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Biotechnological valorization of biodiesel-derived glycerol: Trials with the non-conventional yeasts *Yarrowia lipolytica* and *Rhodosporidium* sp

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ABSTRACT

Four non-conventional yeast strains belonging to the genera *Yarrowia* and *Rhodosporidium* were cultivated under nitrogen-limited conditions in shake flasks with biodiesel-derived glycerol employed as sole substrate. The strains *R. toruloides* DSM 4444 and *Y. lipolytica* ACA-DC 5033 displayed better performances and were further studied. At initial glycerol (Gly_0) ≈ 50 g/L, the strain ACA-DC 5033 produced dry cell weight (DCW) ≈7.0 g/L, and also secondary extra-cellular metabolites as citric acid (*CA*) at ≈16 g/L and polyols (*Pol*) (mannitol, erythritol and arabitol) at ≈21 g/L. In double-media (besides nitrogen also Mg was limiting) for Gly_0 ≈ 50 g/L, a shift towards *CA* production occurred (CA_{max} ≈ 33 g/L). The strain DSM 4444 at Gly_0 ≈ 50 g/L produced DCW = 18.1 g/L containing lipids = 30.3% in DCW. In single nitrogen-limited media with Gly_0 ≈ 90 g/L, the strain ACA-DC 5033 produced *Pol* ≈ 48 g/L and *CA* ≈ 20 g/L, while the strain DSM 4444 produced DCW = 27.3 g/L containing lipid = 54.5% w/w. At the late growth phases, metabolites were re-consumed. Balanced growth phase (trophophase) and phase of secondary metabolite synthesis (idiophase) were successfully simulated with the aid of a modified Velhlust-Aggelis model. Lipid extraction process was studied for these two strains by using two different extraction methods. Yeast lipids contained mostly oleic acid, constituting suitable precursors for the synthesis of 2nd generation biodiesel.

1. Introduction

The main side-product of biodiesel production process is a rich glycerol-containing water (with concentration of glycerol ranging between 65–85% w/w) called “crude” or “industrial” glycerol” (“crude/industrial glycerin”) [1,2]. The synthesis of 10 kg of biodiesel generates *c.* 1 kg of glycerol (purity ≈90% w/w) as side-product of the process [1,3]. Besides biodiesel production process, significant quantities of glycerol-containing water can be generated through bioethanol and alcoholic beverages production units; for instance, during bioethanol production process, ethanol is separated *via* distillation while the liquid fraction of the remaining material (the so-called thin stillage) contains *c.* 2% w/v of glycerol [4]. Likewise, liquid waste streams containing high levels of glycerol (glycerol quantities of 55–90% w/v) are generated in oleochemical facilities in which transformations of vegetable or animal fats are implicated [4,5]. Finally, even larger quantities of glycerol feedstocks into the market volume may be generated due to the very high intra-cellular accumulation of glycerol (in quantities up to *c.* 85% w/w) in several algal species like *Dunaliella* sp. [6,7]. In some of the above-mentioned cases, quantities of glycerol into the water up to 7.8 M (equivalent to *c.* 720 g of glycerol per L in water) can occur [6]. For all these reasons, glycerol over-production and disposal may cause important environmental problems in the near future. The last years therefore, one of the most important topics that have been developed in both the Chemical and Microbial Technology, refers to the utilization of glycerol as starting or supplementary material that would be converted into various final added-value compounds [3,8–11].

Strains of the non-conventional yeasts *Yarrowia lipolytica* and *Rhodosporidium* sp. have been employed as microbial cell factories capable to convert glycerol into a plethora of useful microbial metabolites [9,12,13]. *Y. lipolytica* yeast was employed in order to produce microbial mass, microbial lipids (single-cell oils; SCOs), citric acid and polyols (*viz.* mannitiol, arabitol and erythritol) [14–19]. Strains of the genus *Rhodosporidium* sp. were employed for the synthesis of microbial mass, SCO and endopolysaccharides [7,20–25]. Aim of the present study was to assess the biochemical potential of four non-conventional yeast strains, two belonging to *Y. lipolytica* and two to the genus *Rhodosporidium*, concerning glycerol assimilation performances. Trials were carried out

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under nitrogen-limited media, formulated in order to enhance to production of cellular lipids and/or the secretion of extra-cellular compounds (citric acid and polyols) [2,10,13,26]. The most promising of the selected yeasts were further cultivated under different culture conditions whereas lipid extraction process was studied too. Numerical models simulating the kinetic behavior of the yeasts during the trophophase (*viz*. the balanced growth phase) and the idiophase (*viz*. the phase in which the production of the secondary metabolites occurred) were applied. Considerations concerning the yeast physiological behavior were critically assessed.

2. Materials and methods

2.1. Microorganisms and media

The strains of the current investigation belonged to the genus *Rhodosporidium* sp. (strains *R. kratochvilovae* FMCC Y71 and *R. toruloides* DSM 4444) and to the species *Yarrowia lipolytica* (strains ACA-DC 5033 and LFMB Y19). Strains with the codes FMCC and LFMB Y were indigenous strains isolated from fish-origin products [27] and deposited in the collection of the Laboratory of Food Microbiology and Biotechnology of the Agricultural University of Athens. Strain ACA-DC 5033 was isolated from sourdoughs [28] and deposited in the ACA-DC culture collection of the Agricultural University of Athens. These strains, in previous screening tests have been revealed capable to consume interesting glycerol quantities [24,29]. The strain *R. toruloides* DSM 4444 was provided from the German Collection of Microorganisms and Cell Cultures. In previous studies, this strain has been revealed capable to present interesting growth and variable lipid accumulation on media composed of blends of crude glycerol and commercial xylose [25] while it presented indeed high dry cell weight (DCW) and lipid production on glucose-based nitrogen-limited bioreactor experiments [30,31]. Maintenance of the strains was conducted as previously indicated [25]. All strains were maintained at $T = 4\,^{\circ}C$ and sub-cultured every three months. Prior to any inoculation in the liquid growth medium the strains were regenerated in slants, so that the initial slant culture would be of three days old.

Liquid cultures were performed, in a medium in which the salt composition was as in Papanikolaou et al. [32]. Nitrogen-limited media were employed, in which yeast extract (at 1.0 g/L) and peptone (at 2.0 g/L) were used as nitrogen sources. Nitrogen limitation was employed in order to "boost" the microbial metabolism towards the synthesis of secondary metabolites [10,26,33]. Yeast extract contained *c.* 7% w/w of nitrogen, while peptone contained *c.* 14% w/w of nitrogen. Crude glycerol was provided by the Hellenic biodiesel-producing facility "ELIN VERD SA" (Velestino, Magnissia Prefecture, Greece). The purity of the feedstock was ≈88% w/w, and the major impurities were composed of salts of potassium and sodium (6%, w/w), free-fatty acids (1%, w/w) and water (5%, w/w). In the first set of experiments, all employed strains were cultivated in shake-flask fermentations at media in which initial glycerol (Gly_0) concentration was ≈50 g/L. The initial C/N molar ratio in this set of trials was ≈65 moles/moles. For *Y. lipolytica* ACA-DC 5033, another experiment in which double limitation was also carried out in which Gly_0 was ≈50 g/L; besides nitrogen, limitation in Mg was also imposed (instead of $MgSO_4 \times 7H_2O$ at 1.5 g/L, 0.2 g/L were used) since previous experiments have indicated that both nitrogen and Mg limitation induced lipid accumulation in another *Y. lipolytica* strain (ACA-DC 50109) [34]. Likewise, the presence or absence of various mineral salts (i.e. sulfur, potassium, etc) has important impact upon lipid accumulation or citric acid secretion in other *Y. lipolytica* strains [3]. Thereafter, the most promising strains, namely *Y. lipolytica* ACA-DC 5033 and *R. toruloides* DSM 4444 were cultured at higher Gly_0 concentration (≈90 g/L) in which all other culture parameters (yeast extract at 1.0 g/L and peptone at 2.0 g/L, etc) remained constant (in this set of trials single nitrogen limitation was employed). In this set of experiments, the initial C/N molar ratio was ≈120 moles/moles.

Experiments were carried out in 250-mL conical flasks, filled with the 1/5 of their volume, sterilized (at $T = 115\,^{\circ}C$, 20 min) and inoculated as in Diamantopoulou et al. [25] 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 (mostly for *Y. lipolytica*) presented a drop throughout culture due to the accumulation into the medium of organic acids, and pH value was maintained to a value > 4.7 ± 0.1 by aseptically adding 2.0 M NaOH [25]. For the case of *Rhodosporidium* sp. strain, medium pH remained almost in all fermentation phases within the range 5.0–6.0, therefore no addition of NaOH occurred.

2.2. Analyses and determinations

Yeast biomass was recovered and was quantified through dry cell weight (DCW) determination [25]. Extra-cellular compounds (*viz.* glycerol, polyols, citric acid) were quantitatively determined with the aid of HPLC analysis as previously described [16]. Total intra-cellular polysaccharides were quantified as in Diamantopoulou et al. [35] and were expressed as glucose equivalents. pH in the medium was measured with the aid of a Jenway 3020 pH-meter (Cole-Parmer, UK). Dissolved oxygen tension (DOT) was off-line measured using a selective electrode (HI 9146, Hanna Instruments, Woonsocket-Rhode Island, USA) according to Palaiogeorgou et al. [36]. DOT was always ≥ 20% v/v during all phases for all experiments and microorganisms, therefore trials were carried out under full aerobic conditions [36]. Free amino-nitrogen (*N*) concentration into the medium was determined as in Kachrimanidou et al. [37].

Cellular lipids were extracted with the aid of a modified "Folch" method; specifically yeast dried biomass (up to 300 mg) was put in a McCartney vial and was covered with chloroform/methanol (C/M) 2:1 (v/v) blend (up to 25 mL). The whole was closed with an aluminum screw cap and was left for at least 6 days in the darkness. Occasionally, the content of the vial was gently mixed with the aid of a glass stick. Then, cell debris was removed through filtration (Whatman® filter n° 3), the solvent mixture was collected in a pre-weighted evaporator flask and was completely evaporated in a rotary evaporator (R-144, Büchi Labortechnik, Flawil, Switzerland), with total lipid being determined gravimetrically [38]. In *Y. lipolytica* ACA-DC 5033 and *R. toruloides* DSM 4444, another lipid extraction method, proposed by Tchakouteu et al. [7] was also used; specifically, a precisely weighted yeast DCW quantity (up to 300 mg) was put in a McCartney vial and was acidified by adding 4.0 mL HCl (2.0 M), the whole being placed at $T = 100\,^{\circ}C$ for 40 min. After boiling, vials were left to cool in ambient temperature. Thereafter, C/M 2:1 (v/v) blend (up to 25 mL) was added into the vials, stirring with the aid of a glass stick was carried out, and the vials were closed with an aluminum screw cap and were left for 24 hours in the dark. Then, the lower organic (chloroform) phase was collected with the aid of a pipette Pasteur, anhydrous $MgSO_4$ was added to remove traces of water, this phase was filtered through a Whatman® n° 3 filter paper to remove cell debris and salts precipitates and was finally collected in pre-weighted evaporator flask. Then, organic phase was evaporated in the rotary evaporator and total lipid was quantitatively determined gravimetrically [7]. Irrespective of how total lipids were recovered and quantified, they were trans-methylated and their total fatty acid (FA) composition was evaluated with the aid of GC analysis, according to previously published procedure [38].

