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Original Article

Studies of polyol production by the yeast *Yarrowia lipolytica* growing on crude glycerol under stressful conditions

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ABSTRACT

Crude glycerol, the principal by-product of biodiesel production process, was employed as substrate by three wild-type *Yarrowia lipolytica* strains (ACA-YC 5030, LMBF 20 and NRRL Y-323). Stressful conditions (low pH value = 2.0 ± 0.3, low incubation temperature $T = 20 \pm 1$ °C, non-aseptic conditions) were employed. Interesting production of yeast biomass and polyols (viz. erythritol, mannitol and arabitol) was noted at pH = 2.0 ± 0.3 and $T = 20 \pm 1$ °C. Strains failed to produce significant quantities of cellular lipid, while variable quantities of intra-cellular polysaccharides were produced. Fermentations under previously pasteurized media supported significant biomass and polyols production for most of the tested strains, while only one strain (NRRL Y-323), managed to produce polyols at media that were not previously thermally treated at all. The production of mannitol was favored at low initial glycerol ($Glol_0$) concentrations, whereas higher $Glol_0$ quantities favored the biosynthesis of erythritol. For the strain NRRL Y-323, highly aerated / agitated bioreactor trials showed different physiological profiles as compared to the respective flask experiments. Finally, in flask experiments with the strain NRRL Y-323 at high $Glol_0$ amounts (≈140 g/L) at low medium pH (=2.0 ± 0.3), a significant production of polyols (=84.2 g/L) with the corresponding remarkable conversion yield on glycerol consumed = 62 % w/w was achieved.

Practical application: Renewable and biodegradable fuels, such as biodiesel, are safer and environmentally friendlier than the conventional petroleum diesel. Glycerol is a cost-effective substrate obtained as the main side-product from biodiesel production process and is currently being employed in the realm of Industrial Microbiology and Biotechnology to produce metabolic products with added value. Current research focuses on using glycerol as a starting substrate for biotechnological conversions aiming at producing, amongst other compounds, polyols, microbial biomass, citric acid, etc. from selected strains of the Generally Recognized As Safe (GRAS) yeast *Yarrowia lipolytica*. In the current investigation therefore, we examined the capacity of new wild-type non-extensively studied strains of this yeast to grow and assimilate this inexpensive substrate. Specifically, we have performed the acclimatization of the mentioned strains to stressful environments (i.e., low pH, low incubation temperature, non-aseptic conditions, etc.) and remarkable quantities of the added-value compounds (polyols, yeast mass, citric acid) were produced.

1. Introduction

Due to population growth and the human activities including but not limited to agriculture, industrialization, and transportation, the need for energy has significantly increased the last decades. Moreover, the latter developments and the very important financial events that have occurred the last years (i.e., the economic crisis of the last decade, the war in the Eastern Europe, etc.) resulted in a significant rise in the cost of all types of fuels used for the various types of machines and heating systems. Consequently, the availability of all fuel sources is constrained and dwindling, and their price is constantly increasing, resulting, therefore, in significant energy shortage, and this event occurs

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specifically the last years [1–4]. The pursuit of low-cost alternative energy sources that can be utilized consistently is, therefore, imperative for meeting the increasing energy demands and safeguarding oil reserves, specifically if these energy sources are renewable, sustainable, non-toxic, highly degradable, low in carbon monoxide emissions, widely accessible, and environmentally friendly. Amongst renewable energy sources currently implicated in industrial uses, biodiesel is an attracting and promising biofuel, presenting all of the previously mentioned characteristics [3,5–7]. Thus, the constant increase in biodiesel production results in excess synthesis and subsequent disposal of condensed glycerol-containing water, the so-called crude glycerol (crude glycerin), which is the main by-product generated through biodiesel production process. The continuously increasing accumulation of crude glycerol into the market volume is likely to result in serious environmental issues in the near future [3,7,8]. Therefore, the conversion of concentrated glycerol-containing water into higher added-value products is currently a fundamental issue and one of the "hottest" topics developed in the current Industrial Biotechnology and Green Chemistry [8–10], while besides biodiesel, glycerol is a by-product generated in substantial quantities during several other agro-industrial activities (i.e., the facilities manufacturing alcoholic beverages, bioethanol or potable ethanol, soaps, etc.) [8–10].

The present investigation deals with the microbial valorization of crude glycerol through cultures performed by the non-conventional yeast *Yarrowia lipolytica*, in order to primarily synthesize erythritol and mannitol. Recently, it has been demonstrated that the low-calorie sweetener erythritol might be produced from waste resources by several wild or mutant strains of the yeast *Y. lipolytica* [3,10,12]. Erythritol is a natural sweetener and tastes almost like the table sugar, but it does not cause obesity. Erythritol belongs to the class of compounds called sugar-alcohols; they are found in natural foods like fruits, honey, or wine. This is a four-carbon sugar-alcohol produced as an osmoprotectant by several microorganisms, including but not limited to *Y. lipolytica* [3]. Like other polyols, it has sweetened properties with the texture and taste similar to these of table sugar. Due to its molecular structure, this sugar-alcohol stimulates the sweet taste receptors but does not increase the insulin level into the blood [10]. For people who are overweight and suffering from diabetes, or presenting other problems related to metabolic syndrome, erythritol seems to be an excellent alternative to table sugar. Up to date, most industrial production of polyols is achieved through chemical hydrogenation of the corresponding sugars that are employed as starting materials of the syntheses, with reactions necessitating harsh operating conditions (i.e., temperatures ranging between $T = 100$–200 °C and pressures ranging between 40.0 and 120.0 bar respectively) and presenting relatively low conversion yields [3,10]. On the other hand, recently published data have showed that erythritol might be produced from glycerol by safe yeasts [3,10,11], while the produced sweetener could be used in pilot-scale operations in various food formulations [11].

Y. lipolytica is a non-conventional species that can have various morphological forms, from those with a typical spherical shape to the form of pseudo-hyphae, and even it can be presented in the characteristic form of the true mycelium [12–14]. This yeast species can present a high tolerance to several extreme environmental conditions like the low incubation temperatures, the high salinity into the medium and the potential of growing in a wide range of medium pH values [10,15,16]. It is interesting to indicate that in several cases, the biochemical and physiological response of the microorganism (i.e., its potential to secrete metabolic compounds) is closely related to the governance of different operating conditions (i.e., in trials performed at low pH values mostly polyols are produced while at neutral / slightly acidic conditions, and provided that all other parameters remain the same, shift towards the synthesis of citric acid is performed) [3,10,12].

In the present investigation, several wild-type non-previously extensively studied strains of the yeast *Y. lipolytica* were evaluated for their capacity to convert crude glycerol under nitrogen-limited conditions to produce yeast biomass, intra-cellular metabolites (i.e., microbial lipids and polysaccharides), and polyols. As far as the polyols are concerned, as previously mentioned, their main industrial synthesis way is through catalytic hydrogenation of the corresponding sugars but with harsh operating conditions, and low yields while costly purification steps are required after the accomplishment of the chemical processes [3,17]. The biotechnological production of polyols by *Y. lipolytica* has been emphasized the last years, being advantageous due to the GRAS nature of the implicated yeast [2,4,12], the valorization of residues and the non-harmful to the environment operating conditions employed. In the current investigation thus, newly isolated *Y. lipolytica* strains were used under "stressful" environmental conditions, and the parameters of the growth and the production of polyols were critically evaluated. Considerations concerning the yeast physiology under these conditions were assessed and discussed.

2. Materials and methods

2.1. Raw materials and microorganisms

Industrial (crude) glycerol was obtained from the Hellenic biodiesel-producing facility "ELIN VERD SA" (Velestino, Magnesia Prefecture, Greece). Crude glycerol deriving exclusively from transesterification of used/cooked oils was used as microbial substrate in the performed trials. This feedstock contained approximately on a mass basis (%, w/w): 74 % glycerol, <0.1 % lipids (monoglycerides, diglycerides and free fatty acids), <0.1 % methanol, 12 % potassium and sodium salts, 3 % ash, 11 % water (the pH of the feedstock was = 1.0 ± 0.2). The biological materials used in this investigation were the strains of yeast *Y. lipolytica* ACA-YC 5030, LMBF 20, and NRRL Y-323. The strains with the code characteristics ACA-YC and LMBF were provided by the culture collections of the Department of Food Science and Human Nutrition (Agricultural University of Athens, Athens, Greece), while the strain with the code characteristic NRRL was kindly provided by the NRRL culture collection (Peoria, USA).

2.2. Inoculum preparation and fermentation media

All strains were maintained at $T = 4 \pm 1$ °C on glass slopes containing 10 g/L glucose, 10 g/L yeast extract, 10 g/L peptone, and 20 g/L agar. Prior to any inoculation in liquid growth media, strains were regenerated under aseptic conditions so that the inocula would be of three days old. Following, two Erlenmeyer flasks of 250 mL filled with 50 ± 1 mL of the pre-culture medium were aseptically inoculated from the principal freshly regenerated strain and were incubated in an orbital shaker (Zhicheng ZHWY 211C; Shanghai, China) for 24 h at 180 ± 5 rpm, $T = 30 \pm 1$ °C. The pre-culture medium consisted of 10 g/L of glycerol used as a carbon source (crude glycerol was employed as substrate in order to perform better adaptation of the strains), 10 g/L of peptone, and 10 g/L of yeast extract, the latter ones being used as nitrogen and trace elements source. Microscopic observations of the yeast strains were conducted to verify the strain's purity before inoculation into the principal media.

The cultures were done under aseptic conditions in media sterilized at $T = 121$ °C for 20 min. Certain trials were conducted in non-thermally treated media (*viz*. inoculation from the pre-culture was performed to the principal medium that had not previously undergone any heat treatment at all), while others were conducted in previously pasteurized media (viz. the medium had been heat treated at $T = 65 \pm 1$ °C / 30 min before inoculation). The carbon source used in the principal culture was that of crude glycerol with the initial concentrations of the substrate ($Glol_0$) being adjusted to ≈40 g/L and ≈80 g/L. Moreover, for the strain NRRL Y-323 that presented the best performances, supplementary trials with $Glol_0$ concentrations adjusted to *c*. 120 and 140 g/L were also carried out. Appropriate calculations were performed by taking into consideration the purity of the feedstock in order to achieve the mentioned $Glol_0$ concentrations in all fermentations performed. All

trials of the principal culture were conducted under nitrogen-limited conditions employed in order to enhance the production of secondary metabolites like polyols in *Y. lipolytica* [3,7,9]. The nitrogen source comprised of 2 g/L bacteriological peptone and 1 g/L yeast extract. Nitrogen remained constant throughout the experiments in the increasing $Glol_0$ concentrations, therefore, besides the effect of the increasing $Glol_0$ concentration, also the effect of increasing carbon-excess conditions upon the physiological behavior of *Y. lipolytica* was assessed. The media contained the following minerals: 0.06 g/L $MnSO_4$ H_2O, 1.5 g/L $MgSO_4$ $7H_2O$, 0.02 g/L $ZnSO_4$ $7H_2O$, 0.15 g/L $CaCl_2$ $2H_2O$, 0.15 g/L $FeCl_3$ $6H_2O$, 7.0 g/L KH_2PO_4 and 2.5 g/L Na_2HPO_4.

