of either Jatropha or palm oil, two commonly used starting materials for biodiesel production. As recently suggested by Gardeli et al. (2017), the carbon source profile can markedly affect lipids' typology (e.g., neutral lipids, glycolipids and phospholipids) and FAME profiles in *M. isabellina* and, above all, its lipid accumulation capacity, which, in turn, is affected by the concurrent accumulation of co-products (e.g., storage polysaccharides and polyols). All this gives in perspective, and in view of economic feasibility of the production process, wide margins for further improvements of lipid accumulation performances. The lipid-accumulating capacity of good oleaginous strains, such as that of *M. isabellina*, might be optimized *via* over-expression of genes encoding for key pathways involved in fatty acid and triacylglycerol synthesis and inhibiting, till blocking if possible, the accumulation of organic acids and other storage compounds. A possible methodology suggested in this respect by Dourou et al. (2018) is the adaptive laboratory evolution of the selected strains.

Declarations of interest

None.

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