2.3. Data analysis and modeling

Each experimental point of all of the kinetics presented in the tables and figures is the mean value of two independent determinations; in fact, for each experiment presented, two lots of independent cultures using different inocula were conducted and the standard error (SE) was < 15%. Data were plotted using Kaleidagraph 4.0 Version 2005 showing the mean values with the standard error mean. In the trials

Table 1
Quantitative data of *Yarrowia lipolytica* and *Rhodosporidium* sp. cultivated on crude glycerol under nitrogen-limited conditions ($Gly_0 \approx 50$ g/L, initial C/N ratio ≈65 moles/moles). Three different points in the fermentations are represented: (a) when the maximum quantity of intra-cellular total sugars per DCW ($Y_{IPS/DCW}$ %, w/w) was observed; (b) when the maximum quantity of total lipid per DCW ($Y_{L/DCW}$ %, w/w) was observed; (c) when the maximum dry cell weight quantity (DCW, g/L) was achieved. Fermentation time (h) and quantities of dry cell weight (DCW, g/L), total lipid (*L*, g/L), intra-cellular polysaccharides (*IPS*, g/L) and glycerol consumed (Gly_{cons}, g/L) are also depicted for all the above-mentioned fermentation points. Culture condition as in "Section 2".

Strains		Time (h)	Gly_{cons} (g/L)	DCW (g/L)	*L* (g/L)	$Y_{L/DCW}$ (%, w/w)	*IPS* (g/L)	$Y_{IPS/DCW}$ (%, w/w)
Yarrowia lipolytica ACA-DC 5033	b	56	17.2	4.8	2.3	47.9	1.5	31.2
	a	96	36.8	6.2	2.3	37.0	2.0	32.2
	c	291	50.0	7.0	0.4	5.7	1.2	17.1
Yarrowia lipolytica LFMB Y19	b	144	39.1	11.2	0.9	8.0	5.0	44.6
	a	193	41.0	12.9	0.6	4.6	6.2	48.0
	c	216	43.3	13.8	0.5	3.6	5.9	42.8
Rhodosporidium toruloides DSM 4444	a	67	17.5	7.4	1.9	25.6	2.2	29.7
	b	140	33.1	13.0	5.0	38.5	2.0	15.3
	c	230	57.5	18.7	5.0	26.7	2.4	12.8
Rhodosporidium kratochvilovae FMCC Y71	a	80	37.1	13.2	2.3	15.1	5.2	39.3
	b,c	192	48.9	17.1	2.8	16.3	4.9	28.7

where kinetics were simulated with the aid of numerical models, the governing differential equations were solved in Microsoft Office EXCEL Version 2007, using an explicit finite difference scheme with a time step of 0.1 h [39]. The kinetic parameters involved were determined by minimizing the Sum of Squared Errors (SSE) between the experimental and theoretical concentrations values though the SOLVER add-in program of EXCEL. Graphs of the simulations were done using EXCEL Version 2007.

3. Nomenclature

DCW: Dry cell weight (total microbial mass) (g/L); *X*: Biomass (g/L) – (this term was employed only in the sub-chapter of the modeling; specifically, in *R. toruloides*, *X* was the lipid-free biomass calculated after subtraction of the total cellular lipids from the DCW, whereas in *Y. lipolytica*, *X* was the total microbial mass); *Gly*: Glycerol (g/L); *L*: Total cellular lipids (g/L); *IPS*: Total cellular polysaccharides (g/L); *CA*: Total citric acid (g/L); *Ml*: Mannitol (g/L); *Ery*: erythritol (g/L); *Ara*: Arabitol (g/L); *Pol*: Total polyols (=*Ara* + *Ery* + *Ml*) (g/L); *t*: Fermentation time (h); *N*: free amino nitrogen concentration (mg/L or g/L); q_{CA}: specific citric acid formation rate, g of citric acid /(g of biomass × h); q_L: specific lipids formation rate, g of lipids/(g of biomass × h); q_{Pol}: specific polyols formation rate, g of polyols/(g of biomass × h); $Y_{X/N}$: yield coefficient for (lipid-free) biomass production with respect to nitrogen consumption, (g_X/g_N) (g of *X* per g of free amino nitrogen); $Y_{Pol,CA/Gly}$: yield coefficient for polyols and/or citric acid production with respect to glycerol consumed, ($g_{Pol} + g_{CA}$)/g_{Gly} (g of polyols and/or g of citric acid per g of glycerol); $Y_{X/Gly}$: yield coefficient for (lipid-free) biomass production with respect to glycerol consumed, g_X/g_{Gly} (g of biomass per g of glycerol); $Y_{L/Gly}$: yield coefficient for total lipid production with respect to glycerol, g_L/g_{Gly} (g of lipids per g of glycerol); $Y_{DCW/Gly}$: yield coefficient for total DCW production with respect to glycerol consumed, g_{DCW}/g_{Gly} (g of total DCW per g of glycerol); $Y_{L/DCW}$: total cellular lipids in DCW (%, w/w); $Y_{IPS/DCW}$: total cellular polysaccharides in DCW (%, w/w); indexes 0, max and cons show the initial and maximum and the consumed quantity of the elements in the experiments carried out.

4. Results and discussion

4.1. Initial trials on crude glycerol

In the first part of the study, all available strains were cultivated on crude glycerol ($Gly_0 \approx 50$ g/L, C/N ≈ 65 moles/moles) in order to assess their potential towards glycerol assimilation under nitrogen limitation. Cellular lipids were extracted using the modified "Folch" method (see Section 2). The obtained results are shown in Table 1. All tested strains presented appreciable quantities of consumed glycerol and variable quantities of DCW production, whereas from a given fermentation point and thereafter (i.e. after $t \geq 45$ h) all trials were conducted under nitrogen-limited conditions (initial free amino nitrogen; $N = 65 \pm 10$ mg/L, *N* at $t \geq 45$ h $= 10 \pm 6$ mg/L). Concerning DCW, strains *Rhodosporidium* sp. presented DCW_{max} quantities ranging between 17.1 and 18.7 g/L (simultaneous $Y_{DCW/Gly}$ values = 0.33–0.35 g/g) that were higher than the respective ones of the strains of *Y. lipolytica* (see Table 1). *Rhodosporidium* sp. strains produced small concentrations of extra-cellular metabolites (mostly citric acid, at maximum concentration ≈2.0–3.0 g/L at the middle of the culture, which was re-consumed as the fermentations proceeded). On the other hand, strains belonging to *Y. lipolytica* presented lower DCW_{max} quantities; the strain LFMB Y19 showed $DCW_{max} = 13.8$ g/L, while the strain ACA-DC 5033 showed a respective quantity of 7.0 g/L. These strains, contrary to *Rhodosporidium* sp., secreted non-negligible quantities of extra-cellular metabolites during idiophase (*viz.* after nitrogen depletion from the medium); the strain LFMB Y19 at $t = 216$ h had produced almost exclusively citric acid (*CA*) at concentration = 18.6 g/L (simultaneous yield $Y_{CA/Gly} = 0.43$ g/g), while the strain ACA-DC 5033 at $t = 167$ h had produced *CA* at concentration = 16.1 g/L ($Y_{CA/Gly} = 0.32$ g/g), *Ml* at concentration = 11.0 g/L, *Ery* at concentration = 7.4 g/L and *Ara* at concentration = 2.4 g/L (therefore *Pol* concentration = 20.8 g/L, $Y_{Pol/Gly} = 0.42$ g/g). Further incubation of the strain ACA-DC 5033 after $t = 167$ h, resulted in complete glycerol uptake and remarkable re-consumption of polyols previously synthesized in favor of *CA* synthesis (at $t = 291$ h, *CA* ≈ 21 g/L while *Pol* ≈ 4.0 g/L).

In contrast to the theory indicating that lipid production in the oleaginous microorganisms occurs only during idiophase [5,12,33] *Y. lipolytica* ACA-DC 5033 produced noticeable lipid quantities ($L = 2.3$ g/L, simultaneous $Y_{L/DCW} = 47.9\%$ w/w) at the end of trophophase (see i.e. $t = 56$ h; Table 1). The L_{max} quantity for this trial was recorded relatively more late in the fermentation and slightly after nitrogen depletion from the medium ($t = 80$ h – at that point L_{max} was = 2.7 g/L, simultaneous $Y_{L/DCW}$ value was = 46.5% w/w). Thereafter, and despite the non-negligible concentration of glycerol into the medium, *L* and $Y_{L/DCW}$ values were significantly depleted indicating storage lipid degradation, whereas simultaneously extra-cellular metabolites (*CA* and *Pol*) where accumulated into the medium in noticeable concentrations. The strain LFMB Y19 presented a relatively analog physiological pattern, since the higher *L* and $Y_{L/DCW}$ values were reported at the relatively early fermentation steps. Nevertheless, these values were drastically lower than these of the strain ACA-DC 5033 (Table 1). Quite similar physiological behavior with the strain ACA-DC 5033, has been reported by a number of wild-type or genetically engineered *Y. lipolytica* strains, during their batch-wise growth on glycerol or