2.3. Culture conditions

The fermentation efficiency was investigated initially in shake-flask experiments when the employed strains were exposed to various stressful conditions. The carbon source used was crude glycerol deriving from transesterification of used/cooked oils. The strains were thereafter examined for their capability to withstand temperatures and pH values below those cited in the literature as optimal. Two different incubation temperatures of $T = 20 \pm 1$ °C and $T = 30 \pm 1$ °C (the latter used as optimal – see [4,9,10]) and two different medium pH values, 2.0 ± 0.3 and 6.0 ± 0.3 (the latter used as optimal – see [3,14,16]) were tested. Furthermore, strains were cultivated in previously sterilized, previously pasteurized, and in non-thermally at all treated media. The most promising strain (NRRL Y-323) was cultured in batch bioreactor fermentations and in higher $Glol_0$ concentrations in shake-flask trials aiming at more efficient production of the target metabolites (*viz.* polyols).

Shake-flask experiments of the principal culture were conducted in 250-mL Erlenmeyer flasks, containing 50 ± 1 mL of growth medium and inoculated with 1 mL of a 24-h exponential pre-culture (*c.* 2.0×10^6 cells, initial biomass concentration at the flasks $X_0 \approx 0.12$ g/L; see pre-culture composition previously). As in the case of the pre-cultures, principal flask cultures were performed in an orbital shaker (Zhicheng ZHWY 211C; Shanghai, China) at 185 ± 5 rpm and incubation temperature $T = 30 \pm 1$ °C or $T = 20 \pm 1$ °C. Besides flask cultures, batch bioreactor experiments were conducted in previously sterilized (sterilization performed at $T = 121$ °C / 30 min) or non-thermally treated conditions. Bioreactor trials were carried out in a 3-L bioreactor (INFORS HT Labfors; Bottmingen, Switzerland), with a working volume of 1.5 L and incubation temperature of $T = 30 \pm 1$ °C. Agitation rate of 750 ± 5 rpm and aeration rate of 0.5–1.5 vvm were employed to maintain the dissolved oxygen tension (DOT) at a saturation value $\geq$ 20.0 %, v/v. In the bioreactor experiments, $Glol_0$ concentration was $\approx$40 g/L and the composition of the mineral solution, as well as the initial nitrogen quantity, were as reported previously. The pH was automatically regulated at 2.0 ± 0.3 using sterile 5 M NaOH. Inoculation was performed with 10 % v/v of 24-h yeast pre-culture. During fermentations in both flasks and bioreactor, samples were taken periodically for the determination of extra-cellular compounds (polyols, glycerol citric acid), dry cell weight (DCW), intra-cellular polysaccharides (IPS) and lipid (L).

2.4. Analytical methods

Yeast cell biomass (viz. the whole content of the flasks or a content of $\approx$20 mL from the bioreactor) was harvested by centrifugation in a Universal 320R - Hettich centrifuge (Vlotho, Germany) at $9{,}000 \pm 5$ rpm for 10 min at $T = 4.0 \pm 0.1$ °C, washed once with distilled water, and centrifuged again. Biomass concentration was determined gravimetrically from dry cell weight at $T = 80 \pm 1$ °C to constant weight. A pH meter (Jenway 3020, Cole-Parmer, Eaton Socon, UK) was used to determine off-line the pH of the medium in the flask experiments. The pH correction of the medium was maintained at the desired value by aseptically adding the necessary amounts of NaOH or HCl solution to the flasks. Glycerol consumption and production of polyols and acid were determined using high-performance liquid chromatography (HPLC) analysis as previously indicated [5].

Total intra-cellular polysaccharides (IPS) were measured based on a modified protocol of Argyropoulos et al [18]. Briefly, 0.05 g of DCW was acidified by adding 10 mL of HCl (2 M). The acidified solution was then hydrolyzed at $T = 100 \pm 1$ °C for 30 min, neutralized to pH 7.0 ± 0.3 with 10 mL NaOH (2 M), filtered through Whatman filter paper, and subjected to determination of reducing sugar content (glucose equivalent), according to the method of 3,5 dinitrosalicylic acid. Intra-cellular polysaccharide content ($Y_{IPS/X}$) was expressed as g of IPS produced per 100 g of DCW (%, w/w).

Total intra-cellular lipids (L) were quantified according to previously published procedure (Folch, [19,20]) with some modifications; after mechanical disruption of the dried biomass, Folch mixture (chloroform: methanol at 2:1, v/v) was added for total lipid extraction and quantification. Specifically, yeast-dried biomass (around 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) [19–21]. The whole was closed with an aluminum screw cap and left in the darkness for at least 5 days. Occasionally, the vials' content was gently mixed with the aid of a glass stick. Then, cell debris was removed through filtration (Whatman® filter n°3), and the extract was collected in a pre-weighted evaporator flask. It was utterly evaporated in a rotary evaporator (R − 144, Büchi Labortechnik, Flawil, Switzerland) *via* vacuum evaporation, and lipids were determined gravimetrically in pre-weighed round bottom flasks. Intra-cellular lipid content ($Y_{L/X}$) was expressed as g of lipid produced per 100 g of DCW (%, w/w). To study the fatty acid (FA) composition of the cellular lipids of the used strains, total lipids were *trans*-methylated and were converted to their respective FA methyl-esters (FAMEs) in a two-step reaction with methanolic sodium and hydrochloric methanol as previously described [19,21]. GC analysis of the produced FAMEs was carried out on a Fisons 8060 device (Biostad, Canada) according to Dritsas and Aggelis [19]. FAMEs were identified by reference to authentic standards of C16:0, C18:0, $^{\Delta 9}$C16:1, $^{\Delta 9}$C18:1 and $^{\Delta 9,12}$C18:2.

2.5. Abbreviations and units

Ara: Arabitol (g/L); X: Biomass (dry cell weight – DCW, g/L); CA: Citric acid (g/L); Ery: Erythritol (g/L); L: Intra-cellular lipids; IPS: Intra-cellular polysaccharides; Glol: Glycerol (g/L); MI: Mannitol (g/L); Pol: Total polyols (g/L); $Y_{L/X}$: Intra-cellular lipids in DCW (% w/w); $Y_{IPS/X}$: Intra-cellular polysaccharides in DCW (% w/w); $Y_{Pol/Glol}$: Yield of total polyols produced per glycerol consumed (%, w/w); indices 0 and max correspond to the initial and maximum quantities of the elements in the performed trials.

3. Results and discussion

3.1. Growth of yeast strains at low incubation temperature

All yeast strains were cultivated on industrial-type glycerol. The effect of the incubation temperature upon the biosynthesis of metabolites and specifically the formation of sugar-alcohols has not been extensively discussed in the literature [22,23]. It is recommended that growth temperatures of *Y. lipolytica* strains would be ranging between 24 and 33 °C [21,24,25]. The efficiency of the strains employed in the present study at low temperatures ($T = 20 \pm 1$ °C) that normally does not allow sufficient microbial growth in *Y. lipolytica* [21], was compared at both very low and slightly acidic medium pH values [25]. Besides the obvious academic interest, successful fermentation achieved by the newly isolated *Y. lipolytica* strains at these low incubation temperatures (i.e. $T = 20 \pm 1$ °C), could potentially have as result the process scale-up in these incubation temperatures that do not allow sufficient microbial growth by many microorganisms grown on glycerol-based media (in most cases the optimal temperatures for these strains vary between 25 and 37 °C

Table 1
Quantitative data of cultures of *Yarrowia lipolytica* strains on glycerol-based media ($Glol_0$≈40 g/L) under different pH values. Culture conditions: shake-flask fermentation in 250.0-mL conical flasks at 180 ± 5 rpm and incubation temperature $T = 20 \pm 1$ °C. Each experimental point presented is the mean value of two independent determinations (SE ≤ 15.0 %).

	Stains	Time (h)		Glol consumed (g/L)	X (g/L)	MI (g/L)	Ery (g/L)	Ara (g/L)	Pol (g/L)	CA (g/L)	$Y_{IPS/X}$ (% w/w)	$Y_{L/X}$ (% w/w)
pH 2.0 ± 0.3	ACA-YC 5030	45	f	12.6	4.7	8.2	0.7	1.0	10.0	nd	7.5	18.3
		95	e	33.7	9.6	9.6	4.5	2.7	16.3	nd	14.0	2.2
		120	a,b,c,d	38.0	9.8	10.5	6.4	2.7	19.6	nd	12.1	1.9
	LMBF 20	70	f	18.2	6.1	8.6	0.6	1.1	10.3	nd	9.8	4.9
		95	e	25.0	6.7	9.4	1.1	1.6	12.1	nd	15.5	3.9
		120	a,b,c,d	31.8	7.4	10.0	1.5	1.9	13.5	nd	12.8	2.4
	NRRL Y-323	48	b,d	18.5	6.0	0.6	1.6	0.2	2.4	nd	4.0	0.5
		168	a,c,f,e	20.6	6.0	0.5	1.8	0.0	2.3	nd	4.0	0.8
pH 6.0 ± 0.3	ACA-YC 5030	70	e	18.7	9.4	8.7	1.3	1.3	11.3	nd	24.6	12.2
		95	f	26.6	10.2	8.7	2.0	1.4	12.2	nd	20.1	19.7
		120	a,b,c,d	34.7	12.4	10.2	4.5	2.1	16.8	nd	14.8	19.9
	LMBF 20	70	f	20.9	9.0	9.1	0.5	1.3	10.8	nd	13.3	36.2
		95	e	31.5	11.1	9.1	0.6	1.5	11.2	nd	17.7	16.5
		120	a,b,c,d	31.5	11.2	10.6	0.6	1.9	13.1	nd	19.2	4.6
	NRRL Y-323	48	e	7.6	1.5	0.5	1.5	0.0	1.9	3.2	8.3	1.5
		120	b,c	8.9	1.7	1.6	2.0	0.3	3.9	3.9	7.7	2.0
		168	a,d	9.1	3.0	1.6	2.0	0.4	4.0	4.5	4.6	2.1

Representations: (a) When the maximum concentration of biomass is achieved; (b) When the maximum concentration of mannitol is achieved; (c) When the maximum concentration of erythritol is achieved; (d) When the maximum concentration of arabitol is achieved; (e) When the maximum percentage of intra-cellular polysaccharides on dry matter ($Y_{IPS/X}$, % w/w) is achieved; (f) When the maximum percentage of lipid on dry matter ($Y_{L/X}$, % w/w) is achieved.
nd: Not determined (≤0.3 g/L).

Table 2
Quantitative data of cultures of *Yarrowia lipolytica* strains on glycerol-based media ($Glol_0$≈40.0 g/L) under different pH values. Culture conditions: shake-flask fermentation in 250.0-mL conical flasks at 180 ± 5 rpm and incubation temperature $T = 30 \pm 1$ °C. Each experimental point presented is the mean value of two independent determinations (SE ≤ 15.0 %).

