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Research Letter

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Production of added-value microbial metabolites during growth of yeast strains on media composed of biodiesel-derived crude glycerol and glycerol/xylose blends

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One sentence summary: Value-added metabolites synthesized by fermentation of glycerol and glycerol-xylose blends by yeasts.

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ABSTRACT

A total of 11 yeast strains of *Yarrowia lipolytica*, *Metschnikowia* sp., *Rhodotorula* sp. and *Rhodosporidium toruloides* were grown under nitrogen-limited conditions with crude glycerol employed as substrate in shake flasks, presenting interesting dry cell weight (DCW) production. Three of these strains belonging to *Metschnikowia* sp. accumulated significant quantities of endopolysaccharides (i.e. the strain V.V.-D4 produced 11.0 g/L of endopolysaccharides, with polysaccharides in DCW ≈ 63% w/w). A total of six *Y. lipolytica* strains produced either citric acid or mannitol. Most of the screened yeasts presented somehow elevated lipid and polysaccharides in DCW values at the early steps of growth despite nitrogen appearance in the fermentation medium. Lipid in DCW values decreased as growth proceeded. *R. toruloides* DSM 4444 cultivated on media presenting higher glycerol concentrations presented interesting lipid-accumulating capacities (maximum lipid = 12.5 g/L, maximum lipid in DCW = 43.0–46.0% w/w, conversion yield on glycerol = 0.16 g/g). Replacement of crude glycerol by xylose resulted in somehow decreased lipid accumulation. In xylose/glycerol mixtures, xylose was more rapidly assimilated from glycerol. *R. toruloides* total lipids were mainly composed of triacylglycerols. Total cellular fatty acid composition on xylose presented some differences compared with that on glycerol. Cellular lipids contained mainly oleic and palmitic acid.

Keywords: biodiesel-derived glycerol; endopolysaccharides; microbial lipid; *Rhodosporidium toruloides*; xylose

INTRODUCTION

Biodiesel fuels are produced at constantly increasing quantities the last decade as a 'renewable' response to the decrease of hydrocarbon feedstocks and the continuously rising CO_2 emissions (Papanikolaou and Aggelis 2011, 2019; Bellou *et al.* 2014, 2016a; Patel *et al.* 2016, 2019a; Patel, Mikes and Matsakas

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2018). Biodiesel synthesis is connected with the generation of glycerol-containing water as the process by-product (Athenaki *et al.* 2018; Russmayer *et al.* 2019). The last years, biomass-based fuel production (i.e. biodiesel and bioethanol) is in constant rise (Athenaki *et al.* 2018; Russmayer *et al.* 2019) and it would be expected to increase up to 36.0 billion gallons by 2022 (Moser 2009), with glycerol-containing water being also, besides biodiesel, the principal waste stream of bioethanol production units (Russmayer *et al.* 2019). With trans-methylation of triacylglycerols (TAGs) in order to yield in the synthesis of biodiesel, *c.* 1 kg of glycerol (purity ≈ 90% w/w) per 10 kg of biodiesel is generated (Moser 2009; Russmayer *et al.* 2019). The expected increase of worldwide biodiesel production from edible vegetable oils to 30×10^6 t by 2021 will result in the generation of $\sim 3 \times 10^6$ t of glycerol, only from 1st generation biodiesel production, without taking into consideration 2nd generation biodiesel and other possible industrial routes of glycerol accumulation into the market volume (Koutinas *et al.* 2014). Therefore, conversion of this low-value product into several types of compounds currently attracts enormous significance; concerning the implication of the fermentation technology, many recent reports indicate the potential of (wild or mutant) microbial strains to convert glycerol into various interesting materials like lipids, organic acids, microbial mass and polyols (Rymowicz *et al.* 2006; Papanikolaou *et al.* 2008, 2017a, 2017b; André *et al.* 2009; Rymowicz, Rywińska and Marcinkiewicz 2009; Makri, Fakas and Aggelis 2010; Rymowicz *et al.* 2010; Kamzolova *et al.* 2011; Bellou *et al.* 2012, 2016b; Taccari *et al.* 2012; Morgunov, Kamzolova and Lunina 2013; Rywińska *et al.* 2013; Santamauro *et al.* 2014; Tomaszewska *et al.* 2014; Egermeier *et al.* 2017; Egermeier, Sauer and Marx 2019; Filippousi *et al.* 2019; Russmayer *et al.* 2019).

Lignocellulosic biomass (i.e. agricultural residues, grasses, forestry wastes, sawdust, woody biomass, various types of food residues and wastes, etc) represents the largest and probably the most attractive biomass resource worldwide that can serve as cheap feedstock amenable to be converted into a plethora of added-value compounds through the use of chemical, enzyme and microbial technology (Patel *et al.* 2016; Sarris and Papanikolaou 2016; Qin *et al.* 2017; Diwan, Parkhey and Gupta 2018; Dourou *et al.* 2018; Patel, Mikes and Matsakas 2018). Lignocellulosic materials, particularly abundant in the nature, have a significant potential for bioconversion; in fact, glucose and xylose found in several ratios, are the most important sugars of lignocellulosic biomass, and are produced *via* chemical and/or enzymatic hydrolytic treatment processes (Qin *et al.* 2017; Diwan, Parkhey and Gupta 2018). Specifically, xylose is found in several ratios into these materials (Patel *et al.* 2016, 2018; Patel, Mikes and Matsakas 2018), and the capability of microorganisms to utilize this sugar is very important (Saini *et al.* 2020).

Microbial lipids, produced by the so-called 'oleaginous' microorganisms possess similar fatty acid (FA) composition with various edible oils, and, thus, can be employed as perfect precursors of '2nd generation' biodiesel synthesis. They can also be employed in the generation of several added-value oleochemical compounds (Bellou *et al.* 2014; Patel *et al.* 2016, 2019a; Qin *et al.* 2017; Diwan, Parkhey and Gupta 2018; Dourou *et al.* 2018). On the other hand, the biotechnological interest of polysaccharides synthesized by yeasts and molds is very high, since these mycoproducts are very important for the pharmaceutical industries and the food-processing facilities (Philippoussis and Diamantopoulou 2012). Finally, citric acid and polyols present significant importance as compounds used in food industries due to their very interesting properties (Rywińska *et al.* 2013).

In the current investigation, a number yeasts belonging to *Yarrowia lipolytica*, *Metschnikowia* sp., *Rhodosporidium toruloides* and *Rhodotorula* sp. (in total 11 strains) were tested as regards their potential to assimilate biodiesel-derived crude glycerol. With the exception of the strains *R. toruloides* DSM 4444 and *Y. lipolytica* ATCC 20 460 that have been obtained from foreign official culture collections, all other yeast strains studied in the current investigation were indigenous ones, and most of them have not been previously studied as regards their quantitative physiological behavior on glycerol-based media. Moreover, only in a relatively scarce number of reports, the production of endopolysaccharides under various fermentation configurations and several employed sugars used as microbial carbon sources has been studied in yeast species (see i.e.: Tchakouteu *et al.* 2015a; Bhutada *et al.* 2017; Dourou *et al.* 2017), whereas, equally in a restricted number of papers glycerol has been employed as substrate in order for the synthesis of yeast polysaccharides to be carried out (Tchakouteu *et al.* 2015b; Sarris *et al.* 2019a; Tzirita *et al.* 2019). Thereafter, *R. toruloides* DSM 4444, a promising microorganism that was previously indicated as capable to accumulate non-negligible quantities of cellular lipids during the initial screening experiment on glycerol, was further cultivated on blends of glycerol and commercial xylose, in order to assess its potential to produce high lipid quantities on these renewable resources employed as substrates. Despite the abundance of both glycerol and xylose as renewable and low-cost carbon sources, the production of microbial oils during growth of various types of oleaginous microorganisms on blends of these substrates has rarely been reported (Díaz-Fernández *et al.* 2019), and, therefore, besides other issues, the present investigation aimed to fulfill this literature gap on this point. Technological considerations concerning the biotransformation carried out were critically discussed.