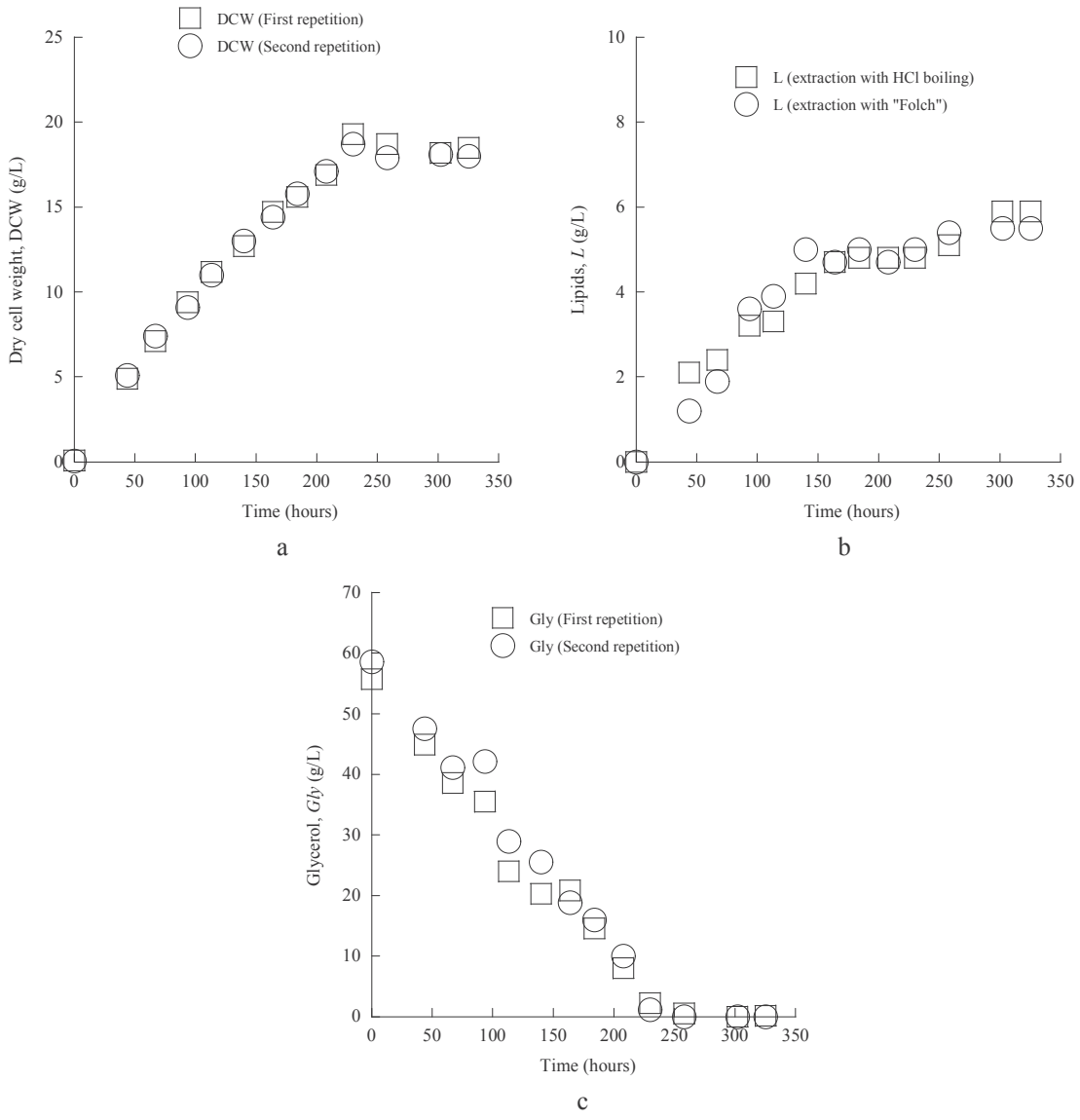

Fig. 1. Kinetics of dry cell weight (DCW, g/L) (a), lipid (*L*, g/L) (b) and glycerol (*Gly*, g/L) (c) evolution by *Rhodosporidium toruloides* DSM 4444, during growth on biodiesel-derived glycerol ($Gly_0 \approx 50$ g/L, initial C/N ratio ≈65 moles/moles). With "first repetition" we term the trial in which lipids were extracted with the method of digestion with HCl; with "second repetition" we term the trial in which lipids were extracted with the modified "Folch" method. Culture conditions as in "Section 2".

similarly metabolized compounds (i.e. glucose) under nitrogen-limited conditions [15,16,29,34,40–42]; in most of the mentioned cases, *Y. lipolytica* strains displayed an "atypical" oleaginous behavior in which lipids were stored at the relatively earlier growth steps (i.e. at the end of trophophase), and were subjected to biodegradation (turnover) as fermentation proceeded, in favor of (mostly) *CA* and (to lesser extent) *Pol* biosynthesis. Nevertheless, as far as we are aware, the current investigation is one of the first in which such high lipid in DCW values ($Y_{L/DCW} \approx 50\%$ w/w) were recorded at the early growth steps. Moreover, both strains presented a non-negligible production of total endopolysaccharides (*IPS*), the relative value of which ($Y_{IPS/DCW}$ in % w/w) was relatively elevated (i.e. > 30% w/w) even for the early growth steps (see results of the strain ACA-DC 5033 in Table 1).

Both *Rhodosporidium* sp. strains presented appreciable DCW production and remarkable assimilation of glycerol. *R. kratochvilovae* FMCC Y71 presented interesting $Y_{IPS/DCW}$ values at the relatively earlier growth phases (at $t = 80$ h, $Y_{IPS/DCW} \approx 40\%$ w/w). These values were somehow reduced as the fermentation proceeded, while simultaneously *L* and $Y_{L/DCW}$ values increased (Table 1). Nevertheless, $Y_{L/DCW}$ never reached the value of 20% w/w that is the lower threshold characterizing a microorganism as oleaginous [5,10]. On the other hand, the strain *R. toruloides* DSM 4444 presented an appreciable lipid production ($L_{max} = 5.0$ g/L, $Y_{L/DCW} = 38.5\%$ w/w), while, as previously, relatively elevated *IPS* in DCW values were recorded at the earlier growth steps (at $t = 80$ h, $Y_{IPS/DCW} \approx 30\%$ w/w – Table 1). Thereafter, $Y_{IPS/DCW}$ values drastically decreased, with concomitant increase of the *L* value (L_{max} value achieved = 5.0 g/L). Similar behavior in which elevated $Y_{IPS/DCW}$ values were reported at the earlier growth steps has been observed for other *Rhodotorula* sp. strains and also for other yeast species like *Cryptococcus curvatus* [24,43] several *Y. lipolytica* strains [24,25,44,45] *Rhodotorula gracilis* [46] and *Naganishia uzbekistanensis* [24]. This feature is considered to be as quite "atypical", since endopolysaccharides, as storage lipids, theoretically request nitrogen limitation in order to be accumulated in significant quantities inside the yeast cells [10,26,33,47]. In contrast, in other cases of yeasts strains (i.e. other *Y. lipolytica* strains, *Apiotrichum curvatum*, other *R. toruloides* strains, *Metschnikowia* sp. etc) grown on glucose or glycerol under nitrogen-limited conditions, typical accumulation of intra-cellular

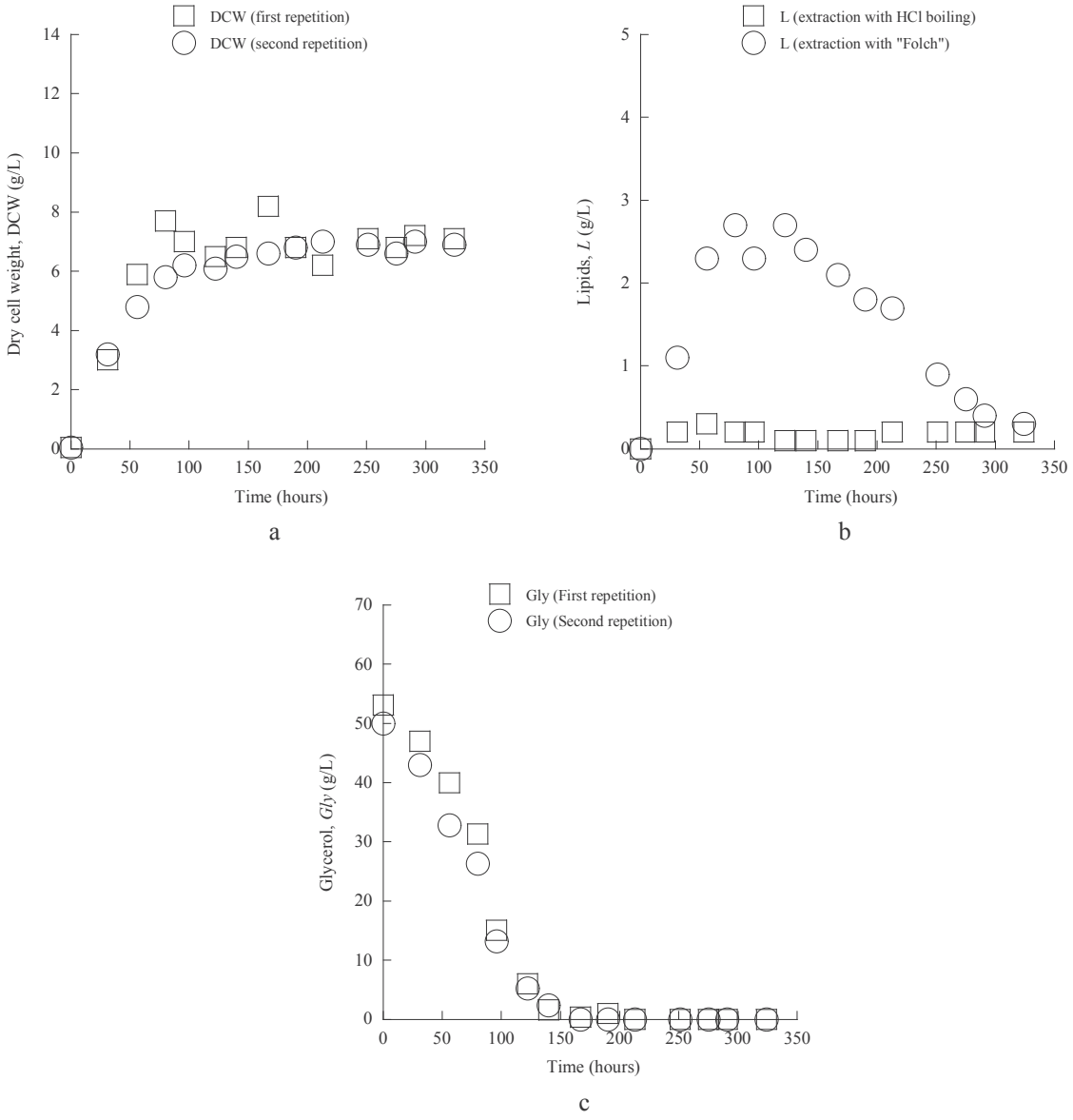

Fig. 2. Kinetics of dry cell weight (DCW, g/L) (a), lipid (*L*, g/L) (b) and glycerol (*Gly*, g/L) (c) evolution by *Yarrowia lipolytica* ACA-DC 5033, during growth on biodiesel-derived glycerol ($Gly_0 \approx 50$ g/L, initial C/N ratio ≈65 moles/moles). With "first repetition" we term the trial in which lipids were extracted with the method of digestion with HCl; with "second repetition" we term the trial in which lipids were extracted with the modified "Folch" method. Culture conditions as in "Section 2".

polysaccharides inside the cells has been reported during idiophase [24,25,48,49].

4.2. Evaluation of two different lipid extraction methods – Repeatability of the experiments

In this part of the study, two different extraction methods of the SCOs produced (see "Materials and Methods") were tested for *R. toruloides* DSM 4444 and *Y. lipolytica* ACA-DC 5033, at $Gly_0 \approx 50$ g/L and C/N ≈ 65 moles/moles. The method in which yeast DCW was "digested" at $T = 100\,^{\circ}C$ with the use of concentrated HCl was applied, since in previous investigations with the use of the strain *Lipomyces starkeyi* DSM 70296, this method had been revealed as adequate for the extraction of cellular lipids, with the modified "Folch" method being completely deficient [7,50]. Moreover, the repeatability of the experiments, at least as regards DCW production and glycerol assimilation could also be considered. The obtained results for both microorganisms are illustrated in Fig. 1(a–c) (culture of *R. toruloides*) and Fig. 2(a–c) (culture of *Y. lipolytica*) [with open squares (called "first repetition") the trial in which lipids were extracted with the method of digestion with HCl is shown; with open circles (called "second repetition"), the trial in which lipids were extracted with the modified "Folch" method is presented]. From the obtained results, it can be indicated that very satisfactorily repetition as regards both DCW production and glycerol assimilation was seen (Fig. 1a; 1c; 2a; 2c). Moreover, concerning *R. toruloides*, lipid extraction with both methods showed almost equivalent results (Fig. 1b). In contrast, much higher lipid recovery was observed with the modified "Folch" method in comparison to the HCl "digestion" method for *Y. lipolytica* lipids.