	Stains	Time (h)		Glolconsumed (g/L)	X (g/L)	MI (g/L)	Ery (g/L)	Ara (g/L)	Pol (g/L)	CA (g/L)	$Y_{IPS/X}$ (% w/w)	$Y_{L/X}$ (% w/w)
pH 2.0 ± 0.3	ACA-YC 5030	119	e,f	36.3	6.2	15.5	3.5	5.7	24.7	nd	14.6	7.3
		141	a,b,c,d	43.3	7.1	16.0	3.9	5.9	25.5	nd	14.3	4.1
	LMBF 20	52	f	20.0	4.3	11.1	2.2	0.0	13.3	nd	18.6	14.5
		121	e	24.7	5.0	12.5	3.6	2.2	18.3	nd	20.5	10.0
		140	a,b,c,d	41.3	6.4	14.2	4.5	3.5	22.2	nd	16.0	3.3
	NRRL Y-323	172	e	28.1	8.2	10.9	4.9	1.1	16.8	nd	9.3	35.2
		216	a,b,c,d,f	42.4	10.5	14.0	9.9	1.8	25.7	nd	7.7	19.4
pH 6.0 ± 0.3	ACA-YC 5030	22	f	21.6	3.7	8.2	0.0	0.0	8.2	nd	15.5	12.9
		119	b,c,d,e	40.3	7.1	16.2	2.6	1.4	20.2	nd	12.3	5.1
		141	a	41.3	9.4	13.7	0.7	1.0	15.4	nd	7.1	3.0
	LMBF 20	75	e	27.3	6.4	12.2	0.8	1.8	14.9	nd	9.0	9.4
		121	a,b,c,d,f	39.8	6.9	16.5	1.0	2.7	20.3	nd	1.6	13.2
	NRRL Y-323	172	f	26.4	7.1	9.7	3.7	0.6	14.1	4.7	2.0	37.4
		216	a,b,c,d, e	40.1	11.1	14.5	6.5	1.1	22.0	6.0	3.1	16.6

Representations: (a) When the maximum concentration of biomass is achieved; (b) When the maximum concentration of mannitol is achieved; (c) When the maximum concentration of erythritol is achieved; (d) When the maximum concentration of arabitol is achieved; (e) When the maximum percentage of intra-cellular polysaccharides on dry matter ($Y_{IPS/X}$, % w/w) is achieved; (f) When the maximum percentage of lipid on dry matter ($Y_{L/X}$, % w/w) is achieved.
nd: Not determined (≤0.3 g/L).

[21,23]. On the other hand, low pH values were chosen, since it has been seen in the literature that the production of polyols was triggered by low pH values (i.e., pH≈3.0) into the growth medium [26–28]. However, in the present investigation, even a lower pH value (pH = 2.0 ± 0.3) was chosen than that recommended for the production of polyols (pH≈3.0), since potential growth and sufficient production of metabolites in this very low pH value, could potentially allow the accomplishment of the bioprocess under previously pasteurized (and not sterilized) media.

The fermentation efficiency of the tested strains in shake-flask experiments with $Glol_0$≈40 g/L under nitrogen limitation, is shown in Tables 1 and 2. The strain NRRL Y-323, upon exposure to low temperatures, was unable to metabolize the entire quantity of substrate in order to produce polyols, DCW, microbial lipids and other metabolites, irrespective of the medium pH value that was imposed. In fact, under these conditions, the production of DCW and the subsequent assimilation of glycerol was remarkably lower compared to the other two strains, *viz.* ACA-YC 5030 and LFMB 20 (see Table 1). It is noted that the polyol production by *Y. lipolytica* ACA-YC 5030 and LMBF 20 for the two pH values to which they were subjected, was remarkably similar under identical low-temperature conditions. On the other hand, the strain NRRL Y-323 as mentioned, failing to utilize a significant portion of the available substrate at $T = 20 \pm 1$ °C, resulted in inadequate Pol production (this was also observed for this strain at $T = 20 \pm 1$ °C and pH = 6.0 ± 0.3). Particularly noteworthy was the production of polyols, specifically Ml, Ery and Ara, which amounted to 25.6 g/L and was achieved by the strain NRRL Y-323 under elevated temperature and low pH value.

As indicated in the previous paragraphs, the biotechnological production of metabolic compounds like sugar-alcohols or citric acid (CA) is currently attracting considerable interest, specifically when wastes or

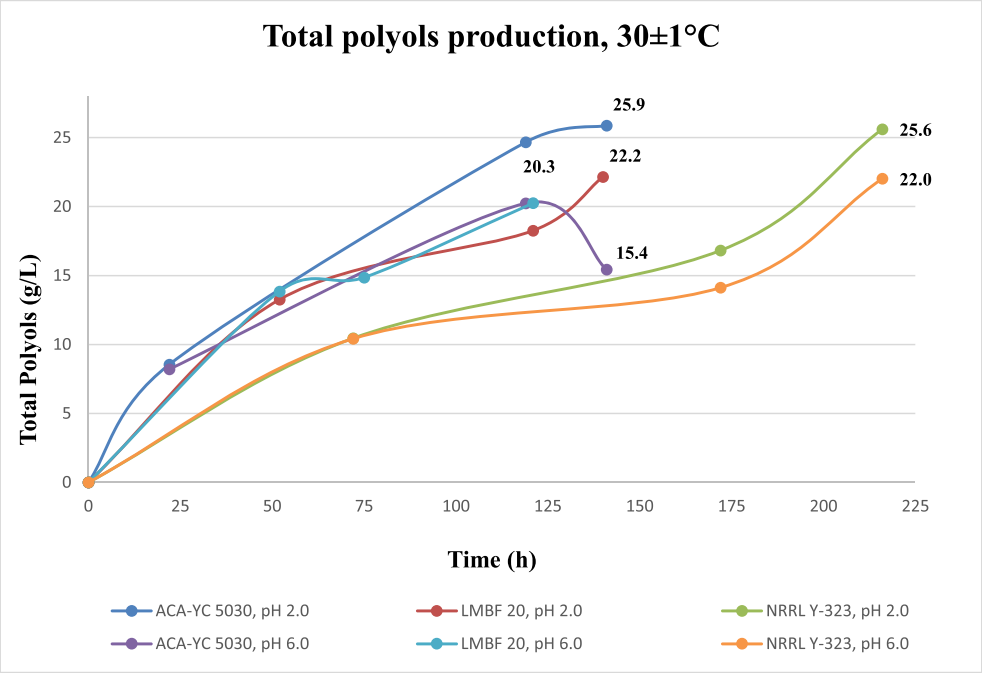

Fig. 1. Time profiles of the production of total polyols by ACA-YC 5030 at pH 2.0 ± 0.3 (●), LMBF 20 at pH 2.0 ± 0.3 (●), NRRL Y-323 at pH 2.0 ± 0.3 (●), ACA-YC 5030 at pH 6.0 ± 0.3 (●), LMBF 20 at pH 6.0 ± 0.3 (●) and NRRL Y-323 at pH 6.0 ± 0.3 (●) during culture on biodiesel-derived glycerol in shake-flask experiments at 180 ± 5 rpm and incubation temperature $T = 30 \pm 1$ °C. Each experimental point is the mean value of two independent determinations (SE ≤ 15 %).

residues (like crude glycerol) are implicated as substrates [31–33]. The dominant polyol synthesized when cultures are performed by *Y. lipolytica* depends on the fermentation conditions to which the respective microorganism is exposed; therefore, in response to high osmotic stress, the flux of the carbon source in polyol biosynthesis changes, a phenomenon known as "osmotic stress response" [28,29]. Under high osmotic stress conditions, yeasts produce more erythritol to reduce the outflow of water from the intra-cellular to the extra-cellular environment [28]. Consequently, the pentose phosphate pathway is in operation, as opposed to the Embden-Meyerhof-Parnas (EMP) pathway or to the pathway fructose-mannitol, through which mannitol is primarily synthesized [3]. Moreover, medium pH plays a crucial role upon the synthesis and secretion of polyols, since it is generally considered that growth under strong acidic conditions (i.e., pH≈3.0 ± 0.2) channels the carbon flow towards the synthesis of polyols, while growth on weak acidic conditions (i.e., pH ranging between 4.8 and 6.0) favors the production of CA, to the detriment of polyols production [26,30,31].

In contrast to many literature reports that indicate that polyol production is enhanced exclusively at significantly low pH values into the medium, all implicated strains in the present study produced noticeable Pol quantities at $T = 30 \pm 1$ °C and an almost neutral medium pH value (=6.0 ± 0.3). In many instances, and despite the mentioned almost neutral pH of the growth medium, low (or even negligible) quantities of CA were synthesized, with polyols being the major metabolic

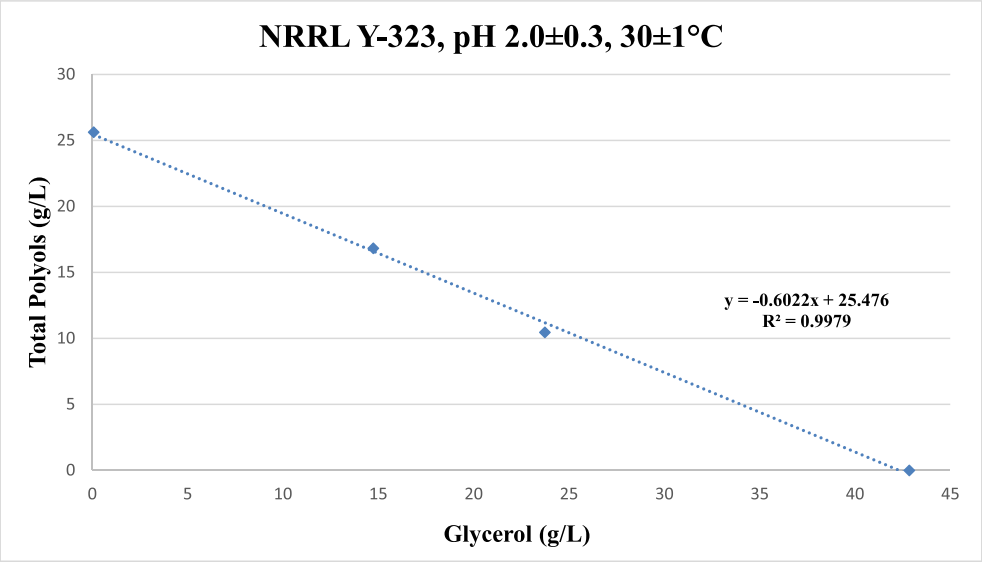

Fig. 2. Kinetics of total polyols (♦) *vs* remaining glycerol by *Yarrowia lipolytica* NRRL Y-323 during cultivation on biodiesel-derived glycerol in shake flasks at 180 ± 5 rpm, pH 2.0 ± 0.3, incubation temperature $T = 30 \pm 1$ °C. Each experimental point is the mean value of two independent determinations (SE ≤ 15 %).