MATERIALS AND METHODS

Microorganisms and media

The strains of the current investigation belonged to the genus *Metschnikowia* sp. (strains P.D.-F1, P.D.-D2 and V.V.-D4), to the species *Yarrowia lipolytica* (strains ACA-DC 50 109, LFMB Y-20, LMBF Y-21, LMBF Y-46, LMBF Y-47 and ATCC 20 460), to the species *Rhodosporidium toruloides* (strain DSM 4444) and to the genus *Rhodotorula* sp. (strain LMBF Y-49). Strains P.D.-F1, P.D.-D2 and V.V.-D4 were isolated from the species *Prunus domestica* (plum) and *Vitis vinifera* (grape vine) (Maina *et al.* 2017), ACA-DC 50 109 and LFMB Y-20 are indigenous *Y. lipolytica* strains isolated from foodstuffs that have been previously reported to produce citric acid and mannitol (Papanikolaou *et al.* 2008, 2017a), strains LMBF Y-21, LMBF Y-46, LMBF Y-47 and LMBF Y-49 are indigenous, equally isolated from foodstuffs, strains (Tryfinopoulou *et al.* 2002), ATCC 20 460 was provided from the American Type Culture Collection, while finally strain DSM 4444 was given from the German Collection of Microorganisms and Cell Cultures. The maintenance of *Metschnikowia* sp. and *Y. lipolytica* strains was done on YPDA medium (1% glucose, 1% yeast extract, 0.5% peptone and 2% agar) while that of *Rhodotorula* sp. and *R. toruloides* on YPDMA medium (1% glucose, 1% yeast extract, 1% malt extract, 0.5% peptone and 2% agar) at $T = 4^{\circ}$C.

Liquid cultures were performed, in a medium in which the salt composition was as in Papanikolaou *et al.* (2002). Yeast extract and peptone were used as nitrogen sources. Yeast extract contained ~7% w/w nitrogen, while peptone contained 14% w/w

Table 1. Quantitative data of *Metschnikowia* sp., *Rhodotorula* sp. and *Rhodosporidium toruloides* strains deriving from kinetics on crude glycerol, in nitrogen-limited shake-flask cultures, in which the initial concentration of the substrate had been adjusted to *c.* 45 g/L. Three different points in the fermentations are represented: (a) when the maximum quantity of intra-cellular total sugars per DCW ($Y_{IPS/X}$ %, w/w) was observed; (b) when the maximum quantity of lipid per DCW ($Y_{L/X}$ %, w/w) was observed; (c) when the maximum quantity of DCW (X, g/L) was observed. Fermentation time (h) and quantities of DCW (X, g/L), total lipid (L, g/L), intra-cellular polysaccharides (IPS, g/L) and glycerol consumed ($Glol_{cons}$, g/L) are also depicted for all the above-mentioned fermentation points. Culture condition: growth on 250-ml conical flasks at 180 ± 5 rpm, initial pH = 6.0 ± 0.1, pH ranging between 5.2 and 6.0, incubation temperature $T = 28^{\circ}C$. Each experimental point is the mean value of two measurements (SE < 15%).

Strains		Time (h)	$Glol_{cons}$ (g/L)	X (g/L)	L (g/L)	$Y_{L/X}$ (%, w/w)	IPS (g/L)	$Y_{IPS/X}$ (%, w/w)
Metschnikowia	b	24	8.9	6.7	1.2	17.9	1.7	25.4
sp. P.D.-F1	a, c	166	39.1	16.4	0.6	3.7	7.2	43.9
Metschnikowia	b	24	9.4	6.5	1.0	15.4	1.7	26.2
sp. P.D.-D2	a, c	181	36.9	16.5	0.5	3.0	7.7	46.6
Metschnikowia	b	24	8.0	6.2	1.0	12.9	1.8	29.0
sp. V.V.-D4	a, c	190	41.7	17.5	0.4	2.4	11.0	62.8
Rhodosporidium	a	72	22.3	7.1	1.4	19.7	2.5	35.2
toruloides DSM	b, c	199	45.0	12.7	4.9	38.6	3.7	29.1
4444								
Rhodotorula sp.	b	48	14.8	5.0	0.8	16.0	1.1	22.0
LMBF Y-49	a, c	162	23.1	8.8	1.0	11.3	2.4	27.3

Table 2. Quantitative data of *Yarrowia lipolytica* strains deriving from kinetics on crude glycerol, in nitrogen-limited shake-flask cultures, in which the initial concentration of the substrate had been adjusted to *c.* 45 g/L. Four different points in the fermentations are represented: (a) when the maximum quantity of citric acid (CA, g/L) was obtained; (b) when the maximum quantity of lipid per DCW ($Y_{L/X}$ %, w/w) was observed; (c) when the maximum quantity of DCW (X, g/L) was observed; (d) when the maximum quantity of mannitol (Ml, g/L) was observed. Fermentation time (h) and quantities of DCW (X, g/L), total lipid (L, g/L), citric acid (CA, g/L), mannitol (Ml, g/L) and glycerol consumed ($Glol_{cons}$, g/L) are also depicted for all the above-mentioned fermentation points. Culture conditions: growth on 250-ml conical flasks at 180 ± 5 rpm, initial pH = 6.0 ± 0.1, pH ranging between 4.5 and 6.0, incubation temperature $T = 28^{\circ}C$. Each experimental point is the mean value of two measurements (SE < 15%).

Strains		Time (h)	$Glol_{cons}$ (g/L)	X (g/L)	L (g/L)	$Y_{L/X}$ (%, w/w)	CA (g/L)	Ml (g/L)	$Y_{CA/Glol}$ (g/g)	$Y_{Ml/Glol}$ (g/g)
Yarrowia lipolytica	b	24	9.9	3.3	0.5	15.1	1.1	Tr.	0.11	–
ACA DC 50 109	a, c, d	191	41.1	6.3	0.4	6.3	16.0	3.9	0.39	0.10
Yarrowia lipolytica	b	24	10.1	3.0	0.4	13.3	1.9	Tr.	0.19	–
LFMB Y-20	a, c, d	195	37.8	5.8	0.4	6.9	11.1	6.0	0.30	0.16
Yarrowia lipolytica	a, b	19	11.1	4.9	0.5	10.2	0.8	0.9	0.07	0.08
LMBF Y-21	c, d	95	46.7	9.0	0.6	6.7	1.0	8.0	0.02	0.17
Yarrowia lipolytica	b	24	11.8	3.4	0.4	11.8	3.2	0.6	0.27	0.05
ATCC 20 460	a, c, d	142	35.8	6.1	0.4	6.6	27.8	2.8	0.78	0.08
Yarrowia lipolytica	a, b	48	29.5	11.5	1.4	12.1	0.6	7.1	0.02	0.24
LMBF Y-45	c, d	120	45.6	13.0	0.6	4.6	Tr.	12.9	–	0.28
Yarrowia lipolytica	*a, b*	24	12.1	6.9	1.3	18.8	1.0	1.2	0.08	0.10
LMBF Y-46	*c, d*	141	48.9	14.0	1.0	7.1	Tr.	14.0	–	0.29

Tr.≤0.5 g/L.

nitrogen, respectively. Crude glycerol was given from the Hellenic biodiesel-producing plant 'P.N. Pettas Fats and Oils SA'. The purity of the feedstock was ≈ 90% w/w, and the major impurities were composed of salts of potassium and sodium (4%, w/w), free-fatty acids (1%, w/w) and water (5%, w/w). In the first series of experiments, all employed strains were shake-flasked at an initial glycerol ($Glol_0$) concentration = 45.0 ± 5.0 g/L, while yeast extract and peptone were employed at concentrations 1.0 and 2.0 g/L respectively. The initial C/N molar ratio in this set of trials was ≈ 60 moles/moles. Experiments were carried out in previously sterilized 250-mL conical flasks, containing 50 ± 1 mL growth medium and inoculated as in Papanikolaou *et al.* (2017b), at an agitation rate of 180 ± 5 rpm and incubation temperature $T = 28 \pm 1^{\circ}C$ (use of an orbital shaker Zhicheng ZHWY 211C; PR of China). Medium initial pH was 6.0 ± 0.1, while pH value (specifically for *Y. lipolytica*) presented a drop throughout culture and pH value was maintained to a value > 4.5 ± 0.1 by aseptically adding 2 N NaOH (Papanikolaou *et al.* 2008).