In most of the cases, methods of extraction of lipid from oleaginous microorganisms are more effective when dried microbial mass (i.e. freeze- or thermally dried) was used than wet one [51–53]. In the case of wet cells, biomass pretreatment (i.e. bead milling, ultrasound treatment, osmotic shock, microwave-induced heating, subcritical water hydrolysis, enzymatic hydrolysis, autolysis, chemical hydrolysis, autoclaving at $T = 120\,^{\circ}C$ for variable time applications, etc) was mandatory before

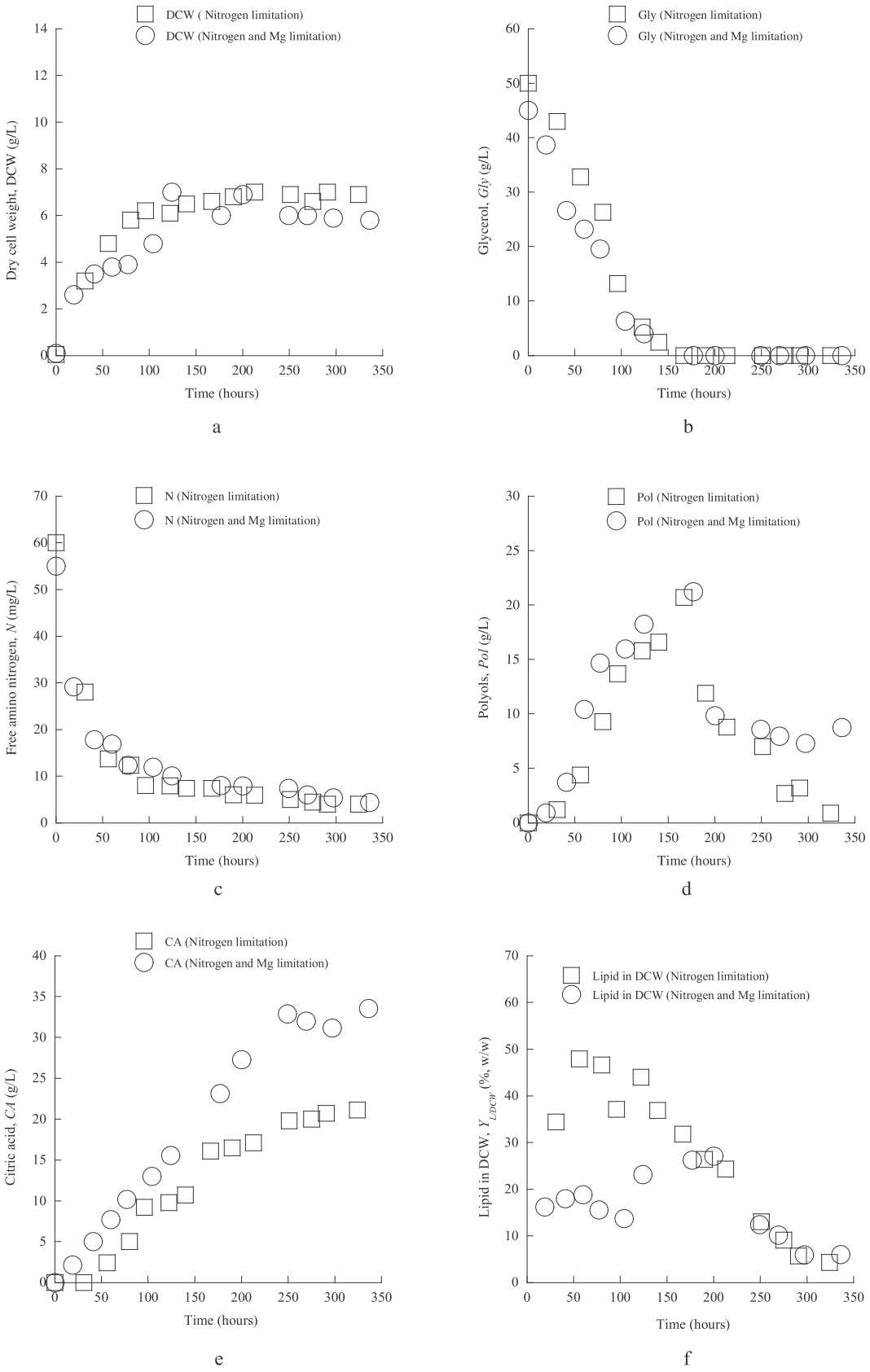

Fig. 3. Kinetics of dry cell weight (DCW, g/L) (a), glycerol (*Gly*, g/L) (b), free amino nitrogen (*N*, mg/L) (c), polyols (*Pol*, g/L) (d), citric acid (*CA*, g/L) (e) and lipid in DCW (%, w/w) (f) evolution by *Yarrowia lipolytica* ACA-DC 5033, during growth on biodiesel-derived glycerol in shake-flask experiments under solely nitrogen- and both nitrogen- and Mg-limited conditions ($Gly_0 \approx 50$ g/L). Culture conditions as in "Materials and Methods".

Table 2
Quantitative data of *Yarrowia lipolytica* ACA-DC 5033 and *Rhodosporidium toruloides* DSM 4444 cultivated on crude glycerol under nitrogen-limited conditions ($Gly_0 \approx 90$ g/L, initial C/N ratio ≈120 moles/moles). Three different points in the fermentations are represented: (a) when the maximum quantity of intra-cellular total sugars per DCW ($Y_{IPS/DCW}$ %, w/w) was observed; (b) when the maximum quantity of total lipid per DCW ($Y_{L/DCW}$ %, w/w) was observed; (c) when the maximum dry cell weight quantity (DCW, g/L) was achieved. Fermentation time (h) and quantities of dry cell weight (DCW, g/L), total lipid (*L*, g/L), intra-cellular polysaccharides (*IPS*, g/L) and glycerol consumed (Gly_{cons}, g/L) are also depicted for all the above-mentioned fermentation points. Culture conditions as in "Materials and Methods".

Strains		Time (h)	Gly_{cons} (g/L)	DCW (g/L)	*L* (g/L)	$Y_{L/DCW}$ (%, w/w)	*IPS* (g/L)	$Y_{IPS/DCW}$ (%, w/w)
Yarrowia lipolytica ACA-DC 5033	a	22	11.0	4.5	0.3	6.6	1.5	33.3
	b	71	39.0	9.7	2.0	20.6	3.0	30.9
	c	211	87.9	13.2	1.9	14.3	3.5	26.5
Rhodosporidium toruloides DSM 4444	a	38	8.5	5.7	1.1	19.3	2.0	35.0
	b	301	87.5	27.3	14.9	54.6	2.7	10.0
	c	348	87.5	27.5	11.5	41.8	2.8	10.2

lipid extraction. Thereafter C/M blends, or utilization of several non-polar solvents were implicated as extracting systems [2,51,53]. Lipid extraction was studied in the microorganisms *C. curvatus* ATCC 20509, *Mortierella isabellina* NRRL 1757 and *Chlorella sorokiniana* UTEX 1602 cultivated under nitrogen limitation on media composed of glucose, and various methods of biomass pretreatment and lipid extraction were performed (in all cases the extracted solvent was a C/M blend 1/1 v/v) [52]. For the case of *M. isabellina* and *C. sorokiniata*, the best method of lipid extraction was that of using Soxhlet apparatus for dried microbial mass (a method somehow comparable with the modified "Folch" method), whereas the method of HCl "digestion" of biomass before SCO extraction was slightly less efficient. By contrast, SCOs were clearly better recovered when HCl "digestion" of yeast DCW had been done in comparison to Soxhlet apparatus for *C. curvatus* [52]. Other methods employed for the pretreatment of the wet microbial mass before lipid extraction (i.e. autoclaving at $T = 121$ °C for 15 min, bead-beating with a bead beater, microwaving with a microwave oven at a high temperature) were less efficient [52]. Li et al. [54] studied lipid extraction from the DCW of the marine microalga *Tetraselmis* sp. M8, using Soxhlet apparatus and applying various solvent combinations (i.e. C/M 1/2, dichloromethane/methanol 2/1, propan-2-ol/hexane 1/1.25 accompanied by ultrasonication, etc). Significant differences as regards the $Y_{L/DCW}$ values were recorded in comparison to the lipid extraction protocol employed. Finally, as stressed previously, the SCO produced by *L. starkeyi* DSM 70296 was drastically better recovered when HCl "digestion" of the DCW had been conducted before lipid extraction compared to the modified "Folch" method [7]. All these elements, as well as the results indicated in the present study suggest that different oleaginous species respond differently to cell disruption and solvent extraction methods used in terms of lipid recovery yields, and no systematic effect of lipid extraction process exists for each oleaginous microorganism employed. In the forthcoming experiments, the modified "Folch" method was used for the quantification of cellular lipids produced.

4.3. Cultures under double limitation or higher initial glycerol concentration

Previous investigations using other *Y. lipolytica* strains have indicated that double limitation in both nitrogen and magnesium induced lipid accumulation [34]. Also, the presence or absence of various mineral salts (i.e. sulfur, potassium, etc) seemed to have impressive impact upon the process of lipid accumulation or citric acid secretion by *Y. lipolytica* [3]. Therefore, it was decided to perform a shake-flask experiment ($Gly_0 \approx 50$ g/L, C/N ≈ 65 moles/moles), in which besides nitrogen, magnesium (Mg) was also employed as limiting substrate (see Section 2). The obtained results of the various fermentation elements are illustrated in Fig. 3(a–f). The patterns of DCW production, glycerol consumption and *N* disappearance from the growth medium presented almost equivalent profiles (Fig. 3a–c). Likewise, the kinetic profile of total polyols produced (Fig. 3d), at least as regards the part representing their accumulation into the medium, presented similarities for both trials; in accordance with the literature [9,19,55,56] onset of polyols biosynthesis was given after assimilable nitrogen depletion from the medium (that occurred at $t = 45 \pm 5$ h after inoculation). In the case of single nitrogen limitation, mannitol (*Ml*) was the principal *Pol* synthesized, followed by erythritol (*Ery*) and arabitol (*Ara*). At $t = 167$ h, fermentation point in which glycerol had just been depleted, *Ml* was = 11.0 g/L, *Ery* was = 7.3 g/L and *Ara* was = 2.4 g/L (*Pol* = 20.7 g/L, simultaneous $Y_{Pol/Gly} = 0.41$ g/g). On the other hand, when double (both nitrogen and Mg) limitation occurred, *Ery* was the principal polyol synthesized, followed by *Ml* and *Ara*; at $t = 177$ h, fermentation point in which glycerol had been depleted, *Ery* was = 11.7 g/L, *Ml* was = 7.0 g/L and *Ara* was = 2.5 g/L (*Pol* = 21.2 g/L, simultaneous $Y_{Pol/Gly} = 0.47$ g/g). Simultaneously with the biosynthesis of polyols, *CA* production occurred (Fig. 3e). At $t = 167$ h, for the trial with the single nitrogen limitation, *CA* was = 16.1 g/L. At $t = 177$ h, for the trial with the double nitrogen and Mg limitation, *CA* was = 23.2 g/L.