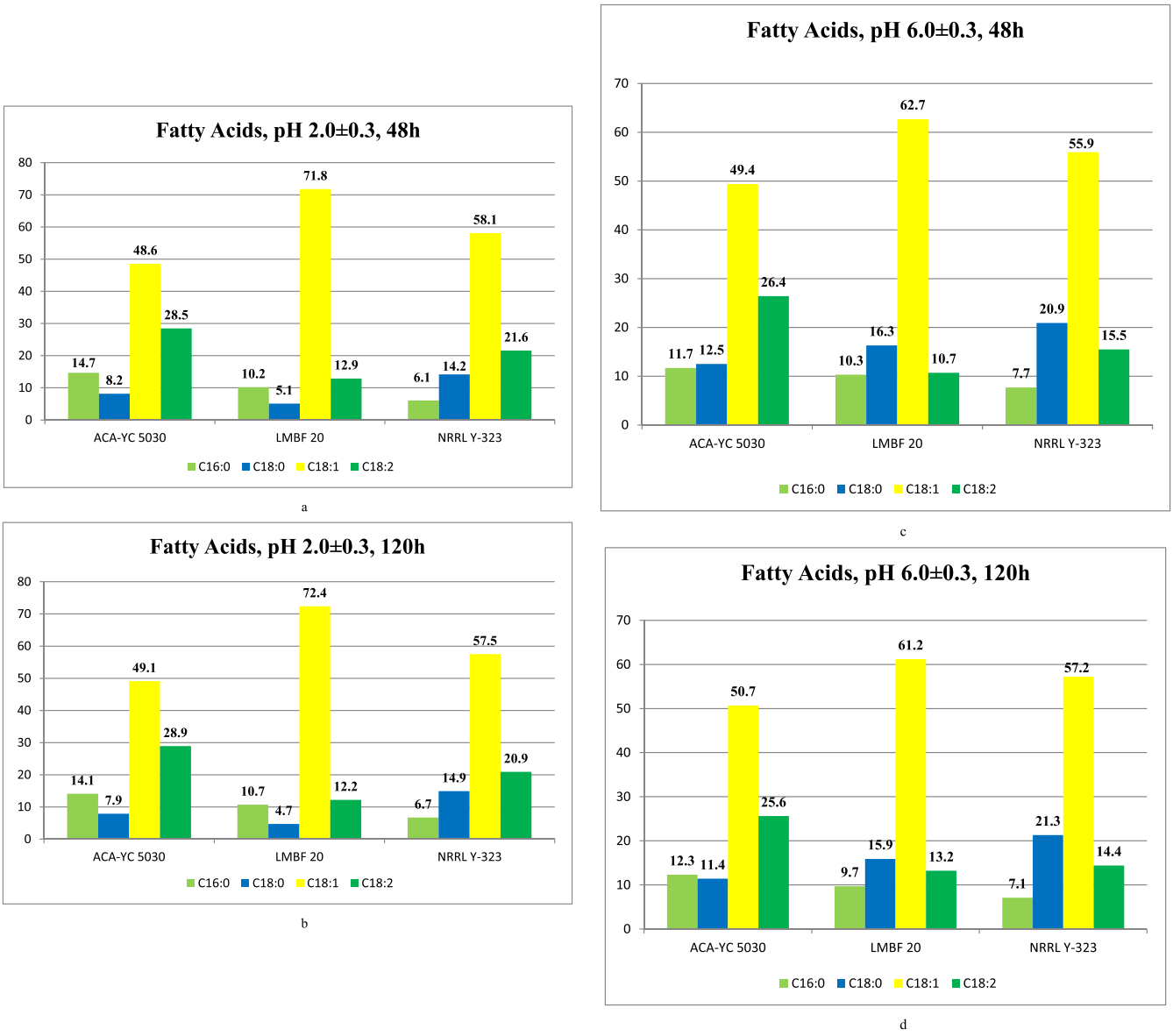

Fig. 3. Fatty acid profile of microbial oil derived from experiments that were carried out with *Y. lipolytica* yeast strains cultivated on glycerol. a: batch fermentations at pH = 2.0 ± 0.3 in 48 h; b: batch fermentations at pH = 2.0 ± 0.3 in 120 h; c: batch fermentations at pH = 6.0 ± 0.3 in 48 h; d: batch fermentations at pH = 6.0 ± 0.3 in 120 h.

compounds for most cases (see Tables 1 and 2). Therefore, at $T = 30 \pm 1$ °C and pH = 6.0 ± 0.3 the strain LMBF 20 produced Pol = 20.3 g/L, the strain ACA-YC 5030 Pol = 20.2 g/L and the strain NRRL Y-323 Pol = 22.0 g/L, while CA production (in low concentrations; *viz*. up to 6.0 g/L) was observed only for the strain NRRL Y-323. These results are in agreement with some literature reports demonstrating that sufficient Pol biosynthesis may occur under slightly acidic or close to neutral pH values into the culture medium when wild-type *Y. lipolytica* strains are used [25,26,32]. On the other hand, for all strains tested in the present investigation, although noticeable Pol synthesis occurred under slightly acidic or close to neutral pH values, higher Pol production was observed at low pH values into the medium (see Tables 1 and 2). Ultimately, the strain ACA-YC 5030 was able to produce a slightly lower quantity of polyols by operating under low pH and temperature conditions, resulting in a total concentration of polyols of approximately 19.6 g/L. The kinetics of total polyols production in the various pH values at incubation temperature $T = 30 \pm 1$ °C is shown in Fig. 1. The global conversion yield of polyols produced per unit of glycerol consumed ($Y_{Pol/Glol}$, % w/w) for the strain NRRL Y-323 that produced the maximum Pol concentration (=25.6 g/L), as illustrated in Fig. 2, was 60.2 % w/w, which, as per the literate analysis, should be considered as a value of high interest [36–38].

The strain NRRL Y-323 presented its maximum production of DCW under elevated pH and temperature conditions, achieving a X_{max} quantity of 11.1 g/L. The other two strains produced the highest amounts of DCW under conditions of low temperature and high pH. Specifically, the strain LMBF 20 produced a DCW quantity = 11.2 g/L and the strain ACA-YC 5030, respectively, a quantity = 12.4 g/L. This significant DCW production at low incubation temperatures (viz. $T = 20 \pm 1$ °C), constitutes a quite original result, taking into consideration the inability of most strains of this species to present significant growth in temperatures $T \leq 24 \pm 1$ °C [4,12,21]. Moreover, it has been observed that the metabolic pathway of the yeast *Y. lipolytica* is shifted towards the production of higher biomass, intra-cellular metabolites (mostly lipids and polysaccharides), and citric acid under elevated (*viz*. slightly acidic or close to neutral) pH values, while, in contrast maximization of polyol productivity occurs at acidic pH values [26,34,35]. On the other hand, according to the achieved results, polyol production, as that of

Table 3
Quantitative data of cultures of *Yarrowia lipolytica* strains on glycerol-based media ($Glol_0 \approx 40.0$ g/L) under different pH values. Culture conditions: shake-flask fermentation in 250.0-mL conical flasks at 180 ± 5 rpm and incubation temperature $T = 30 \pm 1$ °C under previously pasteurized or non-heated media. Each experimental point presented is the mean value of two independent determinations.

		Stains	Time (h)		Glol consumed (g/L)	X (g/L)	MI (g/L)	Ery (g/L)	Ara (g/L)	Pol (g/L)	$Y_{IPS/X}$ (% w/w)	$Y_{L/X}$ (% w/w)	CA (g/L)
Previously pasteurized conditions	pH 2.0 ± 0.3	ACA-YC 5030	24	f	7.5	0.9	1.9	0.5	0.0	2.3	4.0	17.0	nd
			72	e	24.9	10.4	6.8	5.1	2.6	14.5	13.8	9.4	nd
			120	a,b,c,d	38.9	12.9	10.7	9.1	3.2	23.0	12.6	2.7	nd
		LMBF 20	70	f	21.6	4.9	7.9	1.6	1.4	10.9	14.1	13.7	nd
			93	e	29.0	9.6	8.4	2.6	2.5	13.4	15.2	11.0	nd
			146	a,b,c,d	41.6	13.3	12.2	3.7	4.0	19.9	13.0	8.7	nd
		NRRL Y-323	93	f	32.1	9.0	11.3	3.4	3.1	17.7	12.0	12.3	nd
			122	b,c,d,e	41.8	13.5	14.0	4.3	3.6	21.9	12.8	10.2	nd
			146	a	41.8	13.5	13.7	4.0	4.4	22.1	12.4	7.2	nd
	pH 6.0 ± 0.3	ACA-YC 5030	72	e	25.0	7.3	nd	nd	nd	nd	18.3	12.4	nd
			120	a,b,c,d	36.8	7.6	nd	nd	nd	nd	10.4	21.3	nd
		LMBF 20	70	f	22.2	12.9	9.7	1.0	2.0	12.8	24.5	14.6	nd
			93	e	25.0	13.5	11.5	2.0	3.3	16.8	25.2	13.1	nd
			146	a,b,c,d	37.7	16.6	11.6	2.7	2.9	17.3	20.8	9.5	nd
		NRRL Y-323	93	f	35.7	12.1	11.5	1.8	4.0	17.3	19.5	14.0	4.4
			122	d,e	37.6	12.7	11.6	2.1	4.1	17.8	20.1	13.8	5.4
			146	a,b,c	39.6	13.0	12.1	2.3	4.1	18.4	17.9	13.5	5.7
Non-thermally treated conditions	pH 2.0 ± 0.3	NRRL Y-323	196	f	41.0	15.4	8.0	6.9	1.0	15.9	16.0	15.3	nd
			216	e	44.0	16.0	8.5	7.8	1.5	17.7	17.0	7.1	nd
			240	a,b,c,d	44.7	16.1	9.1	8.4	1.5	19.0	16.8	4.6	nd
	pH 6.0 ± 0.3		172	f	25.5	13.1	6.8	3.0	0.3	10.2	12.4	17.6	5.1
			196	e	33.8	16.8	8.0	5.2	0.6	13.7	12.6	10.5	4.7
			240	a,b,c,d	40.2	17.6	9.0	8.0	1.1	18.0	10.5	3.5	3.1

Representations: (a) When the maximum concentration of biomass is achieved; (b) When the maximum concentration of mannitol is achieved; (c) When the maximum concentration of erythritol is achieved; (d) When the maximum concentration of arabitol is achieved; (e) When the maximum percentage of intra-cellular polysaccharides on dry matter ($Y_{IPS/X}$, % w/w) is achieved; (f) When the maximum percentage of lipid on dry matter ($Y_{L/X}$, % w/w) is achieved.
nd: Not determined (≤0.3 g/L).

citric acid, when growth is performed on glycerol or similarly catabolized compounds, is not related to biomass production, occurring at the stationary growth phase, after virtual nitrogen limitation [35–37]. As per the findings of previous studies, it has been demonstrated that pH is the primary factor affecting biomass production [34–36].