To assess the potential of *R. toruloides* DSM 4444 during its growth on higher glycerol concentration and carbon-excess media, this microorganism was cultivated on $Glol_0$ concentrations ≈ 80 g/L, while yeast extract and peptone were employed at concentrations 0.50 and 0.75 g/L respectively. Shake-flask culture parameters (i.e. temperature, agitation) remained as previously. In this experiment the initial C/N molar ratio was ≈ 270 moles/moles, therefore much higher nitrogen limitation compared to the screening experiment was employed. Likewise, to quantify the behavior of the microorganism on media composed of mixtures of glycerol and xylose (two abundant agro-industrial substances amenable for their utilization in the Industrial Microbiology–Patel *et al.* 2016; Russmayer *et al.* 2019), commercial xylose (Xyl) (with a purity of *c.* 95% w/w) and crude glycerol (purity *c.* 90% w/w—see previously) were employed as simultaneous substrates at a total concentration = 92 ± 12 g/L and blends of Glol/Xyl were employed at the ratios 100%–0%, 75%–25%, 50%–50%, 25%–75% and 0%–100%. Equally, in this set of

Table 3. Production of intra-cellular polysaccharides (IPS) by yeast strains cultivated under various fermentation configurations.

Yeast strain	IPS (g/L)	$Y_{IPS/X}$ (%, w/w)	Substrate	Cultivation type	Reference
Cryptococcus curvatus NRRL Y-1511	11.0	41.2	Lactose	Shake flasks	Tchakouteu *et al.* (2015a)
≫	10.9	41.2	Sucrose	≫	≫
≫	11.1	28.8	Cheese-whey	≫	≫
≫	9.9	27.8	Molasses	≫	≫
Lipomyces starkeyi DSM 70 296	6.7	28.9	Crude glycerol	≫	Tchakouteu *et al.* (2015b)
Yarrowia lipolytica H222	n.i.†	≈17.0	Pure glycerol	≫	Bhutada *et al.* (2017)
≫	≫	≈12.0	Glucose		≫
Saccharomyces cerevisiae WT	≫	≈12.0	≫	≫	≫
Yarrowia lipolytica ACA-DC 50 109	≈6	≈45	≫	≫	Dourou *et al.* (2017)
Yarrowia lipolytica FMCC Y75	6.6	51.6	Crude glycerol	≫	Filippousi *et al.* (2019)
Cryptococcus curvatus NRRL Y-1511	8.4	62.2	≫	≫	≫
Rhodosporidium kratochvilovae FMCC Y71	3.7	41.1	≫	≫	≫
Debaryomyces sp. FMCC Y68	7.1	33.0	≫	≫	≫
Debaryomyces sp. FMCC Y69	13.9	45.5	≫	Batch reactor	≫
Yarrowia lipolytica ACA-DC 50 109	≈4.5	≈45††	Glucose	Shake flasks	Daskalaki *et al.* (2019)
≫	≈3	≈32†††	Glucose	≫	≫
Yarrowia lipolytica ACA-DC 5029	3.8	34.0	Crude glycerol	≫	Sarris *et al.* (2019a)
≫	2.4	20.8	Glycerol/O.M.W.&	≫	≫
Yarrowia lipolytica ACA-DC 5031	2.6	31.9	≫	≫	Tzirita *et al.* (2019)
Metschnikowia sp. V.V.-D4	11.0	62.8	Glycerol	≫	Present study

†: n.i.: Not indicated in the relevant manuscript.
††: Early growth phase, starting wild-type strain.
†††: Early growth phase, strain subjected to adaptive laboratory evolution for enhanced lipid production.
&: O.M.W. is the olive-mill wastewater.

Table 4. Quantitative data of *Rhodosporidium toruloides* DSM 4444 cultivated on media composed of mixtures of crude glycerol (Glol) and commercial xylose (Xyl), in nitrogen-limited shake-flask experiments, in which the initial concentration of the substrate (Glol + Xyl) had been adjusted to 92 ± 12 g/L. Four different points in the fermentations are represented: (a) when the maximum quantity of DCW (X, g/L) was obtained; (b) when the maximum quantity of total cellular lipid (L, g/L) was observed; (c) when the maximum quantity of total cellular polysaccharides (IPS, g/L) was observed; (d) when the maximum quantity of xylitol (Xol, g/L) was observed. Fermentation time (h) and quantities of DCW (X, g/L), total lipid (L, g/L), total polysaccharides (IPS, g/L), xylitol (Xol, g/L), consumed xylose (Xyl_{cons}, g/L) and consumed glycerol ($Glol_{cons}$, g/L) are also depicted for all the above-mentioned fermentation points. Culture conditions: growth on 250-mL conical flasks at 180 ± 5 rpm, initial pH = 6.0 ± 0.1, pH ranging between 5.5 and 6.0, incubation temperature $T = 28^{\circ}C$. Each experimental point is the mean value of two measurements (SE < 15%).

Substrate (Glol—Xyl, %w/w)	Time (h)	X (g/L)	L (g/L)	IPS (g/L)	Xol (g/L)	$Glol_{cons}$ (g/L)	Xyl_{cons} (g/L)	$Y_{L/X}$ (%, w/w)	$Y_{Xol/Xyl}$ (g/g)	$Y_{IPS/X}$ (%, w/w)
100%–0%(†)	280 [a,b]	23.1	10.1	4.7	–	78.8	–	43.7	–	20.2
	220 [c]	19.2	8.8	4.8	–	67.7	–	45.9	–	24.9
100%–0%	330 [a,b,c]	28.9	12.5	6.1	–	95.1	–	43.3	–	21.0
75%–25%	325 [a,b,c]	23.0	9.0	4.6	0.0	75.3	26.9	39.1	–	20.0
	142 [d]	10.4	2.1	3.1	5.2	28.7	26.9	20.4	0.19	29.6
50%–50%	336 [a,b]	22.3	8.7	4.4	0.0	39.3	41.8	38.8	–	19.5
	360 [c]	22.3	7.1	4.5	0.0	39.3	41.8	31.9	–	20.0
	97 [d]	9.4	3.7	2.8	5.2	12.2	23.3	39.3	0.22	29.2
25%–75%	359 [a,b,c]	22.5	8.3	7.6	3.0	24.1	75.1	36.9	0.04	20.3
	119 [d]	20.4	2.3	5.0	8.4	14.0	48.5	11.4	0.17	24.4
0%–100%	340 [a,b]	21.1	7.7	1.9	7.0	–	80.8	36.5	0.09	9.1
	214 [c]	13.6	4.9	2.0	8.5	–	55.6	36.2	0.15	14.4
	189 [d]	11.9	4.3	1.7	11.0	–	45.4	36.0	0.24	14.4

†: Trial performed with $Glol_0 \approx 80$ g/L.

experiments, a much higher nitrogen limitation compared to the screening trials was performed (yeast extract and peptone were employed at 0.50 and 0.75 g/L; initial C/N molar ratio in this set was ≈ 315 moles/moles). All other parameters in the shake-flask trials (temperature, rate of agitation, etc) remained as previously.

Analyses

In order to proceed to the various analyses, the whole content of the flasks was harvested by the means of centrifugation [9000 × *g*/15 min at 10°C; use of a Hettich Universal 320R (Germany) centrifuge]. Cells were washed with water once, re-

centrifuged and finally were dried at $T = 80 \pm 5°C$ (wet cells were placed to the above temperature usually for 26 ± 4 h). Biomass concentration (X, g/L) was, therefore determined by dry cell weight (DCW) value. Compounds founds into the supernatant (*viz.* glycerol, mannitol and citric acid) were determined through HPLC analysis (Papanikolaou *et al.* 2017b). Total intracellular polysaccharides (IPS, presented both as g/L and as % w/w in DCW) were measured according to Diamantopoulou *et al.* (2012).