It is interesting to indicate, that promising and comparable (or even higher) quantities of *Pol* compared to *CA* were synthesized, although medium pH remained always > 4.7, a value that normally favors the biosynthesis of citric acid instead of polyols [14,19,57]. In general, medium pH is a crucial culture parameter that may influence the final spectrum of products for *Y. lipolytica* cultures. It has been demonstrated that the very same strain cultivated under nitrogen limitation, can produce almost exclusively *CA* at medium pH values = 5.0–6.0, while metabolic shift and almost exclusive production of polyols may occur when cultures are performed at pH values = 3.0–3.5 [19,57,58]. The fact that interesting *Pol* quantities were synthesized in the current investigation and during growth on media in which pH remained always within the range 4.7–6.0, demonstrates the potential of the studied strain (ACA-DC 5033) concerning the synthesis of these compounds. Moreover, after glycerol had been depleted, *Pol* were degraded in favor of further *CA* production (see Fig. 3d; e). In double-limited media (nitrogen and Mg) notable higher final *CA* production occurred compared with the single nitrogen limitation, ($CA_{max} = 33.6$ g/L against 21.1 g/L for the single nitrogen limitation), suggesting that in the strain ACA-DC 5033, this limitation played a profound role on citric acid biosynthesis. Finally, in contrast to the results reported for the strain ACA-DC 50109 [34] double-limited media (nitrogen and Mg) did not significantly enhance storage lipid accumulation process; in single nitrogen limitation, noticeable quantities of lipid in DCW values were recorded ($Y_{L/DCW} \approx$ 47–49% w/w) barely after assimilable nitrogen was depleted from the medium, with these values drastically being reduced as culture proceeded (Fig. 3c; f). In contrast, lower $Y_{L/DCW\max}$ values were obtained in the trial with the double limitation ($Y_{L/DCW\max} = 27.1\%$ w/w; Fig. 3f). It appears therefore, that there is not any systematic effect upon the physiological behavior of *Y. lipolytica* yeast (i.e. shift towards the production of *CA* or SCO), related with the presence or absence of various salts into the culture medium.

Y. lipolytica ACA-DC 5033 cultivated under double-limited (both nitrogen and Mg) conditions in flask experiments, produced *CA* at a final

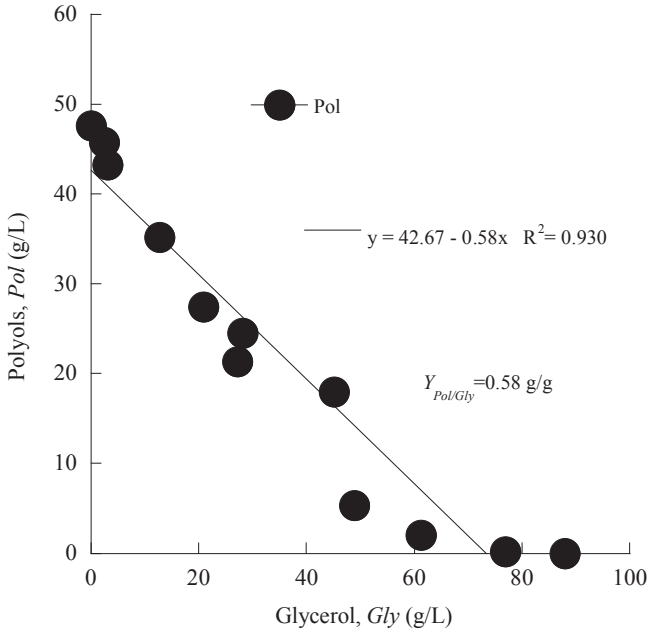

Fig. 4. Total polyols (*Pol*, g/L) produced *vs* glycerol (*Gly*, g/L) during growth of *Yarrowia lipolytica* ACA-DC 5033, at $Gly_0 \approx 90$ g/L under nitrogen-limited conditions. Culture conditions as in "Section 2".

concentration = 33.6 g/L (simultaneous yield $Y_{CA/Gly} = 0.75$ g/g). The CA_{max} quantity produced in the current investigation was lower than the respective values reported in the international literature for flask or bioreactor cultures of *Y. lipolytica* strains cultivated on glycerol (maximum values ranging between 60 and 165 g/L) [14,15,59–66]. In general, with a few exceptions in which indeed high *CA* concentrations (≈102 g/L) have been reported by wild-type strains [67] *CA* quantities > 100 g/L are obtained by mutant (i.e. acetate-negative) or genetically engineered *Y. lipolytica* strains [60–66]. The yield $Y_{CA/Gly}$ achieved in the current investigation (=0.75 g/g) is quite high compared with these reported for both wild-type and mutant strains [16,60,61,63] indicating the potential of the wt strain ACA-DC 5033 to produce *CA* in promising quantities and conversion yields when higher Gly_0 concentrations are employed in double-limited media.

Y. lipolytica ACA-DC 5033 and *R. toruloides* DSM 4444 were cultivated on crude glycerol at higher Gly_0 (≈90 g/L) under single nitrogen limitation, where the initial concentration of nitrogen was as previously (yeast extract at 1.0 g/L and peptone at 2.0 g/L; initial C/N ≈ 120 moles/moles) (see Table 2). It can be seen that much higher DCW quantities were obtained for both microorganisms as compared to the respective trial with $Gly_0 \approx 50$ g/L (C/N ≈ 65 moles/moles) (see Tables 1 and 2). *Y. lipolytica* accumulated lower lipid in DCW quantities in the trial with $Gly_0 \approx 90$ g/L compared to $Gly_0 \approx 50$ g/L, despite the higher nitrogen limitation that was imposed in the former case ($Y_{L/DCWmax} = 20.6\%$ against 47.9% w/w; see Tables 1 and 2). Moreover, as previously, $Y_{L/DCW}$ values presented relatively elevated values at the end of trophophase and beginning of idiophase decreasing afterwards. Concerning total cellular polysaccharides, as in the trial with $Gly_0 \approx 50$ g/L (C/N ≈ 65 moles/moles), somehow elevated $Y_{IPS/DCW}$ values were recorded at the earlier growth steps, that were reduced as fermentation proceeded.

In *Y. lipolytica* ACA-DC 5033, nitrogen limitation and resulting idiophase led to principally polyols and to lesser extent citric acid production. As previously, nitrogen was depleted rapidly (i.e. after $t \geq 44$ h, *N* was = 10 ± 3 mg/L, with initial *N* quantity ≈90 mg/L). From the given time of nitrogen limitation, polyols were gradually accumulated into the medium, with the Pol_{max} concentration being = 47.6 g/L at $t = 211$ h after inoculation. *Ery* concentration was = 25.9 g/L, *Ml* concentration was = 17.5 g/L and *Ara* concentration was = 4.2 g/L. Simultaneously, *CA* in lower concentrations (up to 22.8 g/L) was produced. As previously indicated, it is a quite interesting (and unusual) as result, the fact that remarkable quantities of *Pol* were synthesized in trials performed in pH values ranging between 4.7 and 6.0. As previously indicated, it is possible that one and the same strain when cultivated under nitrogen limitation, can produce almost exclusively citric acid at medium pH = 5.0–6.0, while almost exclusive production of polyols may occur when cultures are performed at pH = 3.0–3.5 [19,57,58]. Moreover, in a scarce number of reports, it has been indicated that *Pol* are the principal metabolic compound of *Y. lipolytica* strains cultured under nitrogen limitation on pH values 4.7–6.0 [16,56,67] as it happens in the current investigation.

Pol biosynthesis occurred as secondary anabolic process, performed after nitrogen had been depleted from the medium (idiophase). At the relatively early steps of the culture thus, (i.e. $t = 0$–44 h after inoculation; trophophase), glycerol assimilation mainly contributed to yeast biomass production and not *Pol* accumulation into the medium. On the other hand, further incubation after glycerol had been depleted from the medium (end of idiophase) resulted in degradation of *Pol* and *CA*, presumably for energy of maintenance requirements. The representation of *Pol* produced *f* (glycerol) (without taking into consideration the late,

Table 3
Representative results concerning production of polyols by several yeasts belonging to the species *Yarrowia lipolytica* when cultivated on glycerol under various fermentation modes.

Strain	*Ery* (g/L)	*Ml* (g/L)	*Ara* (g/L)	*Pol* (g/L)	$Y_{Pol/Gly}$ (g/g)	Cultivation type	Reference
Wratislavia 1.31#†	132.0	23.0	-	155.0	0.52	Fed-batch reactor	Rymowicz et al. [57]
Wratislavia K1†	170.0	12.0	-	182.0	0.60	Fed-batch reactor	Rymowicz et al. [57]
A-15[&]	71.0	8.0	1.8	80.8	0.50	Batch reactor	Tomaszewska et al. [55]
A UV'1†	63.0	8.8	9.2	81.0	0.50	Batch reactor	Tomaszewska et al. [55]
Wratislavia K1†	80.0	2.6	0.3	82.9	0.51	Batch reactor	Tomaszewska et al. [55]
Wratislavia K1†	135.5	3.9	0.1	139.5	0.58	Repeated-batch reactor	Mirończuk et al. [17]
Wratislavia 1.31†	26.2	16.8	3.7	46.7	0.36	Batch reactor	Tomaszewska et al. [19]
Wratislavia K1†	40.7	15.1	2.9	58.7	0.40	Batch reactor	Tomaszewska et al. [19]
MK1†	79.5	2.7	0.4	82.6	0.55	Batch reactor	Mirończuk et al. [18]
MK1†	177.3	2.2	-	179.5	0.67	Repeated-batch reactor	Mirończuk et al. [18]
FCY 218†	80.6	n.i.	n.i.	80.6	0.53	Batch reactor	Carly et al. [68]
HA 1251[&¶]	≈4	≈32	≈5	≈41	n.i.	Batch reactor	Egermeier et al. [56]
ACA YC 5030[&¶]	35.5	32.1	-	67.6	0.49	Batch flasks	Papanikolaou et al. [16]
AIB[&]	56.7	12.6	6.0	75.3	0.49	Fed-batch reactor	Rakicka et al. [69]
AIB pADUTGUT1†	82.2	11.0	7.5	100.7	0.67	Fed-batch reactor	Rakicka et al. [69]
ACA-DC 5029[&¶]	15.6	10.5	3.4	29.5	0.39	Batch flasks	Sarris et al. [29]
ACA-DC 5033[&¶]	25.9	17.5	4.2	47.6	0.58	Batch flasks	Present study

†: Mutant or genetically modified *Yarrowia lipolytica*; [&]: Wild-type *Yarrowia lipolytica*; ¶: Fermentations in which the medium pH remained between 4.5–6.0 throughout the culture (in all other trials, medium pH was = 3.0–3.5).

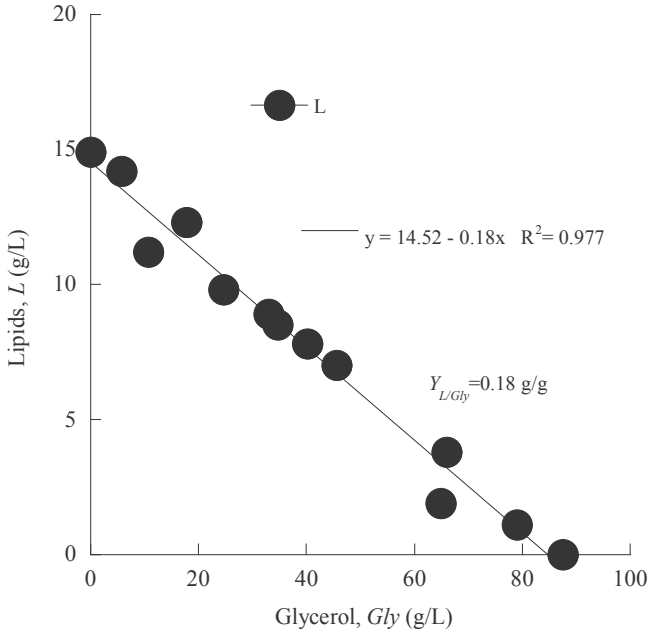

Fig. 5. Lipid (*L*, g/L) produced *vs* glycerol (*Gly*, g/L) during growth of *Rhodosporidium toruloides* DSM 4444, at $Gly_0 \approx 90$ g/L under nitrogen-limited conditions. Culture conditions as in "Section 2".

Table 4
Representative results concerning production of microbial lipid by several yeast species cultivated on glycerol under various fermentation modes.