The production of citric acid as a fermentation metabolite occurred, not in remarkably high concentrations, only at slightly acidic or close to neutral pH conditions and was observed only for the strain NRRL Y-323 (see Tables 1 and 2). In the mentioned fermentation, CA was produced at maximum concentrations of 6.0 g/L and 4.5 g/L at the two tested temperatures, respectively. The findings have led to the conclusion that the reduction in temperature did not significantly enhance its production, while, as mentioned, in all cases, the production of CA is significantly lower than that reported for other wild-type *Y. lipolytica* strains cultured on glycerol or similarly catabolized compounds (i.e., glucose) in media in which the pH value was slightly acidic or close to neutral [22,25,27]. Furthermore, the intra-cellular content of lipids presented significant differentiations related to the strain, the medium pH and the incubation temperature; kinetic analysis showed variations in which cellular lipid in DCW ranged from indeed low values (i.e., 0.5 % in DCW) to noticeable ones (19.0–37.0 % in DCW). In several cases in the literature for wild-type strains of this yeast species, it has been indicated that an "atypical" oleaginous character was seen; in fact, lipid contents of a great number of wild-type strains when cultivated on glucose or similarly catabolized substrates (i.e., glycerol) in culture conditions enabling the *de novo* biosynthesis and accumulation of reserve lipids (*viz.* cultures under nitrogen-limited conditions, as the ones of the present study), usually ranges from 4.0 % (or even lower values in some cases) to 19.0 % in DCW. Rarely for wild-type strains of this species, lipids in DCW values > 20.0 % w/w have been recorded [26,27,31]. It is considered that wild-type *Y. lipolytica* strains are the most untypical examples of the group of oleaginous yeasts that exist, since in many cases in which growth is carried out on the above-mentioned substrates (i.e., glucose, glycerol, etc.) under nitrogen-limited conditions, strains of this microorganism produce some (and in most cases not very high) quantities of lipids at the early growth steps (in the presence or barely after the deprivation of nitrogen from the growth medium), and thereafter and despite the presence of carbon source into the medium, lipid in DCW values decline, while simultaneously low-molecular weight metabolites (i.e., citric acid, polyols) are synthesized and secreted into the medium [31,38,39]. This physiological event has been observed in many cases in the current investigation (see i.e., strain ACA-YC 5030 at $T = 20 \pm 1$ °C at both pH values tested, strain LMBF at $T = 20 \pm 1$ °C and pH = 6.0 ± 0.3, strain NRRL Y-323 at $T = 30 \pm 1$ °C and pH = 6.0 ± 0.3, etc.). In a more restricted number of cases of the present study, $Y_{L/X}$ values remained almost constant, but no matter which the kinetic profile was, lipid content in most cases barely surpassed the value of 25.0 % in DCW (in agreement with many reports in the literature; see i.e., [38–40] and certainly does not compare favorably with lipid accumulation achieved by more "typical" oleaginous genera like *Rhodotorula, Rhodosporidium, Lipomyces*, *Cryptococcus*, and others [41–47].

FA composition of total lipids was investigated for all strains at the early stationary (*viz.* 48 h after inoculation) and the stationary phase (*viz.* 120 h after inoculation) for the trials at $T = 30 \pm 1$ °C at both pH values tested, and the obtained results are demonstrated in Fig. 3a; b; c; b. From the obtained results it can be seen that the main cellular FAs were oleic ($^{\Delta 9}$C18:1) and linoleic ($^{\Delta 9,12}$C18:2), while cellular stearic (C18:0) and palmitic (C16:0) acids were found in lower concentrations.

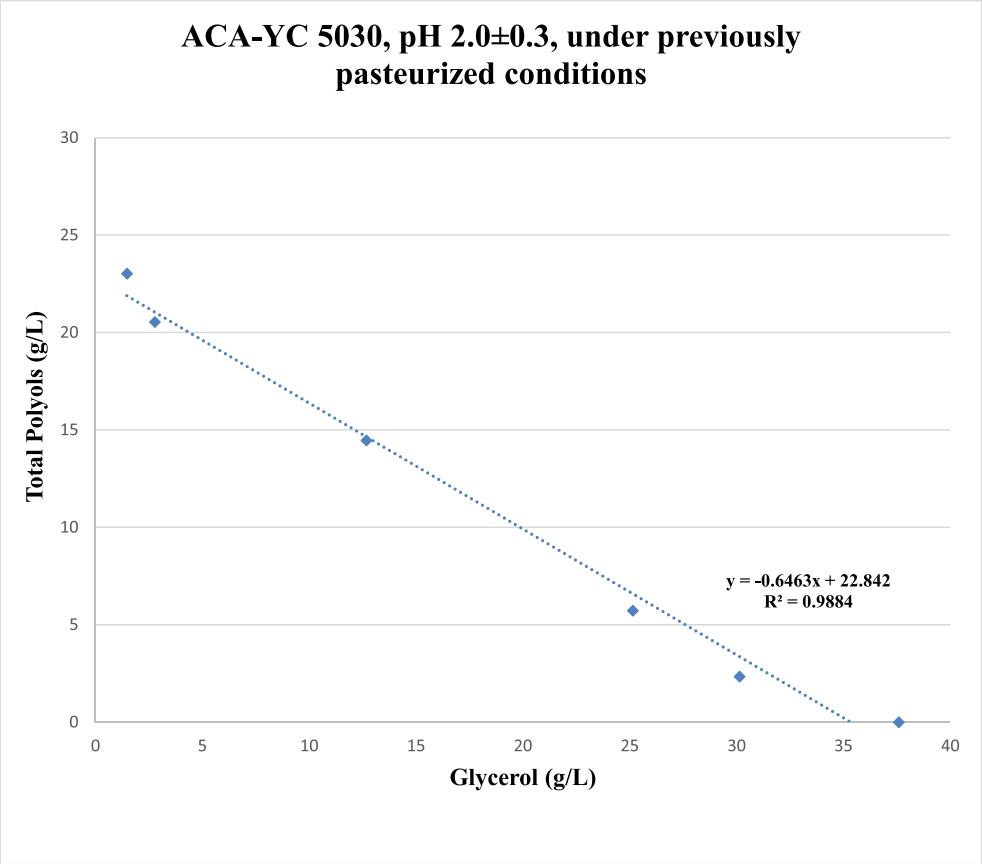

Fig. 4. Kinetics of total polyols (♦) *vs* remaining glycerol by *Yarrowia lipolytica* ACA-YC 5030 during cultivation on biodiesel-derived glycerol in shake flasks at 180 ± 5 rpm, pH 2.0 ± 0.3, incubation temperature $T = 30 \pm 1$ °C in pasteurized medium. Each experimental point is the mean value of two independent determinations (SE ≤ 15 %).

The above FA composition seemed in agreement with many reports in the literature that show similar distribution of FAs compared to the present study [20,26,27]. An interesting result associated with the present study, demonstrated that FA composition for all strains changed as function of the pH value in the culture medium; in cultures performed at pH = 2.0 ± 0.3, the concentration of cellular unsaturated FAs (oleic and linoleic acid) was often higher than that that performed at pH = 6.0 ± 0.3. Potentially, the higher unsaturation degree would demonstrate higher selectivity of the cellular membrane lipids, that would be a pre-requisite response of the cells due to the more inappropriate culture conditions related to the low pH value of the medium [8,31,41]. Moreover, it is interesting to indicate that in some cases (i.e., strain LMBF 20) the concentration of cellular oleic acid was indeed significant (>70 % w/w), demonstrating the capability of the mentioned strain to produce equivalents of high-value plant lipids (i.e., olive oil), in the case that culture optimization towards the significant production of cellular lipids would be performed. Finally, it should be mentioned that in contrast to the FA composition of some other (mostly oleaginous) yeast species (principally we refer to the species *L. starkeyi* and to lesser extent to the species *R. toruloides*), the yeast lipids of the tested *Y. lipolytica* strains seem somehow more unsaturated; yeast lipids of many *L. starkeyi* and *R. toruloides* strains when cultivated on media enabling the *de novo* lipid accumulation process, produce cellular lipids that contain a total saturated FA content (mostly C16:0 and C18:0) that is > 30–35 % w/w [27,32,47], while in some cases (i.e. *L. starkeyi* lipids) this content may reach the value of *c.* 45 % w/w [20].

In the present study, variable quantities of polysaccharides in DCW values were recorded, while the production of IPS reached its highest levels in DCW ($Y_{IPS/X} = 24.6$ % w/w) at pH 6.0 ± 0.3 and low incubation temperature (results achieved with the strain ACA-YC 5030). Not any characteristic trend concerning the synthesis of IPS per unit of DCW can be established as function of the fermentation time (see Tables 1 and 2). On the other hand, it appears that at low incubation temperatures and irrespective of the pH values into the medium, the production of IPS was somehow higher compared to the trials at $T = 30 \pm 1$ °C, while simultaneously the biosynthesis of polyols was somehow impaired. It was also seen that a somehow higher quantities of IPS were observed in the presence of a higher pH values, which is in accordance with the published literature [26,40].

3.2. Growth of *Y. lipolytica* strains under non-aseptic conditions

In the following approach and in order to further evaluate the metabolism of *Y. lipolytica* under stressful conditions, another set of shake-flask cultures was carried out. In this set, trials were carried out under non-aseptic conditions and at incubation temperature $T = 30 \pm 1$ °C. In fact, fermentations were either performed in previously pasteurized media (at $T = 65 \pm 1$ °C / 30 min or in a non-heated media. Fermentations were performed under nitrogen-limited conditions as in the previous experiments, with $Glol_0 \approx 40$ g/L. The physiological behavior of the strains was examined at low and high pH under the above conditions exposed (see Table 3).

DCW synthesis was affected by the non-aseptic conditions when compared with the control experiment (*viz.* the cultures performed under aseptic conditions at $T = 30 \pm 1$ °C). In fact, in almost all cases, the production of DCW was higher compared to the trials performed under previously sterile media, most probably due to the presence of contaminant microorganisms [45,46]. In the previously pasteurized

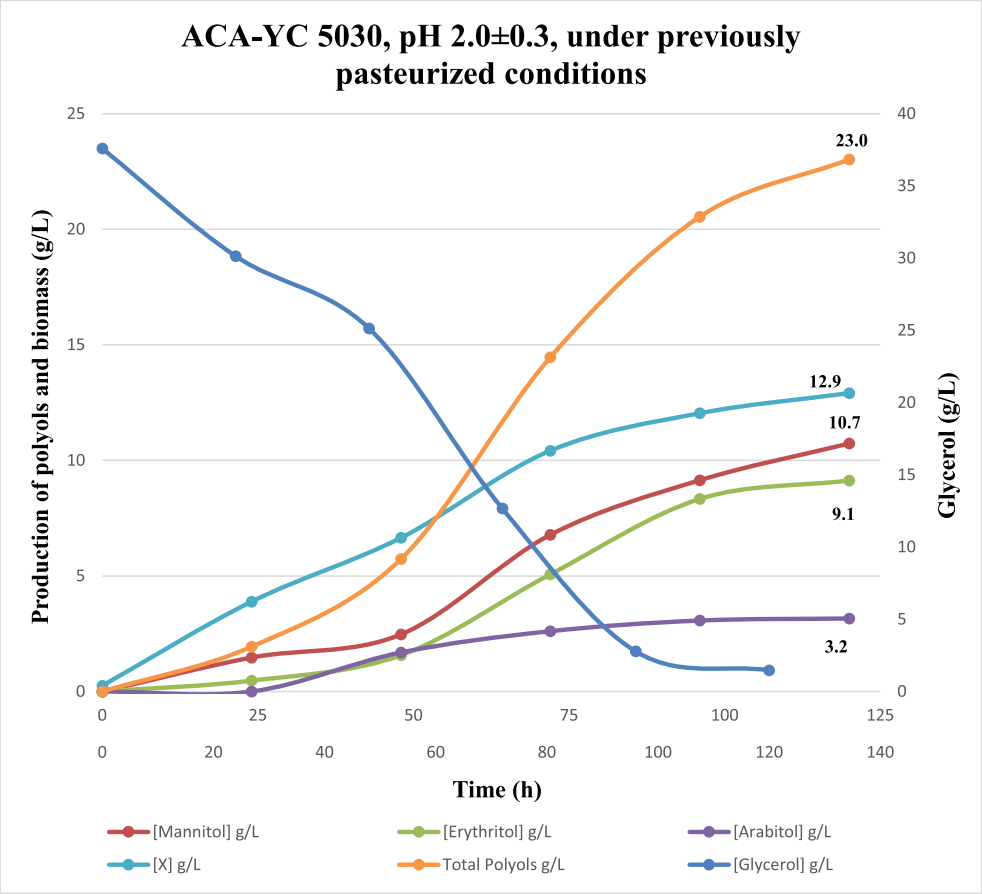

Fig. 5. Time profiles of mannitol (●), erythritol (●), arabitol (●), biomass (●), total polyols (●) and consumption of glycerol (●) by *Yarrowia lipolytica* ACA-YC 5030 during culture in biodiesel-derived glycerol in shake-flask experiments at 180 ± 5 rpm, incubation temperature $T = 30 \pm 1$ °C, pH 2.0 ± 0.3 in pasteurized medium. Each experimental point is the mean value of two independent determinations (SE ≤ 15 %).