Cellular lipids were extracted from yeast dry biomass by virtue of the modified 'Folch' method, in which the blend of chloroform/methanol (C/M) in a 2:1 (v/v) ratio was used as solvent (Gardeli *et al.* 2017). Lipids were esterified and GC analyses were carried according to Gardeli *et al.* (2017). When *R. toruloides* DSM 4444 was cultivated in media containing an elevated $Glol_0$ concentration (≈80 g/L) and higher nitrogen limitation, produced lipids were fractionated into their lipid fractions [glycolipids plus sphingolipids (G + S), neutral lipids (NLs) and phospholipids (PLs)] according to Daskalaki *et al.* (2019). In the same experiment and fermentation point, analysis by the means of TLC was performed for the crude lipid ('Folch' extract) of *R. toruloides* DSM 4444 according to Papanikolaou *et al.* (2017a). Glyceryl trioleate (TAG), cholesterol (CL), cholesteryl linoleate (CE), oleic acid (FFA) and monononadecanoin (MAG) were employed as lipid standards for the identification of the main bands on TLC plates.

Nomenclature

X: Biomass (dry cell weight—DCW); Glol: Glycerol; L: Total lipids; IPS: Total endopolysaccharides; CA: Citric acid; Ml: Mannitol; Xol: xylitol; $Y_{A/B}$: Quantity of element A per quantity of element B; indexes 0 and cons show the initial and the consumed quantity of the elements in the experiments carried out.

RESULTS AND DISCUSSION

Initial screening on crude glycerol

Initially, all available strains were cultivated on crude glycerol at $Glol_0 \approx 45$ g/L (C/N ≈ 60 moles/moles; see results in Tables 1 and 2). Concerning *Metschnikowia* sp. strains, no one from these strains revealed a significant oleaginous character. Total lipid in DCW ($Y_{L/X}$) for all studied strains presented relatively elevated values at the first growth phases (i.e. *c.* 24 h after inoculation, a time in which available nitrogen in non-negligible amounts was found into the fermentation medium or it had barely disappeared; data not presented) ranging between 12.9 and 17.9% w/w, while these values were depleted (to < 4% w/w) when the fermentation proceeded. Lipid in DCW values decreased when nitrogen limitation was imposed into the medium, in contrast to the theory suggesting that the onset of accumulation of cellular lipids is given only when the extra-cellular available nitrogen is no longer available (Athenaki *et al.* 2018; Dourou *et al.* 2018; Papanikolaou and Aggelis 2019). It is interesting to indicate that strains of the genus *Metschnikowia* sp. have been used some times for lipid production during growth on glycerol-based media and high lipid quantities have been achieved (Santamauro *et al.* 2014; Canonico *et al.* 2016), while in some cases *Metschnikowia* sp. have been revealed capable to present significant DCW and lipid production in media presenting excessively high (i.e. ≈25% w/v) $Glol_0$ concentrations (Santamauro *et al.* 2014). It can also been deduced that all newly isolated *Metschnikowia* sp. strains presented very interesting DCW production (X_{max} ranging between 16.4 and 17.5 g/L, with simultaneous biomass yield per glycerol consumed $Y_{X/Glol} = 0.42–0.45$ g/g). Moreover, simultaneously high concentrations of endopolysaccharides (up to 11.0 g/L; simultaneous endopolysaccharides per DCW values $Y_{IPS/X} > 43.0\%$ w/w, reaching the value of 62.8% w/w for the strain *Metschnikowia* sp. V.V.-D4) were stored inside the yeast cells for the entire newly isolated *Metschnikowia* sp. strains tested. It is also noted that in the early growth steps in the above-mentioned microorganisms (0–24 h after inoculation) and although nitrogen was found into the growth medium, remarkable amounts of endopolysaccharides ($Y_{IPS/X} > 25.0\%$ w/w) have been accumulated by the tested strains. Similar behavior has been observed for the newly isolated *Rhodotorula* sp. LMBF Y-49 and also for other yeast species like *Cryptococcus curvatus* (Tchakouteu *et al.* 2015a; Filippousi *et al.* 2019), *Lipomyces starkeyi* (Tchakouteu *et al.* 2015b), *Yarrowia lipolytica* (Bhutada *et al.* 2017; Dourou *et al.* 2017; Daskalaki *et al.* 2019; Filippousi *et al.* 2019), *Rhodosporidium kratochvilovae* (Filippousi *et al.* 2019) and *Naganishia uzbekistanensis* (Filippousi *et al.* 2019) indicating that the theory by virtue of which endopolysaccharides, as storage lipids, need nitrogen limitation in order to be accumulated in significant quantities inside the yeast cells (Ratledge 1988; Papanikolaou and Aggelis 2011) seems incomplete (Dourou *et al.* 2018). Metrics of endopolysaccharides production by yeast species grown in various microbial substrates and culture strategies and their comparison with the present investigation are depicted in Table 3.

All of the 6 *Y. lipolytica* strains presented appreciable quantities of DCW production, indicating the suitability of glycerol as substrate for strains of this yeast in accordance to earlier or more recent studies (Papanikolaou *et al.* 2002, 2008; Rymowicz *et al.* 2006; Rymowicz, Rywińska and Marcinkiewicz 2009; Rymowicz *et al.* 2010; Taccari *et al.* 2012; Sarris *et al.* 2019a, 2019b). As in the previous case of *Metschnikowia* sp., all tested *Y. lipolytica* strains presented somehow elevated lipid in DCW ($Y_{L/X}$) values at the relatively early growth steps in which balanced growth occurred (0–48 h after inoculation), and, thereafter, $Y_{L/X}$ values were drastically reduced even if important glycerol concentrations remained into the medium whereas nitrogen had been exhausted. The period of lipid content decrease (in %, w/w) coincided with the biosynthesis and the accumulation into the medium of low molecular weight secondary metabolic compounds (*viz.* citric acid and/or mannitol) in non-negligible quantities, and, has previously been reported as a principal physiological pattern for several strains of this species (André *et al.* 2009; Makri, Fakas and Aggelis 2010; Chatzifragkou *et al.* 2011; Papanikolaou *et al.* 2017b; Sarris *et al.* 2019a, 2019b). From the 6 screened strains belonging to *Y. lipolytica*, 3 strains (namely LMBF Y-21, LMBF Y-45 and LMBF Y-46) produced promising quantities of mannitol (ranging between 8.0 and 14.0 g/L), while insignificant (i.e. ≤1.0 g/L) quantities of citric acid were simultaneously synthesized. In all cases, medium pH remained always > 4.5, a value that normally favors the biosynthesis and accumulation into the medium of citric acid instead of that of polyols (Rymowicz *et al.* 2006; Rymowicz, Rywińska and Marcinkiewicz 2009; Tomaszewska *et al.* 2014; Sarris *et al.* 2019b). In agreement with the results reported in the current investigation, the last years there have been some reports that deal with the synthesis of principally polyols (in most cases these polyols are mannitol and erythritol) by wild-type *Y. lipolytica* strains cultivated under nitrogen-limitation conditions with glycerol as substrate, in which pH values were always ≥ 4.5 (Egermeier *et al.* 2017; Papanikolaou *et al.* 2017b), indicating that the mechanisms that