Strain	Cultivation type	DCW (g/L)	$Y_{L/DCW}$ (% w/w)	Reference
Cryptococcus curvatus ATCC 20,509¶	Fed-batch reactor	118.0	25.0	Meesters et al. [73]
Yarrowia lipolytica ACA-DC 50,109¶	Batch reactor	4.7	23.1	Makri et al. [74]
Cryptococcus curvatus ATCC 20509#	Fed-batch reactor	32.9	52.9	Liang et al. [75]
Cryptococcus curvatus ATCC 20,509#	Fed-batch reactor	22.0	49.0	Cui et al. [76]
Yarrowia lipolytica MUCL 28,849¶	Fed-batch reactor	42.2	38.2	Fontanille et al. [77]
Rhodosporidium toruloides AS2.1389#	Batch reactor	26.7	69.5	Xu et al. [20]
Trichosporanoides spathulata JU4-57#	Batch flasks	17.1	43.4	Kitcha and Cheirslip [78]
Rhodosporidium toruloides Y4¶	Batch reactor	35.3	46.0	Uçkun Kiran et al. [22]
Rhodosporidium toruloides Y4#	Batch flasks	24.9	48.9	Yang et al. [23]
Lipomyces starkeyi DSM 70,296#	Batch flasks	34.4	35.9	Tchakouteu et al. [7]
Rhodosporidium toruloides DSM 4444¶	Batch reactor	~15	~57	Bommareddy et al. [79]
Rhodosporidium toruloides DSM 4444#	Batch flasks	37.0	37.0	Papanikolaou et al. [16]
Rhodosporidium toruloides AS 2.1389#	Batch flasks	15.5	58.6	Kamal et al. [72]
Rhodosporidium toruloides AS 2.1389#[a]	Batch flasks	18.9	64.5	Kamal et al. [72]
Rhodosporidium toruloides DSM 4444#	Batch flasks	27.3	54.6	Present study

¶: Utilization of pure glycerol; #: Utilization of crude glycerol; [a]: Supplementation with exogenous *L*-proline.

Table 5
Governing equations with the corresponding kinetic parameters for the production of cellular (lipids, *L*) and extra-cellular compounds (citric acid; *CA*, and polyols; *Pol*) during the growth of *R. toruloides* DSM 4444 and *Y. lipolytica* ACA-DC 5033 on biodiesel-derived glycerol.

Equation		Parameter	*Rhodosporidium toruloides*	*Yarrowia lipolytica*
$\frac{dX}{dt} = \mu X$	(1)	μ_{max} (h^{-1})	0.09764	0.1262
		X_{max} (g_X/L)	7.433	9.841
$\mu = \mu_{max}\left(1 - \frac{X}{X_{max}}\right)\left(\frac{Gly}{Gly_o}\right)$	(2)	SSE	0.394	5.52
		R^2	0.991	0.945
$-\frac{dN}{dt} = \mu\frac{1}{Y_{X/N}}X$	(3)	$Y_{X/N}$ (g_X/g_N)	121.10	96.97
		SSE	0.000317	0.00286
		R^2	0.896	0.740
$\frac{dL}{dt} = q_L X$ or $\frac{dCA}{dt} = q_{CA}X$	(4)	q_L ($g_L/(g_X h)$)	0.009570	-
		q_{CA} (g_{CA}/(g_Xh))	-	0.008310
		SSE	0.885	15.2
q_L and q_{CA} = constant	(5)	R^2	0.987	0.956
$\frac{dPol}{dt} = q_{Pol}X$	(6)	q_{PoL} (g_{PoL}/(g_Xh))	-	0.02669
		SSE	-	54.3
q_{Pol} = constant	(7)	R^2	-	0.983
$-\frac{dGly}{dt} = \mu\frac{1}{Y_{X/Gly}}X + q_L\frac{1}{Y_{L/Gly}}X$	(8)	$Y_{X/Gly}$ (g_X/g_{Gly})	0.8585	0.4374
		$Y_{L/Gly}$ (g_L/g_{Gly})	0.1932	-
or		$Y_{Pol,CA/Gly}$ (($g_{Pol}+g_{Po}$)/g_{Gly})		0.8411
$-\frac{dGly}{dt} = \mu\frac{1}{Y_{X/Gly}}X + (q_{Pol}+q_{CA})\frac{1}{Y_{Pol,CA/Gly}}X$	(9)	SSE	81.6	50.2
		R^2	0.966	0.993

"turnover" fermentation steps in which *Pol* were degraded) and the subsequent linear regression for the whole set of data (Fig. 4) can illustrate the global conversion yield of *Pol* synthesized on glycerol consumed ($Y_{Pol/Gly}$), that is = 0.58 g/g (the R^2 value of the regression is quite satisfactory for the linear regression, being = 0.93). The Pol_{max} produced in the current submission (=47.6 g/L), although lower in absolute values that many of the results reported in the literature (Table 3), presents interest given that this value can drastically increase after process optimization. On the other hand, strain ACA-DC 5033 is wild-type novel food-derived yeast strain [28] not previously studied in details concerning its physiological behavior. Also, in Table 3 most studies presented were performed in pH values ≈3.0–3.5, whereas in the current investigation the pH value was ≥ 4.7. Moreover, most strains studied concerning their potential on *Pol* production were mutants (i.e. acetate negative) or genetically modified ones.

R. toruloides DSM 4444 presented a very interesting production of SCO on $Gly_0 \approx 90$ g/L ($L_{max} = 14.9$ g/L, $Y_{L/DCW} = 54.6\%$ w/w; see Table 2). $Y_{IPS/DCW}$ values (%, w/w) decreased as the fermentation proceeded, in accordance with the results under lower nitrogen limitation ($Gly_0 \approx 50$ g/L, C/N≈65 moles/moles; see Table 1). As previously, lipids in appreciable quantities were started to be synthesized at the beginning of idiophase, after nitrogen had been depleted from the medium (i.e. after $t \geq 45$ h when *N* concentration was = 10 ± 3 mg/L, initial *N* quantity ≈70 mg/L). Although SCO production was a secondary anabolic activity and in the initial culture steps glycerol consumption mainly contributed to the formation of (lipid-free) biomass, finally the representation of *L* produced *f* (glycerol) and the subsequent linear regression for the whole set of data was very satisfactory (Fig. 5). This regression can illustrate the global conversion yield of *L* synthesized on glycerol consumed ($Y_{L/Gly}$), that is = 0.18 g/g (the R^2 value of the regression is quite satisfactory for the linear regression, being ≈0.98). Although

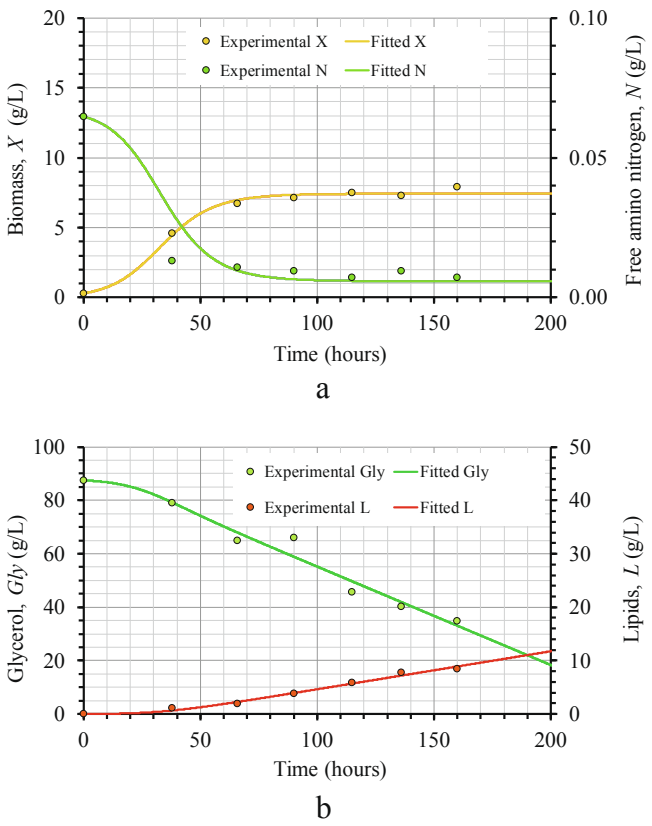

Fig. 6. Experimental data and fitted values showing the evolution of biomass (*X*), free amino nitrogen (*N*) (a), glycerol (*Gly*) and lipids (*L*) (b) concentration during the trophophase and the idiophase of *Rhodosporidium toruloides* DSM 4444 cultivated on glycerol in shake-flask experiments. Culture conditions as in "Materials and Methods".

maximum theoretical yield of lipid produced per glycerol consumed is ≈0.30 g/g [47] this yield (as that on glucose consumed, that is almost equivalent [47]), under real fermentation conditions is rarely ≥ 0.22 g/g, with these values being obtained generally by genetically engineered yeast strains cultivated in highly aerated bioreactors on glucose [10,70]. The obtained results thus, as regards both SCO production in absolute values (in g/L) and conversion yield of SCO produced on glycerol consumed by the strain *R. toruloides* DSM 4444, are quite encouraging. Further incubation of *R. toruloides* after glycerol assimilation from the medium, resulted in lipid biodegradation (end of idiophase and onset of storage compounds turnover [70]), in favor of further DCW and lipid-free material synthesis (Table 3).

In bioreactor experiments with glucose employed as sole substrate, *R. toruloides* DSM 4444 presented a yield of lipid produced glucose consumed = 0.21 g/g, with a simultaneous $Y_{L/DCW}$ value ≈65% w/w [31]. In flour-rich hydrolysates (composed mainly of glucose and maltose) yields of SCO produced per quantity of sugar assimilated was ≈0.17–0.18 g/g, while $Y_{L/DCW}$ values ≥ 50% w/w have been indicated [30] suggesting the suitability of glucose for *R. toruloides* DSM 4444. On the other hand, growth on xylose resulted in shift towards the synthesis of lipid-free material compared with the equivalent trials performed on glycerol, indicating that the former substrate (xylose) is less effective as regards SCO production as compared to glycerol or glucose [25]. Moreover, the same strain cultivated on biodiesel-derived crude glycerol in shake-flask trials, produced significant DCW quantities and higher compared with the present study (i.e. DCW_{max} = 35–39 g/L) that contained remarkably lower quantities of lipids ($Y_{L/DCWmax} \leq 38\%$ w/w) [16]. Presumably this happened due to the fact that in the later case, inorganic nitrogen [$(NH_4)_2SO_4$] was employed as the principal nitrogen source; implication of inorganic nitrogen (NH_4Cl) resulted in drastically lower $Y_{L/DCWmax}$ values compared with the utilization of organic nitrogenous compounds (*L*-glutamate or asparagines) for several types of oleaginous yeasts like *R. toruloides* CBS 14, *R. toruloides* IF0 0559, *R. toruloides* CBS 6016 and *Trichosporon cutaneum* 40 growing on glucose in shake-flask experiments (in all trials, the same initial nitrogen concentration was used) [71]. On the other hand, recent investigations with the strain *R. toruloides* AS 2.1389 cultivated on crude glycerol in flasks have demonstrated a conversion yield $Y_{L/Gly}$ = 0.21 g/g that increased to 0.26 g/g, when small concentrations of *L*-proline were added into the medium [72]. Lipids by various yeast strains cultivated on glycerol and comparison with the present study are all depicted in Table 4.