media, the level of contamination (contaminant cells/total cell population, %), that was mostly performed by aerobic rods, was *c*. 10.0–15.0 % when the cultures were stopped at 130–150 h after inoculation, with the exception of the strain ACA-YC 5030 at pH = 6.0 ± 0.3, that was not allowed at all to produce polyols, due to significant contamination already observed from the first day of the culture. In unheated media the strain NRRL Y-323 was the only one that managed to withstand the stressful conditions and the presence of contaminant microorganisms. The strain NRRL Y-323 managed to produce a Pol_{max} quantity = 19.0 g/L at pH = 2.0 ± 0.3, and Pol_{max} = 18.0 g/L at pH = 6.0 ± 0.3 respectively, quantities that are slightly lower than the ones achieved in the previously sterilized media (see and compare Tables 2 and 3). As mentioned, DCW_{max} was notably higher in the non-aseptic and unheated media compared to the aseptic cultures (see and compare Tables 2 and 3), in agreement with the literature [45,46]. Likewise, in almost all cases, the pH value 6.0 ± 0.3 positively affected cellular growth and improved the final biomass concentration. Crude glycerol consumption did not seem to be affected in the previously pasteurized or (only for the strain NRRL Y-323) in unheated media *vs* the aseptic trials, while, as it was anticipated, the production of the desired extra-cellular metabolites (polyols) was slightly or somehow decreased in the non-aseptic experiments compared to the media in which previous sterilization had been carried out (see and compare Tables 2 and 3).

Total cellular lipids and polysaccharides were quantified, and their production was compared with those recorded in the previously sterilized media. Intra-cellular lipid in DCW values presented similar trend as compared to the axenic cultures, but in general $Y_{L/X}$ values were lower than the ones achieved in the previously sterilized media, which could be attributed to the presence of contaminant microorganisms and the interaction that these very microorganisms could have with the yeast cells. Therefore, maximum concentration of lipids in DCW ≤ 21.3 % w/w was obtained, while in most cases $Y_{L/X}$ values < 15.0 % were recorded (see Table 3). Moreover, $Y_{IPS/X}$ values for all trials performed under non-aseptic conditions ranged in more instances between 12 and 25 % w/w for both medium pH values. In many instances, an increase in the $Y_{IPS/X}$ values was recorded at the middle fermentation steps, demonstrating comparable biosynthetic patterns under nitrogen-limited conditions with other wild-type *Y. lipolytica* strains and other yeast species belonging to *Rhodotorula* sp. and *Metschnikowia* sp. [32,38,39], while it was observed that the higher pH value promoted the production of IPS. Equally, in the trials with the higher pH value, higher $Y_{L/X}$ values were recorded (Table 3).

The total production of polyols reached a maximum value of 23 g/L with a corresponding conversion yield ($Y_{Pol/Glol}$) of 64.6 % w/w when crude glycerol was utilized in previously pasteurized media at pH 2.0 ± 0.3 by the strain ACA-YC 5030 (Fig. 4). This is a very promising value and comparable with the best ones that have appeared in the literature (see i.e., [36–38]), demonstrating the potential of transformation of crude glycerol into added-value compounds by this strain, specifically by taking into consideration the fact that non-aseptic conditions were employed. The strain NRRL Y-323 under previously pasteurized conditions at pH 2.0 ± 0.3 produced Pol quantities = 22.1 g/L with a conversion yield of 52.8 % w/w, and finally, the strain LMBF 20 under the same conditions produced Pol = 19.9 g/L with a yield of 47.8 % w/w. The kinetics of growth, glycerol consumption and production of metabolites for the strain LMBF 20 are presented in Fig. 5.

Table 4
Quantitative data of cultures of *Yarrowia lipolytica* strain NRRL Y-323 on glycerol-based media ($Glol_0$≈80.0 g/L, ≈120.0 g/L and ≈140.0 g/L) under different pH values. Culture conditions: shake-flask fermentation in 250.0-mL conical flasks at 180 ± 5 rpm and incubation temperature $T = 30 \pm 1$ °C. Each experimental point presented is the mean value of two independent determinations.

pH $Glol_0$	Time (h)		Glol consumed (g/L)	X (g/L)	MI (g/L)	Ery (g/L)	Ara (g/L)	Pol (g/L)	$Y_{IPS/X}$ (% w/w)	$Y_{L/X}$ (% w/w)	CA (g/L)
2.0 ± 0.3	192	e,g	68.9	13.1	11.6	21.2	4.6	37.4	14.5	7.0	nd
≈80 g/L	216	a,b,c,d	84.4	15.6	17.4	25.5	7.3	50.2	13.6	4.7	nd
2.0 ± 0.3	24	g	36.6	5.3	10.4	8.2	2.7	21,3	5.8	17.6	nd
≈120 g/L	148	e	91.7	15.1	19.9	27.5	7.4	54,8	7.1	12.1	nd
	214	a,b,c,d	121.4	18.6	26.9	33.1	14.8	74.8	16.3	5.6	nd
2.0 ± 0.3	28	g	37.1	4.9	9.9	6.8	1.9	18.6	9.1	19.8	nd
≈140 g/L	160	e	100.5	14.8	20.3	34.0	8.1	62.4	16.8	15.9	nd
	255	a,b,c,d	135.8	19.0	28.1	38.8	17.3	84.2	14.9	9.8	nd
6.0 ± 0.3	240	e,g	67.3	11.7	11.2	16.4	3.7	31.3	18.0	7.6	10.5
≈80 g/L	312	a,b,c,d,f	80.2	17.2	15.1	22.8	5.2	43.1	14.7	2.7	11.8
6.0 ± 0.3	72	e,g	50.3	9.6	8.3	8.5	2.8	19.,6	19.0	18.4	6.6
≈120 g/L	194	a,b,c,d,f	117.1	18.9	20.5	24.4	10.1	55.0	17.3	6.9	16.1

Representations: (a) When the maximum concentration of biomass is achieved; (b) When the maximum concentration of mannitol is achieved; (c) When the maximum concentration of erythritol is achieved; (d) When the maximum concentration of arabitol is achieved; (e) When the maximum percentage of intra-cellular polysaccharides on dry matter ($Y_{IPS/X}$, % w/w) is achieved; (f) When the maximum concentration of citric acid is achieved; (g) When the maximum percentage of lipid on dry matter ($Y_{L/X}$, % w/w) is achieved.
nd: Not determined (≤0.3 g/L).

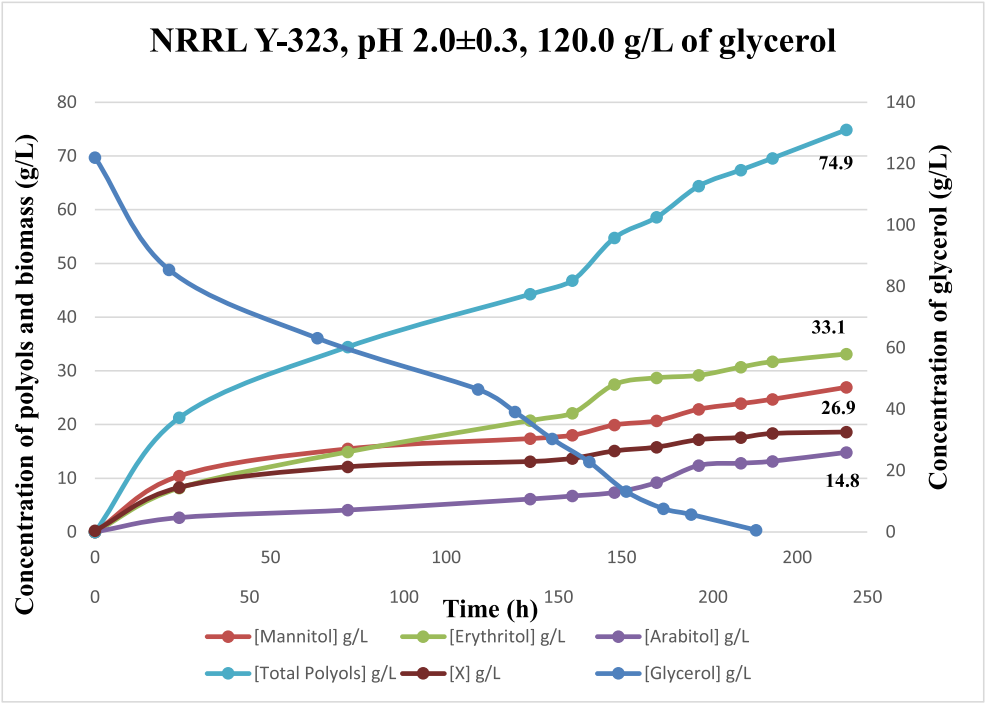

Fig. 6. Kinetics of glycerol consumption (●), biomass (●), mannitol (●), erythritol (●), arabitol (●) and total polyols (●) production by *Yarrowia lipolytica* NRRL Y-323 during culture in biodiesel-derived glycerol at initial glycerol concentration adjusted to *c.* 120 g/L, in shake-flask experiments at 180 ± 5 rpm, incubation temperature $T = 30 \pm 1$ °C, pH 2.0 ± 0.3. Each experimental point is the mean value of two independent determinations (SE ≤ 15 %).