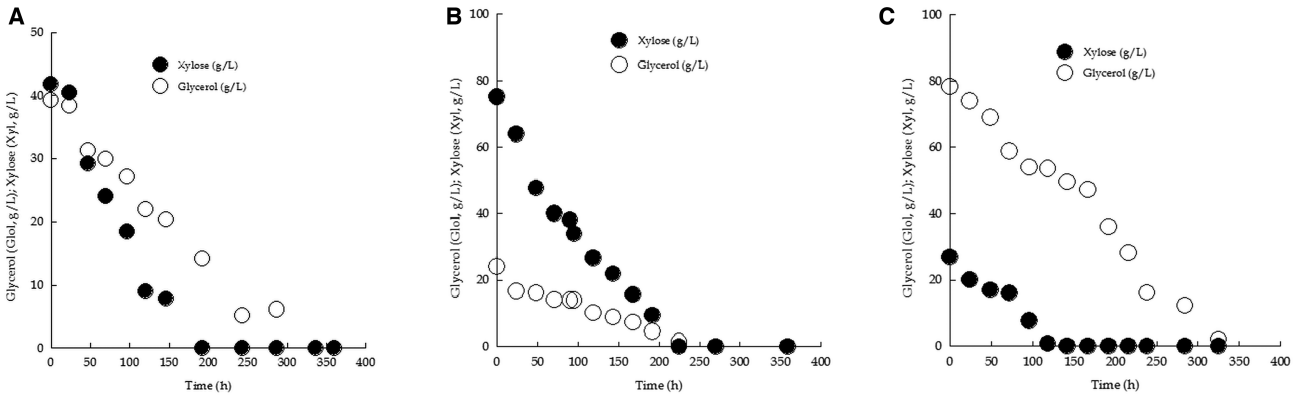

Figure 1. Kinetics of glycerol (Glol, g/L) and xylose (Xyl, g/L) assimilation by *Rhodosporidium toruloides* DSM 4444, during growth on biodiesel-derived glycerol and commercial xylose blends in shake-flask experiments under nitrogen-limited conditions. Culture conditions: growth on 250-mL conical flasks at 180 ± 5 rpm, initial pH = 6.0 ± 0.1, pH ranging between 5.5 and 6.0, incubation temperature $T = 28^{\circ}C$. Initial sugar + polyol concentration ≈ 90 ± 10 g/L, xylose/glycerol blend 50/50 in (1a), xylose/glycerol blend 75/25 in (1b), xylose/glycerol blend 25/75 in (1c). Each experimental point is the mean value of two measurements (SE < 15%).

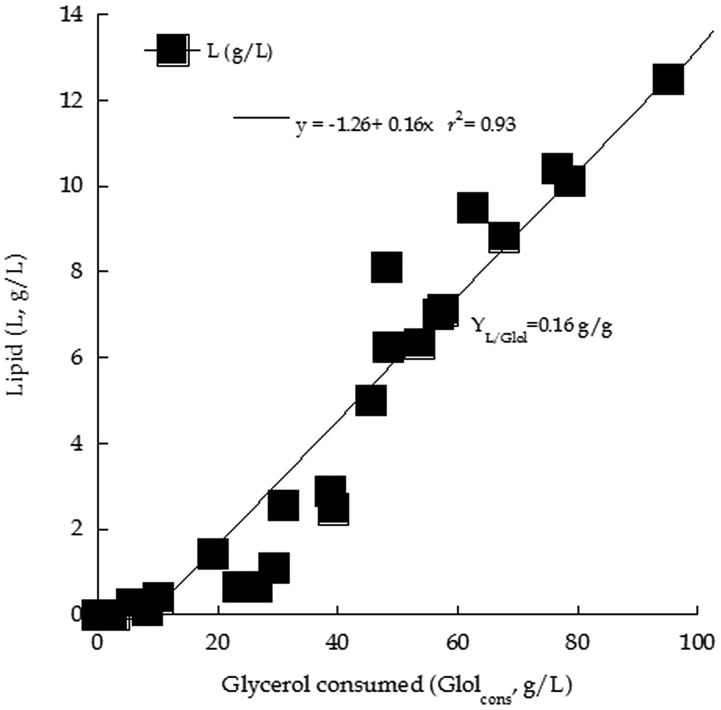

Figure 2. Lipid produced *vs* glycerol consumed in shake-flask experiment during growth on biodiesel-derived glycerol of *Rhodosporidium toruloides* DSM 4444, at elevated initial substrate concentration ($Glol_0 \approx 80$ g/L and $Glol_0 \approx 100$ g/L) under nitrogen-limited conditions. Culture conditions: growth on 250-mL conical flasks at 180 ± 5 rpm, initial pH = 6.0 ± 0.1, pH ranging between 5.5 and 6.0, incubation temperature $T = 28^{\circ}C$. Each experimental point is the mean value of two measurements (SE < 15%).

are governing the synthesis of polyols *vs* those of citric acid are much more complicated than a simple pH regulation in values ≈ 3.0–3.5. Finally, the sole yeast strain that indicated real oleaginous capacities (lipid in DCW values > 20% w/w) was *R. toruloides* DSM 4444 (see Table 1), that was studied in more details in the next chapter.

Lipid production of *Rhodosporidium toruloides* DSM 4444 growing at higher nitrogen limitation conditions on glycerol/xylose blends

In the next stage of the study, *R. toruloides* DSM 4444 was cultured on media composed of commercial xylose and crude glycerol as simultaneous substrates (total sugar + polyol concentration ≈ 90 ± 10 g/L), with blends of glycerol/xylose being employed at various ratios in shake-flask fermentations in which high nitrogen limitation had been imposed. A trial with $Glol_0 \approx 80$ g/L employed as sole substrate was also carried out (see results in Table 4). From the achieved results, it can be concluded that *R. toruloides* presented significant DCW production in all media employed as substrates. Moreover, apparently higher lipid concentrations, both in absolute (g/L) and relative (% w/w in DCW) values, were recorded when glycerol was the sole microbial carbon source, with the relevant maximum values (*viz*: L_{max} and $Y_{L/Xmax}$) being somehow reduced when glycerol had been substituted from xylose. Similarly, mixtures of xylose with glycerol allowed higher lipid production than that attained with xylose utilized as sole substrate in experiments in which genetically engineered *Ashbya gossypii* strains have been employed (Díaz-Fernández *et al.* 2019). Moreover, the presence of

Table 5. Lipid production by *Rhodosporidium* sp. (mostly *Rhodosporidium toruloides* unless it is indicated at the end of the table) yeast strains cultivated under various fermentation configurations.

Strain	Substrate	Cultivation type	DCW (g/L)	$Y_{L/X}$ (% w/w)	Reference
AS2.1389	Glucose	Shake flasks	18.3	76.0	Li *et al.* (2006)
Y4	Glucose	Fed-batch bioreactor	106.5	67.5	Li, Zhao and Bai (2007)
≫	Glucose (phosphate-limited trial)	≫	20.6	51.4	Wu *et al.* (2010)
≫	Jerusalem artichoke extracts	≫	25.5	40.0	Zhao *et al.* (2010)
≫	Glucose (sulfate-limited trial)	≫	14.2	55.6	Wu *et al.* (2011)
CCT 0783	Glucose/xylose blend	Batch bioreactor	13.3	42.0	Bonturi *et al.* (2015)
CBS14	Glucose	Fed-batch bioreactor	35.0	71.4	Wiebe *et al.* (2012)
≫	Glucose/xylose/arabinose blend	≫	27.0	55.5	≫
AS2.1389	Crude glycerol	Shake flasks	19.2	47.7	Xu *et al.* (2012)
≫	≫	Batch bioreactor	26.7	69.5	≫
Y4	≫	≫	35.3	46.0	Uçkun Kiran, Trzcinski and Webb (2013)
2F5	Inulin	Shake-flasks	15.8	62.1	Wang *et al.* (2014)
≫	≫	Batch bioreactor	15.6	70.4	≫
Y4	Crude glycerol	Shake flasks	24.9	48.9	Yang *et al.* (2014)
DSM 4444	Glucose	Batch bioreactor	~22	~40	Bommareddy *et al.* (2015)
≫	Pure glycerol	≫	~15	~57	≫
CCT 0783	Glucose/xylose blend	≫	13.3	42.0	Bonturi *et al.* (2015)
DSM 4444	Crude glycerol/sunflower meal hydrolysate	Shake flasks	27.9	29.0	Leiva-Candia *et al.* (2015)
≫	≫	Fed-batch bioreactor	37.4	51.3	≫
CCT 0783	Sweet sorghum extract	Shake flasks	41.7	33.1	Matsakas *et al.* (2015)
NRRL Y-27 012	Crude glycerol	≫	30.1	40.0	Tchakouteu *et al.* (2015b)
DMKU-RK253†	Crude glycerol	Batch bioreactor	14.1	63.8	Polburee *et al.* (2016)
≫	≫	Shake flasks	23.0	69.5	≫
DSM 4444	FRWH*/glucose	Fed-batch bioreactor	49.0	57.5	Tsakona *et al.* (2016)
≫	Glucose (NaCl-enriched)	≫	37.2	64.5	Tchakouteu *et al.* (2017)
≫	Crude glycerol	Shake flasks	37.0	37.0	Papanikolaou *et al.* (2017b)
AS 2.1389	Crude glycerol	≫	26.5	37.7	Xu *et al.* (2017)
NCYC 1576	BSGH**	≫	18.4	56.5	Patel *et al.* (2018)
FMCC Y70††	≫	≫	8.6	19.8	Filippousi *et al.* (2019)
HIMPA1††	Clarified butter sediment waste	≫	15.5	70.7	Patel *et al.* (2019b)
CBS14	Glucose	Batch bioreactor	13.8	47.5	Tiukova *et al.* (2019)
≫	Xylose	≫	11.1	47.1	≫
NRRL Y-27 012	Molasses	Fed-batch bioreactor	41.0	61.0	Boviatsi *et al.* (2020)
DSM 4444	Crude glycerol	Shake flasks	28.9	43.3	Present study
≫	Commercial xylose	≫	21.1	36.5	≫