4.4. Modeling growth and production of secondary metabolites in *Rhodosporidium toruloides* and *Yarrowia lipolytica*

The governing equations with the corresponding kinetic parameters describing the production of lipids, citric acid, and polyols, during the trophophase and the idiophase of *Y. lipolytica* ACA-DC 5033 and *R. toruloides* DSM 4444 cultivated on crude glycerol (Gly_0≈90 g/L) are presented on Table 5. The equations and the iterative scheme used for their solution were based on previously given details [80]. Biomass specific production rate (μ) was simulated with the aid of a Verhulst-type equation [39,81] where the glycerol decreasing ratio, Gly/Gly_0, was also implicated, according to the approach proposed by Aggelis and colleagues [82–86]. In the models proposed, the last experimental points of the idiophase and the "turnover" phase (for the storage lipids in *R. toruloides* and for the extra-cellular metabolites in *Y. lipolytica*), were not subjected to simulation since in these cases, different approaches taking into consideration also the degradation of the products [82,84] would be requested. In the present work therefore, modeling only the first stages of fermentation (balanced phase and production phase) occurred. Moreover, concerning *R. toruloides* runs, the simulation of biomass (*X*) and the representation of optimized parameter values in which biomass was implicated (i.e. μ, $Y_{X/Gly}$, q_L) referred only to the "active" portion of biomass [82,84] therefore, lipid-free material was considered. In contrast, concerning *Y. lipolytica* runs, and given that lipid

Table 6
Comparison between the optimized parameter values and those calculated from the experimental data, during batch-flask cultures of *R. toruloides* DSM 4444 and *Y. lipolytica* ACA-DC 5033 on biodiesel-derived glycerol. Culture conditions as in "Materials and Methods".*

Parameter	Optimized values, *R. toruloides*	Experimental values, *R. toruloides*	Optimized values, *Y. lipolytica*	Experimental values, *Y. lipolytica*
μ_{max} (h^{-1})	0.09764	0.07**	0.1262	0.11**
X_{max} (g_X/L)	7.433	7.90	9.841	10.94
$Y_{X/Gly}$ (g_X/g_{Gly})	0.8585	0.149	0.4374	0.129
$Y_{X/N}$ (g_X/g_N)	121.10	136.91	96.97	116.17
$Y_{L/Gly}$ (g_L/g_{Gly})	0.1932	0.18		
$Y_{Pol,CA/Gly}$ [$(g_{Pol} + g_{CA})/g_{Gly}$]			0.8411	0.82

*: The experimental values for the parameters q_{CA}, q_L and q_{Pol} could not be accurately determined from data and for this reason they were not included in this table; **: experimental μ_{max} values were calculated by the formula $\mu_{max} = \frac{ln(X_1/X_0)}{t_1 - t_0}$ where X_1 was the first experimental point after inoculation and t_1 was the respective time, while X_0 was the concentration of inoculum and t_0 was = 0 h.

Table 7
Representation of optimized parameter values estimated from models describing lipid, citric acid and polyols production processes from yeasts and fungi cultivated on hydrophilic or hydrophobic carbon sources, and comparisons with the results obtained in the present study.

Parameter	Reference	Present study
μ_{max} (h^{-1})	0.23 (Papanikolaou and Aggelis [84])[1,2], 0.159–0.834 (Vasiliadou et al. [87])[3], 0.32 (Papanikolaou et al. [85])[4], 0.566 (Economou et al. [88])[5], 0.23 (Meeuwse et al. [89])[6], 0.21 (Meeuwse et al. [90])[7], 0.468 (Economou et al. [91])[8], 013 (Aggelis and Sourdis [82])[9], 0.052 (Diamantopoulou et al. [80])[10], 0.093 (Diamantopoulou et al. [80])[11], 0.267 (Karamerou et al. [92])[12], 0.22–0.43 (Valdés et al. [93])[13], 0.10–0.46 (Valdés et al. [93])[14], 0.15–0.54 (Valdés et al. [92])[15], 0.16–0.17 (Antimanon et al. [81])[16]	0.098[a], 0.126[b]
X_{max} (g_X/L)	18.7 (Diamantopoulou et al. [80])[10], 15.4 (Diamantopoulou et al. [80])[11], 13.72–18.39 (Antimanon et al. [81])[16], 4.06–4.23 (Sarris et al. [39])[17], 5.0–6.7 (Papanikolaou et al. [94])[17], 4.09 (Sarris et al. [39])[18], 5.89 (Papanikolaou and Aggelis [86])[19]	7.43[a], 9.84[b]
q_{Lmax} (g_L/(g_Xh))	0.16 (Papanikolaou and Aggelis [84])[1], 0.10 (Papanikolaou and Aggelis [84])[2], 7.57×10^{-4}–2.73×10^{-3} (Papanikolaou et al. [85])[4], 0.785 (Economou et al. [88])[5], 0.023 (Meeuwse et al. [89])[6], 0.298 (Economou et al. [91])[8], 0.06 (Aggelis and Sourdis [82])[9], 4.6×10^{-3} (Diamantopoulou et al. [80])[10], 5.1×10^{-3} (Diamantopoulou et al. [80])[11], 0.05 (Karamerou et al. [92])[12], 1.49×10^{-8}–6.06×10^{-8} (Antimanon et al. [81])[16], 0.03 (Robles-Rodriguez et al. [95])[20]	9.6×10^{-3}[a]
q_{CAmax} (g_{CA}/(g_Xh))	3.92×10^{-3}–8.50×10^{-3} (Papanikolaou et al. [85])[4], 0.110 (Sarris et al. [39])[17], 1.33×10^{-2} (Papanikolaou et al. [94])[17], 0.143 (Sarris et al. [39])[18], 3.03×10^{-3}–3.05×10^{-3} (Papanikolaou and Aggelis [86])[19], 0.053 (Klasson et al. [96])[21], 0.0511 (Klasson et al. [97])[21], 0.146 (Arzumanov et al. [98])[22]	8.3×10^{-3}[b]
q_{Polmax} (g_{Pol}/(g_Xh))	2.51×10^{-2}–4.20×10^{-2} (Papanikolaou et al. [67])[19]	2.67×10^{-2}[b]
Biomass yield (g_X per g of carbon source)	0.86 (Papanikolaou and Aggelis [84])[1], 0.78 (Papanikolaou and Aggelis [84])[2], 1.13–1.45 (Vasiliadou et al. [87])[3], 2.22 (Papanikolaou et al. [85])[4], 0.345 (Economou et al. [88])[5], 0.354 (Economou et al. [91])[8], 0.49–0.51 (Aggelis and Sourdis [86])[9], 0.33 (Diamantopoulou et al. [80])[10], 0.35 (Diamantopoulou et al. [80])[11], 0.20 (Karamerou et al. [92])[12], 0.27–0.35 (Valdés et al. [93])[13], 0.29–0.35 (Valdés et al. [93])[14], 0.19–0.31 (Valdés et al. [93])[15], 0.21–0.26 (Papanikolaou et al. [94])[17], 0.29–0.30 (Papanikolaou and Aggelis [86])[19], 0.12–0.27 (Papanikolaou et al. [67])[19], 0.41 (Klasson et al. [96])[21]	0.859[a], 0.437[b]
Lipid yield (g_L per g of carbon source)	0.63 (Papanikolaou and Aggelis [84])[1], 0.86 (Papanikolaou and Aggelis [84])[2], 1.16–1.39 (Vasiliadou et al. [87])[3], 0.27–0.52 (Papanikolaou et al. [85])[4], 0.242 (Economou et al. [88])[5], 0.298 (Economou et al. [91])[8], 0.58–0.60 (Aggelis and Sourdis [82])[9]; 0.42 (Diamantopoulou et al. [80])[10], 0.22 (Diamantopoulou et al. [80])[11]	0.193[a]
CA and/or *Pol* yield (g_{CA} and/or g_{Pol} per g of carbon source)	0.29–0.71 (Papanikolaou et al. [85])[4], 0.67–0.96 (Sarris et al. [39])[17], 0.82 (Papanikolaou et al. [94])[17], 0.81 (Sarris et al. [39])[18], 0.62–0.63 (Papanikolaou and Aggelis [86])[19], 0.66–1.06 (Papanikolaou et al. [67])[19], 0.71 (Klasson et al. [95])[20]	0.841[b]
$Y_{X/N}$ (g_X/g_N)	47.8–83.1 (Papanikolaou et al. [85])[4], 18.21 (Economou et al. [88])[5], 23.13 (Economou et al. [91])[8], 126.1 (Diamantopoulou et al. [80])[10], 108.7 (Diamantopoulou et al. [80])[11], 38.6 (Karamerou et al. [92])[12], 29.9–33.2 (Sarris et al. [39])[17], 30.6 (Sarris et al. [39])[18], 31.97–33.62 (Papanikolaou and Aggelis [86])[19], 23.3 (Robles-Rodriguez et al. [95])[20], 27.3 (Klasson et al. [97])[21], 19.67 (Arzumanov et al. [98])[22]	121.10[a], 96.97[b]

[1] : *Yarrowia lipolytica* on stearin (industrial derivative of tallow, composed of saturated free fatty acids), batch flask culture; [2]: *Yarrowia lipolytica* on stearin/hydrolyzed rapeseed oil 50/50, batch flask culture; [3]: *Yarrowia lipolytica* on olive oil, representation of values for each individual fatty acid of the substrate, batch flask culture;[4]: *Yarrowia lipolytica* on blend of commercial glucose and stearin, batch flask culture – in the referred model it was considered that for lipid yield only stearin contributed to the formation of cellular lipids, while for citric acid yield only glucose contributed to the formation of citric acid; [5]: *Mortierella isabellina* on sweet sorghum extract, batch flask culture; [6]: *Umbelopsis isabellina* on glucose, single-stage continuous culture; [7]: *Umbelopsis isabellina* on glucose, batch bioreactor culture; [8]: *Mortierella isabellina* on rice hull hydrolysate, batch flask culture; [9]: *Mucor circinelloides* on sunflower oil, batch flask culture; [10]: *Cryptococcus curvatus* on commercial xylose, batch bioreactor culture; [11]: *Cryptococcus curvatus* on commercial xylose, batch flask culture; [12]: *Rhodotorula glutinis* on glycerol, batch flask culture; [13]: *Meyerozyma guillermondii* on pure xylose, or pure mannose or pure xylose, batch flask culture; [14]: *Scheffersomyces coipomensis* on pure xylose, or pure mannose or pure xylose, batch flask culture; [15]: *Sugiyamaella paludigena* on pure xylose, or pure mannose or pure xylose, batch flask culture; [16]: Engineered *Aspergillus oryzae* on glucose, batch bioreactor culture; [17]: *Yarrowia lipolytica* on glucose-enriched olive-mill wastewaters, batch flask culture; [18]: *Yarrowia lipolytica* on glucose-enriched olive-mill wastewaters, batch bioreactor culture; [19]: *Yarrowia lipolytica* on (crude) glycerol, batch flask culture; [20]: *Yarrowia lipolytica* on glucose, fed-batch bioreactor culture; [21]: *Yarrowia* (*Saccharomycopsis*) *lipolytica* on glucose, batch bioreactor culture; [22]: *Yarrowia lipolytica* on technical-grade ethanol, repeated fed-batch bioreactor culture.

[a] : *Rhodosporidium toruloides* on crude glycerol, batch flask culture; [b]: *Yarrowia lipolytica* on crude glycerol, batch flask culture.