3.3. Cultures at high initial glycerol concentrations – bioreactor trials

The most promising strain amongst the previously tested ones, viz. *Y. lipolytica* NRRL Y-323, was selected for further experiments, in media exhibiting an increased concentration of $Glol_0$ (≈80, ≈120 g/L) while maintaining all other parameters constant (*viz.* initial nitrogen at 2.0 g/L of peptone and 1.0 g/L of yeast extract, pH = 2.0 ± 0.3 or pH = 6.0 ± 0.3, $T = 30 \pm 1$ °C). Given that the obtained kinetic results as regards the production of polyols were more interesting in the trials at pH = 2.0 ± 0.3, a final experiment with even higher glycerol concentration (≈140 g/L) was performed in this pH value. The obtained results are demonstrated in Table 4, while the kinetics of growth, glycerol assimilation and production of metabolites at $Glol_0$≈120 g/L (pH = 2.0 ± 0.3 or pH = 6.0 ± 0.3) are seen in Figs. 6 and 7. Compared to the trials at $Glol_0$≈40 g/L (see Table 2) it may be seen that the maximum DCW quantity achieved in the trials with higher $Glol_0$ concentrations was noticeably higher. Specifically, in the experiments with $Glol_0$ adjusted to *c.* 120 or 140 g/L, the noticeable DCW quantity ≈20 g/L was achieved. This could be attributed to the fact that in the latter case, noticeably higher IPS quantities were accumulated inside the yeast cells (see and compare $Y_{IPS/X}$ values in Table 2 and 4). Likewise, despite the elevated $Glol_0$ concentration imposed into the culture medium (specifically when $Glol_0$ was ≈120 and 140 g/L), the enhanced DCW production and the significant glycerol uptake, suggest the absence of inhibition phenomena

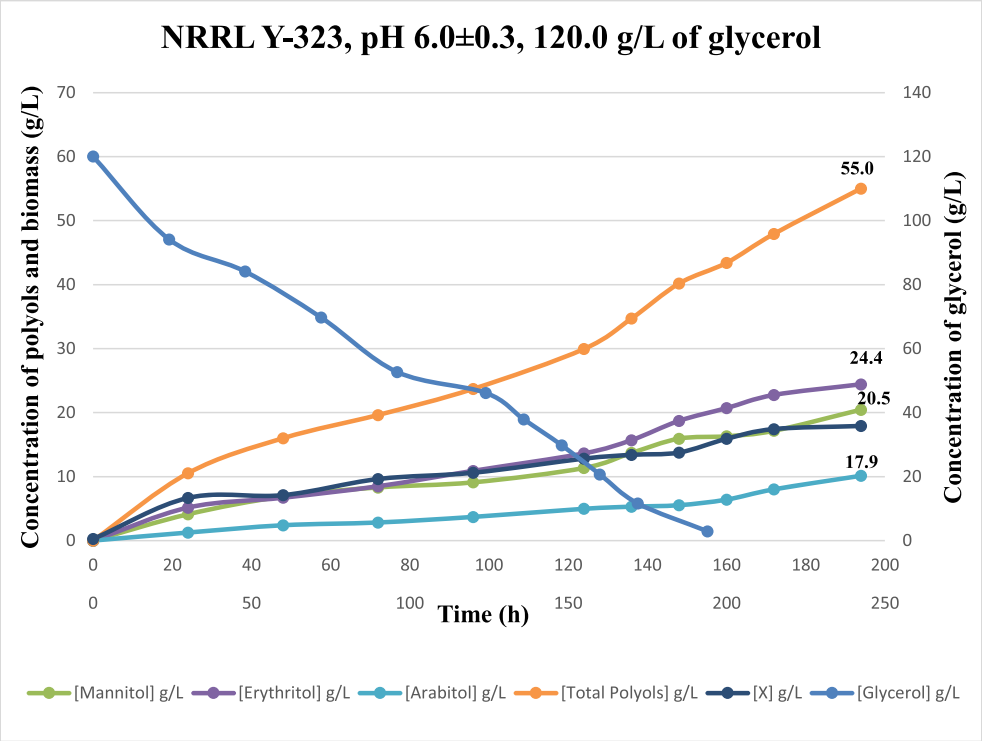

Fig. 7. Time profiles of glycerol consumption (●), mannitol (●), erythritol (●), arabitol (●), total polyols (●), and biomass (●) production by *Yarrowia lipolytica* NRRL Y-323 during culture in biodiesel-derived glycerol at initial glycerol concentration adjusted to *c.* 120 g/L, in shake-flask experiments at 180 ± 5 rpm, incubation temperature $T = 30 \pm 1$ °C, pH 6.0 ± 0.3. Each experimental point is the mean value of two independent determinations (SE ≤ 15 %).

towards the cells of the strain NRRL Y-323 due to the high substrate concentration, or the impurities of the feedstock that were accumulated into the growth medium. However, lipid in DCW values were significantly lower in the experimentσ with the higher $Glol_0$ concentration compared to the one with $Glol_0 \approx 40$ g/L (see and compare $Y_{L/X}$ values in Table 2 and 4), despite the fact that in the former case, much higher initial glycerol quantity and higher initial molar ratio C/N were imposed into the medium, that are prerequisites of significant accumulation of lipid for the oleaginous microorganisms [16,44]. This result, despite the fact that can be considered as "unusual", has already been observed also for other wild-type *Y. lipolytica* strains cultivated under conditions enabling the *de novo* accumulation of reserve lipid [25,31,32] and provides evidence of the untypical oleaginous character of this species [3,8,44].

The nitrogen limitation, the increased $Glol_0$ concentrations (up to 140 g/L) and the elevated osmotic pressure that were all simultaneously imposed into the medium in this experiment, directed the cellular metabolism of *Y. lipolytica* NRRL Y-323 towards the synthesis and the secretion of extra-cellular polyols. As in the previous sets of experiments, here too it has again been proved that although the medium pH remained into slightly acidic to neutral values (pH = 6.0 ± 0.3) the principal extra-cellular metabolites were the polyols erythritol, mannitol and to lesser extent arabitol ($Pol_{max} = 55.0$ g/L), in accordance with some recent literature reports demonstrating the above-mentioned physiological event for a small number of other wild-type *Y. lipolytica* strains [25–27,32]. As it was previously mentioned, batch trials of *Y. lipolytica* performed under nitrogen-limited and glycerol-excess conditions in medium pH values close to neutral (i.e., pH values ranging between 4.8 and 6.0) mostly lead to the accumulation into the medium of CA, with simultaneous low (or even negligible) secretion of Pol [33,35,37], therefore for another one time the present investigation shows that the obtained results by the strain NRRL Y-323 are not frequently met. It is also indicated that despite the non-negligible Pol production that was observed by the strain NRRL Y-323 in pH values close to neutral, significantly higher Pol quantities were produced when the pH into the medium was found in low values (at pH = 2.0 ± 0.3, for $Glol_0 \approx 120$ g/L, the Pol_{max} quantity obtained was = 74.8 g/L). The conversion yield $Y_{Pol/Gly}$ in this experiment was 61.6 % (the respective value for the trial at $Glol_0 \approx 40.0$ g/L was 60.2 % w/w; see Fig. 2), that is, as mentioned in the previous paragraphs, a quite interesting value as compared with the international literature [28,29,36]. This result led us to the necessity to perform another experiment at even higher $Glol_0$ concentration (≈140 g/L) at low pH value of the medium, that resulted to the very interesting production of total polyols of 84.2 g/L (concomitant conversion yield on glycerol consumed = 62 % w/w). Taking into consideration that the strain used as cell factory in these experiments is neither a mutant nor a genetically modified one, these values of production of polyols from glycerol-based media, are amongst the quite high ones of the international literature. Finally, irrespective of the pH value imposed into the medium, in the trials performed with the higher $Glol_0$ concentrations into the medium, the carbon flow was shifted mainly towards the synthesis of erythritol and to lesser extent towards mannitol and arabitol (see Table 4), while, in contrast, at $Glol_0 \approx 40$ g/L by far the most abundant polyol was that of mannitol (see Table 2). The initial glycerol concentration has been revealed to have a significant impact upon the production and distribution of polyols; in accordance with the present study, metabolic shift towards the production of erythritol has been observed in the literature when the $Glol_0$ concentration into the medium increased [27,34,35], most probably due to the already mentioned phenomenon known as "osmotic stress response" [28,29], that seems to favor the shift of the carbon flow towards the pentose phosphate metabolic network, impairing the pathway glycerol → mannitol in favor of the pathway glycerol → erythritol [3,17,28].

The strain was also cultured under highly agitated and aerated conditions, i.e., 750 ± 5 rpm and 0.5–1.5 vvm, respectively, to ensure a DOT ≥ 20.0 % v/v, in batch bioreactor experiments under both aseptic and unheated media and conditions enabling the production of polyols

Table 5
Quantitative data of cultures of *Yarrowia lipolytica* strain NRRL Y-323 on glycerol-based media ($Glol_0 \approx 40.0$ g/L) under pH values of 2.0 ± 0.3. Culture conditions: batch fermentation in bioreactor at 750 ± 5 rpm and incubation temperature $T = 30 \pm 1$ °C. Each experimental point presented is the mean value of two independent determinations.

	Time (h)		Glycerol consumed (g/L)	X (g/L)	MI (g/L)	Ery (g/L)	Ara (g/L)	Pol (g/L)	$Y_{IPS/X}$ (% w/w)	$Y_{L/X}$ (% w/w)
Aseptic conditions	55	e,f	9.9	4.9	1.8	4.7	0.7	7.2	8.1	19.2
	113	a,b,c,d	36.9	7.3	7.6	15.2	2.2	25.0	7.3	14.7
Non-thermally treated conditions	99	e	32.4	11.9	3.2	12.6	1.7	17.5	14.6	9.2
	148	a,b,c,d,f	40.1	12.8	5.0	13.7	2.0	20.6	15.2	7.4

Representations: (a) When the maximum concentration of biomass is achieved; (b) When the maximum concentration of mannitol is achieved; (c) When the maximum concentration of erythritol is achieved; (d) When the maximum concentration of arabitol is achieved; (e) When the maximum percentage of intra-cellular polysaccharides on dry matter ($Y_{IPS/X}$, % w/w) is achieved; (f) When the maximum percentage of lipid on dry matter ($Y_{L/X}$, % w/w) is achieved.

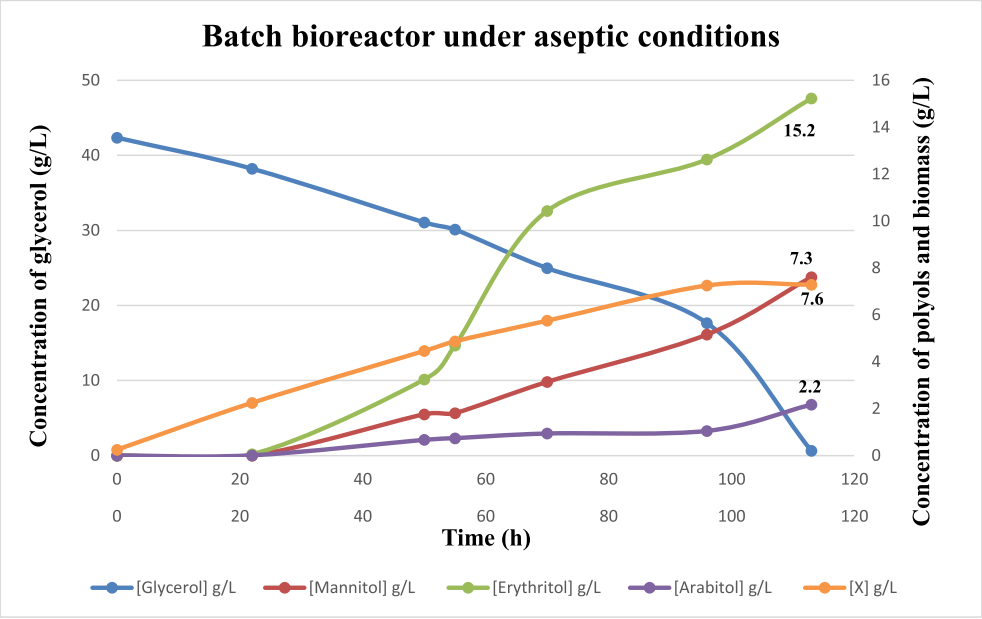

Fig. 8. Kinetics of glycerol consumption (●), mannitol (●), erythritol (●), arabitol (●) and biomass (●) production by *Yarrowia lipolytica* NRRL Y-323 during culture with biodiesel-derived glycerol, in bioreactor experiments at 750 ± 5 rpm, incubation temperature $T = 30 \pm 1$ °C, pH 2.0 ± 0.3 in sterilized medium. Each experimental point is the mean value of two independent determinations (SE ≤ 15 %).