†: *Rhodosporidium fluviale*.
††: *Rhodosporidium kratochvilovae*
*: FRWH is a flour-rich waste hydrolysate
**: BSGH is the brewers' spent grain hydrolysate

xylose into the medium was accompanied by secretion of xylitol, the maximum produced quantity of which was positively related with the increase of the initial xylose amount into the mixture (see Table 4). Xylitol was mostly accumulated into the growth medium at the relatively early stages of the culture, and was partially or completely re-consumed by *R. toruloides* in order for biomass and cellular lipids to be synthesized. Xylitol secretion has been already reported for other oleaginous microorganisms cultivated on xylose as microbial substrate (or co-substrate) like *Thamnidium elegans* (Zikou *et al.* 2013), *Mortierella isabellina* (Gardeli *et al.* 2017) and *A. gossypii* (Díaz-Fernández *et al.* 2019) and might reflect the saturation of the activity of the enzyme xylitol dehydrogenase, that is a very important and crucial one for the catabolism of xylose in the xylose-consuming microorganisms (Papanikolaou and Aggelis 2011, 2019; Díaz-Fernández *et al.* 2019).

The somehow lower lipid accumulation by *R. toruloides* on media in which xylose had been employed as the principal or the sole carbon source compared with that achieved on glycerol-rich media, suggests the utilization of the pentose phosphate pathway by the above-mentioned microorganism and not that of phosphoketolase, concerning xylose assimilation and breakdown; it is known that xylose catabolism can be either performed through the phosphoketolase reaction (synthesis of c. 1.2 moles of acetyl-CoA per 100 g of xylose assimilated), or the pentose phosphate pathway (synthesis of c. 1.0 mole of acetyl-CoA is formed per 100 g of xylose utilized) (Ratledge 1988; Papanikolaou and Aggelis 2011). Thus, due to the lower lipid production on xylose-rich media compared with the trials on glycerol, potentially the later pathway seems to be used for the catabolism of xylose in *R. toruloides*. Additionally and in agreement with the findings reported for the previously screened yeasts (see Table 1),

Table 6. Fatty acid composition of the cellular lipids produced by yeast strains cultivated on crude glycerol in shake-flask experiments ($Glol_0$ concentration adjusted at *c.* 40 g/L). Time of fermentation for the determination of the fatty acid composition was between 150 and 200 h after inoculation. Culture conditions as in the Tables 1 and 2.

	Fatty acid composition of yeast lipids (%, w/w)					
Yeast strain	C16:0	C18:0	C18:1 (ω-9)	C18:2 (ω-6)	C18:3 (ω-3)	Others
Metschnikowia sp. P.D.-F1	20.0	5.0	50.6	3.2	Tr.	21.2*
Metschnikowia sp. P.D.-D2	11.4	3.4	39.2	31.3	Tr.	14.7**
Metschnikowia sp. V.V.-D4	9.4	Tr.	33.9	41.8	Tr.	14.9**
Rhodosporidium toruloides DSM 4444	25.9	10.1	50.3	9.9	1.2	2.6§
Rhodotorula sp. LMBF Y-49	Tr.	Tr.	8.3	68.1	2.6	21.0**
Yarrowia lipolytica LFMB Y-20	14.0	8.1	41.4	28.9	2.5	5.1#
Yarrowia lipolytica ACA-DC 50 109	14.0	9.9	45.5	21.0	3.5	6.1#
Yarrowia lipolytica ATCC 20 460	22.4	9.0	49.2	6.4	4.1	8.9#
Yarrowia lipolytica LMBF Y-45	17.6	8.0	46.9	19.3	5.0	3.2#
Yarrowia lipolytica LMBF Y-46	16.9	4.0	44.2	22.3	6.9	5.7#
Yarrowia lipolytica LMBF Y-21	12.1	5.9	44.1	21.8	5.0	11.1#

*: C10:0, C12:0, C14:0, C16:1 (ω-7).
**: C12:0, C14:0, C16:1 (ω-7).
§: C14:0, C16:1 (ω-7), C22:0.
#: C14:0, C16:1 (ω-7).
Tr.: < 0.5%.

Table 7. Fatty acid composition of the cellular lipids produced by *Rhodosporidium toruloides* DSM 4444 cultivated on crude glycerol and commercial xylose blends in shake-flask experiments in which the initial concentration of the substrate (Glol + Xyl) had been adjusted to 92 ± 12 g/L. Culture conditions as in Table 4.

	Fatty acid composition of yeast lipids (%, w/w)				
Culture time	C16:0	C18:0	C18:1 (ω-9)	C18:2 (ω-6)	Others§
Substrate: (Glol—Xyl 100%–0%)					
95 h	24.6	6.7	47.4	8.3	13.8
167 h	28.6	6.1	43.6	7.9	13.8
216 h	31.1	6.6	38.6	8.1	15.6
265 h	31.7	5.2	36.9	6.7	19.5
Substrate: (Glol—Xyl 75%–25%)					
72 h	21.0	Tr.	53.6	25.4	Tr.
142 h	29.0	6.3	52.2	8.5	4.0
216 h	27.6	8.1	51.9	8.8	3.6
284 h	29.3	8.2	51.4	9.4	1.7
Substrate: (Glol—Xyl 50%–50%)					
47 h	23.7	Tr.	56.9	13.9	5.5
146 h	26.3	6.1	51.4	7.9	8.3
243 h	26.4	7.5	47.2	9.9	9.0
336 h	25.7	7.7	46.4	11.3	8.9
Substrate: (Glol—Xyl 25%–75%)					
71 h	25.4	5.1	57.7	5.0	6.8
143 h	26.0	5.6	59.5	4.2	4.7
192 h	26.5	5.4	56.3	5.3	6.5
359 h	25.1	5.4	54.9	9.8	4.8
Substrate: (Glol—Xyl 0%–100%)					
70 h	25.2	5.1	62.1	7.6	0.0
119 h	25.2	6.6	59.5	8.7	0.0
168 h	25.4	8.0	58.4	8.2	0.0
214 h	25.1	7.9	56.2	8.3	2.5
Rapeseed Oil	2–7	1–3	50–66	18–28	6–14

Tr.:<0.5%.
§: C14:0, C16:1 (ω-7), C18:3 (ω-3), C22:0.

for all *R. toruloides* cultures performed, slightly higher quantities of polysaccharides per DCW ($Y_{IPS/X}$, in % w/w) seemed to be synthesized at the earlier stages of growth, whereas the lowest $Y_{IPS/X}$ values were recorded in the trial in which xylose was employed as the sole substrate.

In all cases in which glycerol has been employed as co-substrate of xylose, and irrespective of the initial concentrations of the carbon sources employed into the mixture, remarkably higher consumption rate of xylose occurred compared with that of glycerol (Fig. 1). In general, utilization of glycerol and

Table 8. Fatty acid composition of the cellular lipids produced by *Rhodosporidium toruloides* DSM 4444 for selected fermentation points of the kinetics, during growth on crude glycerol at initial substrate concentration ($Glol_0$) ≈80 g/L) (a); Quantities (in %, w/w) of neutral (NL), sphingolipid and glycolipid (G + S) and phospholipid (PL) fractions during lipid accumulation phase of *Rhodosporidium toruloides* DSM 4444 during growth on crude glycerol at $Glol_0 \approx 80$ g/L, *c.* 100 h after inoculation, and fatty acid composition (in %, w/w) of lipid fractions as compared with the fatty acid composition of total lipid for the relevant fermentation point (b). Culture conditions as in Table 4.