Table 8
Fatty acid composition of cellular lipids produced by yeasts during their growth on crude glycerol (Gly_0≈50 g/L, initial C/N ratio ≈65 moles/moles). Time of fermentation for the determination of the fatty acid composition was between 150 and 200 h after inoculation. Culture conditions as in "Materials and Methods".

	Fatty acid composition of yeast lipids (%, w/w)					
Yeast strain	C16:0	C18:0	$^{\Delta 9}$C18:1	$^{\Delta 9,12}$C18:2	$^{\Delta 9,12,15}$C18:3	Others
Yarrowia lipolytica ACA-DC 5033	11.6	10.8	60.9	6.8	Tr.	9.9
Yarrowia lipolytica LFMB Y19	15.6	7.6	49.2	14.9	2.2	10.5
Rhodosporidium toruloides DSM 4444	33.2	10.0	45.1	8.9	1.5	1.3
Rhodosporidium kratochvilovae FMCC Y71	14.1	2.0	24.9	38.1	8.6	12.3

Table 9
Fatty acid composition of the cellular lipids of *Rhodosporidium toruloides* DSM 4444 (a) and *Yarrowia lipolytica* ACA-DC 5033 (b) during growth on crude glycerol ($Gly_0 \approx 50$ and ≈90 g/L). Culture phase was the early (E) (20–60 h after inoculation), late (150–200 h after inoculation) and very late (250–300 h after inoculation). Two different cellular lipid extraction methods were applied (modified "Folch" and HCl digestion). Culture conditions as "Materials and Methods".

a)

Extraction method – Gly_0 (g/L)	Culture phase	C16:0	C18:0	$^{\Delta 9}$C18:1	$^{\Delta 9,12}$C18:2	$^{\Delta 9,12,15}$C18:3
Gly_0≈50 g/L	E	29.4	13.4	44.5	11.0	Tr.
Modified	L	33.2	10.0	45.1	8.9	1.5
"Folch"	VL	31.9	9.8	44.6	10.6	2.0
Gly_0≈50 g/L	E	31.8	9.4	48.0	9.0	Tr.
HCl	L	30.0	9.5	48.0	9.6	1.0
Boiling	VL	32.4	9.0	47.0	9.0	2.5
Gly_0≈90 g/L	E	30.7	7.9	50.1	8.2	0.9
Modified	L	28.7	6.9	50.0	9.0	1.5
"Folch"	VL	28.7	4.9	52.0	10.0	1.5

b)

Extraction method – Gly_0 (g/L)	Culture phase	C16:0	C18:0	$^{\Delta 9}$C18:1	$^{\Delta 9,12}$C18:2	$^{\Delta 9,12,15}$C18:3
Gly_0≈50 g/L	E	12.5	9.1	60.7	7.8	Tr.
Modified	L	11.6	10.8	60.9	6.8	Tr.
"Folch"	VL	9.0	12.0	62.0	5.0	Tr.
Gly_0≈50 g/L	E	10.1	8.8	57.9	9.0	1.0
HCl	L	9.1	10.0	59.7	7.2	1.0
Boiling	VL	8.0	11.4	60.4	6.0	Tr.
Double limitation; Gly_0≈50 g/L	E	19.0	12.5	51.9	9.0	1.0
Modified	L	11.3	13.0	64.5	7.9	Tr.
"Folch"	VL	10.1	12.0	65.9	5.0	Tr.
$Gly_0 \approx 90$ g/L	E	12.5	9.2	62.0	7.0	1.5
Modified	L	9.5	10.0	62.0	6.8	1.5
"Folch"	VL	9.0	11.0	68.9	9.0	Tr.

accumulation during growth on media with $Gly_0 \approx 90$ g/L was not dominant (always $Y_{L/DCW} \leq 20.6\%$ w/w), for the simulation of biomass (X) and the representation of the relevant optimized parameter values in which biomass was implicated it was decided to take into account the whole DCW value. Moreover, all specific formation rates, which are, q_{CA}, q_L and q_{Pol}, were assumed constant [85,86] while a single yield coefficient for both polyols and citric acid production with respect to glycerol consumed, $Y_{Pol,CA|Gly}$, expressing the combined mass (in $g_{CA} + g_{Pol}$) of polyols and citric acid produced per g of glycerol was assumed for the case of *Y. lipolytica*. Comparisons between experimental data and theoretical curves are depicted in Fig. 6 (a; b, case of *R. toruloides*) and 7 (a; b, case of *Y. lipolytica*), demonstrating a very satisfactory fitting for all runs performed. On the other hand, comparison of optimized model parameter values (μ_{max}, X_{max}, $Y_{X/N}$, $Y_{X/Gly}$, $Y_{L/Gly}$, $Y_{Pol,CA/Gly}$) with the experimental ones (Table 6) was quite satisfactory, providing evidence of the accuracy of the implicated models. Representation of optimized parameter values estimated from numerical models describing the process of production of metabolic compounds (i.e. storage lipid, citric acid, polyols) in various types of microorganisms (yeasts and fungi) cultivated on hydrophilic and/or hydrophobic carbon sources and their comparisons with the present study are all depicted in Table 7.

4.5. Yeast lipids analysis

All screened strains were analyzed concerning their total FA composition in the late growth phase (t = 150–200 h after inoculation) (Table 8). In agreement with the literature [2,12,70] the principal FAs found in variable quantities were mainly the oleate ($^{\Delta 9}$C18:1), the palmitate (C16:0) and the linoleate ($^{\Delta 9,12}$C18:2) (Table 9). Polyunsaturated FAs presenting double bonds ≥ 3 were not detected in high concentrations, since these compounds are the principal storage lipophilic compounds in oleaginous fungi and algae [2,8,33] and, in general, they can be produced in significant quantities inside the yeast cells only after appropriate genetic modifications [33]. Interestingly, *R. kratochvilovae* FMCC Y71 produced cellular lipids that contained high concentrations of $^{\Delta 9,12}$C18:2, in contrast to recent literature reports [99,100]. Moreover, *R. toruloides* presented cellular lipids containing mostly the FAs $^{\Delta 9}$C18:1 and C16:0. The method of extraction did not significantly influence the FA composition of the cellular produced lipids, in accordance with the results reported for the microorganisms *C. curvatus*, *M. isabellina* and *C. sorokiniata* [52]. The FA composition of *R. toruloides* lipids was not drastically influenced by the initial glycerol concentration or the fermentation time, in agreement with the results reported for strains of this species or even for other "red" yeasts (i.e. *R. kratochvilovae, R. minuta*) growing on glycerol or sugars [16,24,25,72,99–101]. Finally, *Y. lipolytica* ACA-DC 5033 presented in its cellular lipids slightly higher $^{\Delta 9}$C18:1 quantities (up to 60% w/w or even higher) compared with many other strains of this species cultivated on glycerol-based media that in the various growth phases contained the above-mentioned FA in quantities ranging between 40–50% w/w [15,25,29,42,74]. Only in a relatively scarce number of reports, some strains of this species presented concentrations of the FA $^{\Delta 9}$C18:1

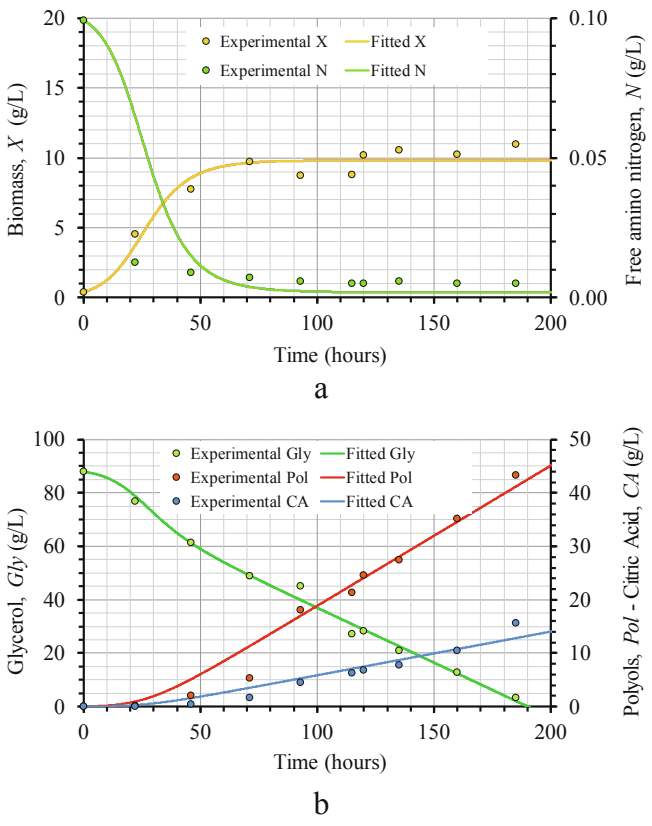

Fig. 7. Experimental data and fitted values showing the evolution of biomass (*X*), free amino nitrogen (*N*) (a), glycerol (*Gly*), polyols (*Pol*) and citric acid (*CA*) concentration during the trophophase and the idiophase of *Yarrowia lipolytica* ACA-DC 5033 cultivated on glycerol in shake-flask experiments. Culture conditions as in "Section 2".

reaching to 60% w/w [16,41] whereas the fermentation time, the addition of natural compounds, or the increase of substrate concentration into the medium can modify the FA composition of the lipids of this microorganism without any systematic effect of these parameters upon the FA composition of this microorganism [15,15,42,74] (See Fig. 7).

5. Concluding remarks

Four strains belonging to *Y. lipolytica* and *Rhodosporidium* sp. demonstrated interesting DCW production and glycerol uptake on nitrogen-limited media based on crude glycerol. Two amongst these, namely *Y. lipolytica* ACA-DC 5033 and *R. toruloides* DSM 4444 were further studied, as regards the process implicated in their cellular lipids extraction, and their potential to produce metabolites under higher initial glycerol concentration media, or double-limited media. Two different extraction methods were studied as regards the recovery of lipids from the DCW and no differences were observed for the strain *R. toruloides* DSM 4444. On the other hand, for the strain *Y. lipolytica* ACA-DC 5033, much higher lipid recovery was observed through the extraction with a mixture of chloroform/methanol without the step of DCW acidification and boiling. Moreover, *Y. lipolytica* accumulated noticeable quantities of lipids at the relatively earlier growth steps, which were degraded as fermentation proceeded in favor of polyols and citric acid production. In double-limited media (besides nitrogen also Mg was limiting factor for cell growth), a shift in favor of citric acid production occurred. In single nitrogen-limited media in which glycerol was adjusted to ≈90 g/L, the quite promising total polyols (arabitol + mannitol + erythritol) production of *c.* 48 g/L (simultaneous yield on glycerol consumed = 0.58 g/g) was obtained. Finally, *R. toruloides* DSM 4444 growing on glycerol at *c.* 50 g/L produced DCW = 18.1 g/L containing lipids = 30.3% in DCW, while at *c.* 90 g/L produced DCW = 27.3 g/L containing lipid = 54.5% w/w. Trophophase and idiophase of both yeasts was successfully simulated with the aid of numerical models. Yeast lipids contained mostly oleic acid and to lesser extent palmitic acid.

CRediT authorship contribution statement

Sofia Sarantou: Investigation, Methodology, Data curation, Formal analysis. **Nikolaos G. Stoforos:** Investigation, Data curation, Formal analysis, Software. **Ourania Kalantzi:** Methodology, Data curation. **Seraphim Papanikolaou:** Conceptualization, Project administration, Writing - review & editing.

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