(pH = 2.0 and $T = 30 \pm 1$ °C). The obtained results are demonstrated in Table 5, while the kinetics of growth, glycerol assimilation and production of metabolites are seen in Figs. 8 and 9. Comparing the batch bioreactor experiment with the equivalent shake-flask trial (pH = 2.0 ± 0.3 and $T = 30 \pm 1$ °C; see Table 2), it may be assumed that much higher assimilation rate of glycerol occurred in the former case, in agreement with similar types of cultures performed by other *Y. lipolytica* strains cultivated on glycerol or similarly catabolized compounds like glucose under nitrogen-limited conditions [25,27,31]. Likewise, similar higher uptake rates of the substrate (i.e., sugar) have also been reported in bioreactor experiments in comparison to shake-flask trials for other non-conventional yeasts like *Rhodosporidium toruloides* cultured under nitrogen-limited conditions [46,47]. On the other hand, in the present investigation, shake-flask experiments were accompanied by higher DCW production and, interestingly, higher accumulation of lipids as compared to the bioreactor trial, in disagreement with some reports [27,46,47]. As far as the typical oleaginous microorganisms are concerned, their cultivation under highly aerated conditions is considered an important prerequisite that, in many cases, ensures significant production of lipid and the successful accomplishment of the single-cell oil production process [41,43,44]. Moreover, in the present investigation, the production of total polyols was similar in the two trials (Pol_{max} = 25 g/L with conversion yield 67.7 % w/w in the bioreactor experiment against 25.6 g/L and 60.2 % w/w in the flask culture). In contrast, in the bioreactor experiment, it seemed that the carbon flow was directed towards the synthesis of erythritol, that was the main polyol produced, while in the respective flask culture the production of erythritol was much lower (see Tables 2 and 5). It appears therefore, that the higher aeration / agitation imposed in the bioreactor experiment favored the pathway glycerol → erythritol. Finally, when growth was carried out on a unheated medium, and in accordance to the previously achieved results in the flask experiments, DCW production was higher (presumably due to the presence of the contaminant rods) while the production of polyols was somehow decreased as compared to the experiment with the axenic culture (Pol_{max} = 20.6 g/L with conversion yield 51.4 % w/w in the non-aseptic experiment against 25 g/L and 67.7 % w/w in the axenic culture; see Table 5).

The maximum Pol production achieved in the present experiment (84.2 g/L, $Y_{Pol/Glol}$ = 62.0 % w/w) is very interesting, specifically by taking into consideration that a wild-type (and not a mutant or a genetically engineered) strain was employed. Comparisons with the literature are seen in Table 6. Although the Pol_{max} value obtained in the present study was somehow lower in absolute values compared to many of the results reported in the literature (see Table 6), as mentioned, in many of the presented cases mutant of genetically engineered strains (i.e., strains Wratislavia K1, Wratislavia 1.31, etc.) have been used. On the other hand, the conversion yield of total polyols produced per glycerol consumed obtained in the present study (=62.0 % w/w) compares

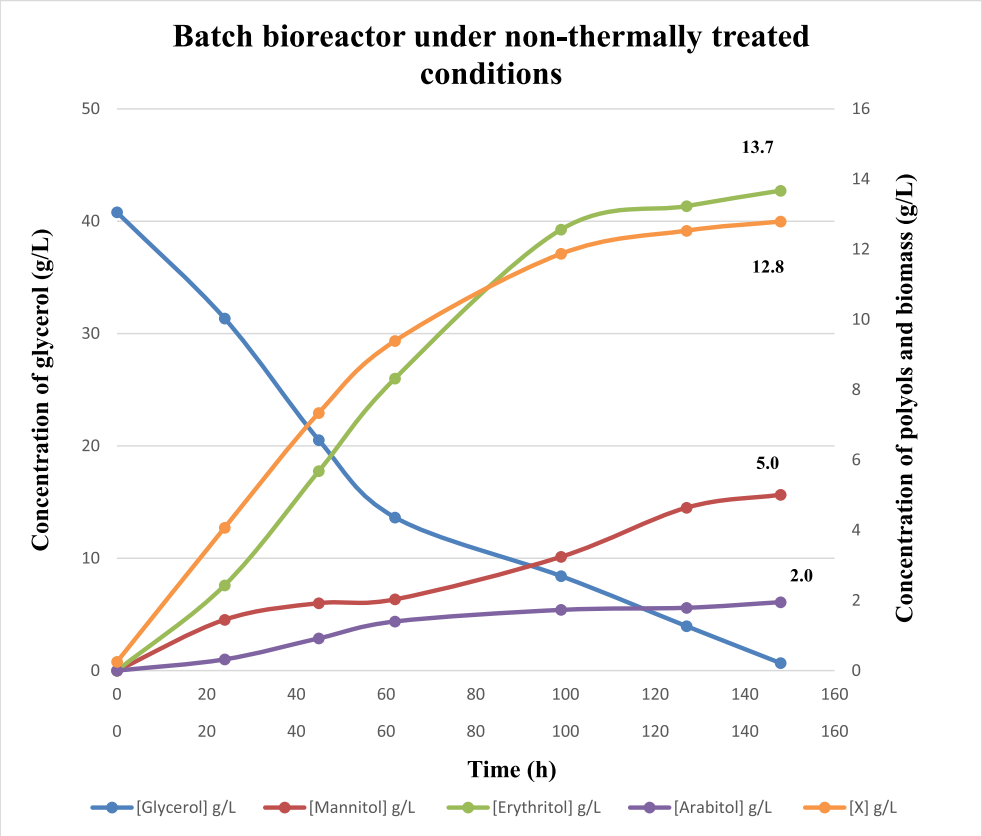

Fig. 9. Time profiles of glycerol consumption (●), mannitol (●), erythritol (●), arabitol (●) and biomass (●) production by *Yarrowia lipolytica* NRRL Y-323 during culture with biodiesel-derived glycerol, in bioreactor experiments at 750 ± 5 rpm, incubation temperature $T = 30 \pm 1$ °C, pH 2.0 ± 0.3 in non-heated medium. Each experimental point is the mean value of two independent determinations (SE ≤ 15 %).

Table 6
Comparative data on the biotechnological production of polyols through valorization of glycerol when various *Yarrowia lipolytica* strains were employed.

Strain	Fermentation mode	MI (g/L)	Ery (g/L)	Ara (g/L)	Pol (g/L)	$Y_{Pol/Glol}$ (% w/w)	References
MK1	bioreactor	6.9	165.0	3.0	174.9	58.3	[48]
Wratislavia K1	bioreactor	6.1	89.0	0.70	95.8	63.9	[35]
Wratislavia K1	flasks	3.9	135.5	0.1	139.5	58.0	[49]
FCY 218	bioreactor	nd	80.6	nd	80.6	53.0	[50]
Wratislavia K1	bioreactor	3.0	132.0	nd	135.0	45.0	[33]
AIB	bioreactor	12.6	56.7	6.0	75.3	49.1	[51]
Wratislavia 1.31	bioreactor	23.0	132.0	nd	155.0	51.7	[30]
A-101	bioreactor	27.0	137.0	nd	164.0	54.7	[30]
8661 UV1	bioreactor	40.5	113.0	nd	153.5	51.2	[30]
LMBF Y-74	flasks	21.7	24.6	10.3	56.6	54.3	[26]
ACA-DC 5029	flasks	10.5	15.6	3.4	29.5	38.9	[52]
ACA-DC 5029	flasks	6.5	65.8	3.4	75.7	44.4	[53]
NRRL Y-323	flasks	28.1	38.8	17.3	84.2	62.0	Present study

favorably with the highest values achieved in the international literature, irrespective of the use of mutant or wild-type *Y. lipolytica* strains implicated in the mentioned bioprocess. In any case, further process optimization of the strain NRRL Y-323 (specifically as regards the utilization of stirred tank bioreactors in fed-batch mode) can even more increase the final Pol quantity achieved, while as mentioned this value was achieved by a wild-type novel yeast strain, not previously systematically studied concerning its bio-production potential.

4. Concluding remarks

Three wild-type *Y. lipolytica* yeast strains were tested for glycerol valorization under various cultivation conditions. All strains exhibited a significant potential for the production of polyols at low pH values (pH = 2.0 ± 0.3). Remarkable growth and polyols' biosynthesis was observed in indeed low incubation temperatures ($T = 20 \pm 1$ °C). All strains demonstrated untypical oleaginous features, failing to produce significant quantities of lipid, although culture conditions enabling the *de novo* accumulation of lipids were employed. Therefore, irrespective of the pH values imposed, the cellular lipid in DCW values remained in almost all cases at a level ≤ 20.0 % w/w, thereby demonstrating this atypical oleaginous nature of the species employed. By contrast, variable quantities of intra-cellular polysaccharides were produced. Pasteurized media can support significant DCW production and polyols biosynthesis for most of the tested strains. Interestingly, the strain NRRL Y-323 managed to produce significant polyol quantities even at media that

were not previously heated. The $Glol_0$ concentration seems crucial for the production and distribution of polyols by the studied strains. The production of mannitol was favored at low $Glol_0$ concentrations, whereas higher concentrations of $Glol_0$ primarily boosted the biosynthesis of erythritol, albeit at the expense of mannitol synthesis. For the most promising strain, namely NRRL Y-323, highly aerated / agitated bioreactor trials showed different physiological profiles as compared to the respective shake-flask experiments. A significant production of polyols of 84.2 g/L with a corresponding conversion yield of 62 % w/w was achieved in shake-flask by the mentioned strain. In conclusion, despite stressful conditions employed, wild-type and not excessively previously studied *Y. lipolytica* strains managed to produce non-negligible quantities of yeast cell biomass and polyols. This investigation offers an alternative approach for converting biodiesel-derived glycerol into important added-value compounds.

CRediT authorship contribution statement

Eleni-Stavroula Vastaroucha: Writing – original draft, Validation, Methodology, Investigation, Data curation. **Nikolaos G. Stoforos:** . **George Aggelis:** . **Seraphim Papanikolaou:** Writing – review & editing, Writing – original draft, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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