8a

Fatty acids—Culture time	24 h	40 h	48 h	100 h	120 h	144 h	167 h	280 h
C16:0	20.6	22.4	22.8	27.5	28.6	28.3	29.2	30.0
C18:0	7.6	8.1	8.2	8.5	9.7	9.0	9.2	10.2
C18:1 (*ω*-9)	53.5	52.0	49.0	51.0	47.6	50.3	46.7	45.9
C18:2 (*ω*-6)	10.5	8.5	10.5	8.1	2.3	5.0	2.3	3.1
Others*	6.8	9.0	9.5	4.9	11.8	7.4	12.6	10.8

8b

	% w/w	C16:0	C18:0	C18:1 (*ω*-9)	C18:2 (*ω*-6)
Total lipid		27.5	8.5	51.0	8.1
NL	86.0	29.9	7.5	50.9	8.0
G + S	12.1	27.5	7.9	54.4	9.1
PL	1.9	22.1	9.0	59.0	9.5

*: C14:0, C16:1 (*ω*-7), C18:3 (*ω*-3), C22:0.

xylose as simultaneous substrates by various types of microorganisms has been seen a number of times, and in accordance with the current study, both substrates seemed to be assimilated simultaneously (Kurosawa *et al.* 2015; Zhou, Xu and Yu 2016; Díaz-Fernández *et al.* 2019; Stoklosa, Nghiem and Latona 2019), whereas in most cases (utilization of genetically engineered *Rhodococcus opacus* MITXM-61 and *Gluconobacter oxydans* NL71 strains and wild-type *Phaffia rhodozyma* ATCC 74 219) xylose uptake rate was seen to be higher than that of glycerol (Kurosawa *et al.* 2015; Zhou, Xu and Yu 2016; Stoklosa, Nghiem and Latona 2019). In contrast, for the genetically engineered *A. gossypii* strains A729 and A877 cultivated on blends of glycerol and xylose, glycerol uptake rate was slightly or remarkably higher than that of xylose (Díaz-Fernández *et al.* 2019). Finally, in other cases (i.e. *Candida mogii* NRRL Y-17 032), uptake rates of glycerol and xylose seemed to be similar (Stoklosa, Nghiem and Latona 2019). Concerning the case of *R. toruloides*, the strain ATCC 204 091 cultivated on blends of glucose/xylose/glycerol presented a sequential consumption of these substrates with a reported 'triauxic' growth (Singh *et al.* 2018) whereas the same strain used in the present investigation (DSM 4444) during its culture on glucose/glycerol blends consumed rapidly glucose and onset of glycerol assimilation occurred only when the concentration of glucose was essentially low, due to catabolite repression of glycerol uptake by glucose (Bommareddy *et al.* 2015; Bommareddy, Sabra and Zeng 2017). Finally, the strain *R. toruloides* CCT 0783 cultivated on sugars blends was found to assimilate both glucose and fructose at similar rates (Matsakas *et al.* 2015).

R. toruloides DSM 4444 presented appreciable lipid production during growth on glycerol-based media ($L_{max} = 12.5$ g/L, $Y_{L/X} = 43.3\%$ w/w; see Table 4), with a global conversion yield of lipid on consumed substrate ($Y_{L/Glol}$) = 0.16 g/g (data obtained from trials with $Glol_0 \approx 80$ and ≈ 100 g/L) (Fig. 2). Whereas the maximum theoretical conversion yield of microbial lipid produced per glycerol is = 0.30 g/g (Ratledge 1988; Papanikolaou and Aggelis 2011), this yield (as the one of lipid produced on glucose consumed, being a little higher, *viz.* ≈0.32 g/g; see: Ratledge 1988; Papanikolaou and Aggelis 2011) in real fermentation conditions is seldom ≥ 0.20 g/g (Papanikolaou and Aggelis 2011, 2019), with these values being obtained generally in highly aerated bioreactors (Papanikolaou and Aggelis 2019). It may be assumed therefore, that the obtained results, specifically the ones obtained on crude glycerol, are quite satisfactory. However, it must be indicated that in aerated fed-batch bioreactors with glucose employed as carbon source, *R. toruloides* DSM 4444 presented a yield of total lipid produced per sugar consumed = 0.21 g/g, with a simultaneous $Y_{L/X}$ value ≈ 65% w/w (Tchakouteu *et al.* 2017). Equally, in flour-rich hydrolysates (mostly composed of glucose and maltose) conversion yields ≈ 0.17–1.18 g/g with simultaneous lipid in DCW values ≥ 50% w/w have been recorded (Tsakona *et al.* 2016) indicating that glucose is a more appropriate substrate than glycerol for this particular strain. The production of lipid for *Rhodosporidium* sp. strains, compared with the findings of the present study are depicted in Table 5.

Lipid analysis

All screened strains were analyzed concerning the FA composition of their total lipids at the stationary growth phase (FA composition analysis performed between 150 and 200 h after inoculation) (Table 6). In agreement with the literature (Papanikolaou and Aggelis 2011, 2019; Wu *et al.* 2018; Saini *et al.* 2020) the principal FAs found in variable quantities were mainly the oleate [C18:1 (*ω*-9)], the palmitate (C16:0) and the linoleate [C18:2 (*ω*-6)] (Table 6). Poly-unsaturated FAs were not detected in high concentrations, since these compounds are the principal storage lipophilic compounds in oleaginous fungi and algae (Bellou *et al.* 2014, 2016a; Dourou *et al.* 2018), and, in general, they can be produced in significant quantities inside the yeast cells only after appropriate genetic modifications (Bellou *et al.* 2016a).

In most *Metschnikowia* sp. strains, the principal cellular FA detected was oleic acid, in agreement with the literature (Chatzifragkou *et al.* 2011; Santamauro *et al.* 2014; Canonico *et al.* 2016). On the other hand, the strain V.V.-D4 presented higher concentrations of the FA C18:2 (*ω*-6) compared with those of oleic acid. Significant quantities of cellular C18:2 (*ω*-6) were also reported for *Rhodotorula* sp. LMBF Y-49 (*viz.* ≈68% w/w of total lipids), in disagreement with the results reported for other *Rhodotorula* sp. strains, in which cellular FAs are mainly composed of C18:1 (*ω*-9) (Chatzifragkou *et al.* 2011; Patel *et al.* 2016; Filippousi *et al.*

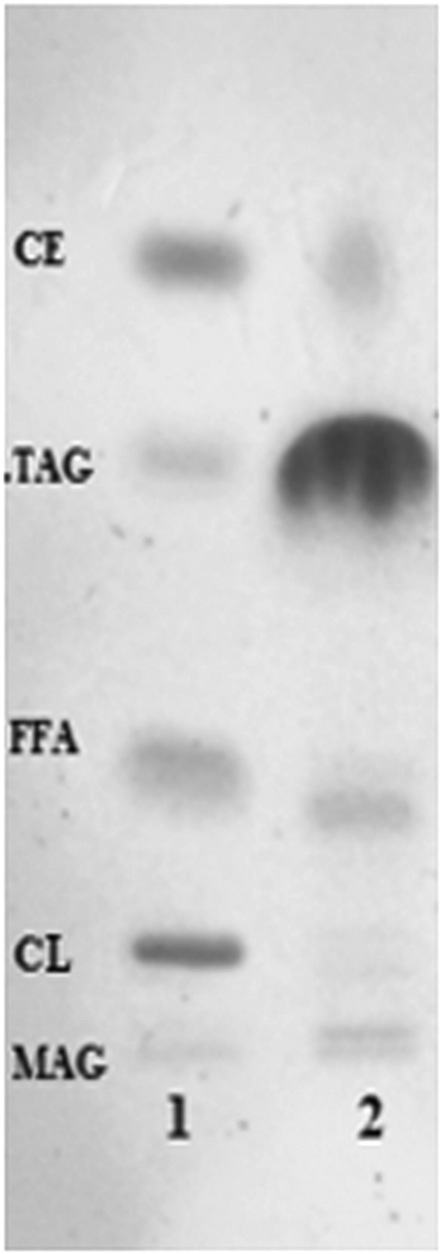

Figure 3. TLC analysis of the 'crude' lipid ('Folch' extract—C/M 2/1) produced by *Rhodosporidium toruloides* DSM 4444 during growth on crude glycerol, at elevated initial substrate concentration ($Gly_0 \approx 80$ g/L) c. 100 h after inoculation. Lane 1: mix of neutral lipid standards (cholesterol-CL, oleic acid-FFA, glyceryl trioleate-TAG, cholesteryl linoleate-CE and monononadecanoin-MAG). Lane 2: Total lipid. Deposition of 2 μl was performed for both the lanes. Total lipid was diluted to C/M 2/1 at 25 mg/mL while standards were diluted to C/M 2/1 at 10 mg/mL.

2019). For *Y. lipolytica* strains, FA composition presented similarities with that reported for other strains cultured on glycerol under similar culture configurations with the current report (Papanikolaou *et al.* 2002, 2008; Makri, Fakas and Aggelis 2010; Chatzifragkou *et al.* 2011; Daskalaki *et al.* 2019; Filippousi *et al.* 2019; Sarris *et al.* 2019a).

R. toruloides DSM 4444 cultivated on glycerol/xylose blends produced lipids mainly composed of the FAs C18:1 (ω-9) and C16:0. Stearic acid (C18:0) and C18:2 (ω-6) were also detected into the cellular lipids in lower concentrations, whereas α-linolenic [C18:3 (ω-3)], palmitoleic [C16:1 (ω-7)], lauric (C14:0) and behenic (C22:0) acids were found in even lower concentrations (sum of these four FAs ranging between 0.0 and 19.5% w/w of the total lipids produced) (Table 7). Although the intra-cellular catabolism of both glycerol and xylose is not directly linked with the pathways implicated in the FA composition of the cellular lipids (Bellou *et al.* 2014; Sargeant *et al.* 2016; Dourou *et al.* 2018), and there would not be any evident reason for which the FA composition of the cellular lipids would be significantly different when xylose or glycerol would have been used as substrates, *R. toruloides* DSM 4444 presented somehow different FA composition during growth on the above-mentioned compounds; trials on xylose-rich media were accompanied by cellular lipids that were more rich in oleate while on glycerol-rich media the concentration of the sum of C18:3 (ω-3), C16:1 (ω-7), C14:0 and C22:0 was always higher than that on xylose-based fermentations (see Table 7). Also, cellular FA composition seemed to change as function of the culture time, specifically in the glycerol-rich blends, since it appeared that lipids presenting somehow higher oleic acid and lower palmitic acid quantities were found at the initial growth steps, and *vice versa* (see Table 7). In order to further prove this assumption, FA composition of *R. toruloides* was studied over the whole range of the culture time in the trial with $Glol_0 \approx 80$ g/L, and, indeed, it was seen that the concentration of the cellular C18:1 (ω-9) was slightly reduced while that of C16:0 increased with the time (Table 8a).

The FA composition of *R. toruloides* cultivated on glycerol/xylose mixtures is consistent with the literature; Wu *et al.* (2011) reported a FA composition of 30% palmitate, 14% stearate and 49% oleate of the cellular lipids for the strain Y4 cultured on glucose. In accordance with the current study, non-negligible oleic acid quantities (38–50% w/w in total lipids) were reported for the strain CBS 14 cultured on glucose or xylose in batch bioreactor trials (Tiukova *et al.* 2019). Equally, Tchakouteu *et al.* (2015b) reported significant cellular C18:1 (ω-9) quantities (44–55% w/w of total lipids) for the strain NRRL Y-27 012 cultivated in shake-flask trials with glycerol employed as substrate, with the higher values of cellular oleic acid reported at the earlier growth steps. Moreover, Wu *et al.* (2018) reported very high quantities of the cellular oleate (>57% w/w of total lipids) for the thermotolerant L1–1 and the wild-type TK16 strains, cultivated on glucose in shake flasks. In contrast to the present study, the strain ATCC 204 091 grown on mixtures of glucose/xylose/glycerol produced lipids with high C18:2 (ω-6) quantities (≈23% w/w of total lipids) (Singh *et al.* 2018). Finally, other 'red' yeasts (i.e. *R. kratochvilovae, R. minuta*) presented similar FA composition (*viz.* elevated cellular oleic and palmitic acid quantities) with the present study (Patel *et al.* 2015, 2016; Sargeant *et al.* 2016).

R. toruloides lipid was mainly composed of neutral lipids (NLs) (86% w/w of cellular lipids) whereas small quantities of glycolipids plus sphingolipids (G + S) (c. 12% w/w of cellular lipids) and mostly phospholipids (PLs) (c. 2% w/w of cellular lipids) were identified (analysis performed at the early 'oleaginous' phase, *viz.* 100 h after inoculation) (Table 8b). In accordance with the present investigation, NLs have been revealed to be the major component of the cellular lipids irrespective of the fact of significant or insignificant accumulation of lipids in the yeasts *R. toruloides*, *Y. lipolytica* and *Pichia membranifaciens* (André *et al.* 2009; Makri, Fakas and Aggelis 2010; Chatzifragkou *et al.* 2011; Papanikolaou *et al.* 2017a; Tiukova *et al.* 2019). Interestingly and in accordance with the present study, several yeasts tended to produce slightly more unsaturated PLs compared with their NLs (André *et al.* 2009; Makri, Fakas and Aggelis 2010; Chatzifragkou *et al.* 2011). For the strain *R. toruloides* CBS 14, the quantity of PLs

was very low (3.9–4.1% w/w of total lipid classes) (Tiukova *et al.* 2019) in agreement with the present investigation. Finally, *R. toruloides* DSM 4444 produced total lipids that were mainly composed of triacylglycerols (TAGs) and contained very low quantities of free FAs (Fig. 3) in accordance with the results reported for the strain CBS 14 (Tiukova *et al.* 2019) and the strain NCYC 1576 (Patel *et al.* 2018) during their growth on glucose/xylose blends.

CONCLUSIONS

Crude glycerol, an important liquid residue stream deriving from the oleochemical and biofuel production industries has been revealed as a competitive and very interesting substrate for yeasts belonging to the species/genera *Yarrowia lipolytica*, *Metschnikowia* sp., *Rhodosporidium toruloides* and *Rhodotorula* sp. (in total 11 strains). In several cases, metabolic compounds that present interest for the chemical, biofuel and food industries (i.e. cellular lipids, endopolysaccharides, polyols, citric acid) were synthesized by the yeasts tested, and, interestingly often in non-negligible concentrations, that are comparable or even higher than those in the current literature. For instance, the strain *Metschnikowia* sp. V.V.-D4 produced the appreciable quantity of 11.0 g/L of endopolysaccharides (with simultaneous polysaccharides in DCW value ≈ 63% w/w) in shake-flask glycerol-based experiments. Thereafter, and given the potential of lipid production by the strain *R. toruloides* DSM 4444, this yeast was cultured on media presenting higher initial glycerol concentrations and enhanced nitrogen limitation, in order to stimulate lipid production, and indeed, significant lipid production (lipid up to 12.5 g/L, lipid in DCW up to 46% w/w) was recorded. Xylose, another low-cost abundant bioresource was implicated as substrate of *R. toruloides*, and somewhat lower lipid quantities were synthesized compared with the equivalent trial performed on glycerol employed as the sole substrate. Fermentations performed on blends of glycerol and xylose resulted in appreciable total dry yeast biomass and interesting cellular lipid production, but, in any case, the more xylose was found into the substrate mixture, the less storage lipids were produced. Yeast lipids synthesized, containing mostly neutral fractions (TAGs), could constitute a perfect starting material for the synthesis of high-quality biodiesel. The current study therefore, suggested various alternative ways related with the valorization abundant low- or almost zero-cost carbon sources (*viz.* crude glycerol and xylose), by using these compounds as substrates of natural yeast strains, in order to produce added-value metabolic compounds of importance of the food, biofuel and chemical industries.

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