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Physiologo-biochemical characteristics of citrate-producing yeast *Yarrowia lipolytica* grown on glycerol-containing waste of biodiesel industry

Igor G. Morgunov[1] · **Svetlana V. Kamzolova**[1]

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Abstract In this study, physiologo-biochemical characteristics of citrate-producing yeast *Yarrowia lipolytica* grown on glycerol-containing waste of biodiesel industry were studied by an investigation of growth dynamics, the consumption of glycerol, and the fatty acid fractions from waste as well as by measuring the activities of enzymes involved in the metabolism of waste. It was shown that *Y. lipolytica* realizes concurrent uptake of glycerol and the fatty acid fractions during conversion of glycerol-containing waste, although glycerol was utilized at a higher rate than fatty acids. Under optimal feeding of glycerol-containing waste by portions of 20 g l^{-1}, the citric acid production and the ratio between citric acid and isocitric acid depended on the strain used. It was revealed that wild strain *Y. lipolytica* VKM Y-2373 produced citrate and isocitrate with a ratio of 1.7:1, while the mutant strain *Y. lipolytica* NG40/UV7 synthesized presumably citric acid (122.2 g l^{-1}) with a citrate-to-isocitrate ratio of 53:1 and the yield of 0.95 g g^{-1}.

Keywords Glycerol-containing waste (GW) · Biodiesel industry · Citric acid (CA) production · Isocitric acid (ICA) production · *Yarrowia lipolytica* · Glycerol · Fatty acids

✉ Igor G. Morgunov
morgunovs@rambler.ru

[1] G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Moscow 142290, Russia

Introduction

In recent years, the conversion of glycerol-containing waste (GW) of biodiesel industry into practically valuable compounds has attracted increased interest because a current price of waste varied from US$0.06 to US$0.11 per pound (Nicol et al. 2012). Glycerol-based microbiological processes have been developed which aimed at production of 1,3-propanediol (Papanikolaou et al. 2008), poly-3-hydroxybutyrate (Mothes et al. 2007; Ibrahim and Steinbüchel 2010), lipids (Papanikolaou and Aggelis 2010; Papanikolaou and Aggelis 2002; Dedyukhina et al. 2012), polyols (Rywińska et al. 2013; Mirończuk et al. 2014), arachidonic acid (Dedyukhina et al. 2014), pyruvic acid (Morgunov et al. 2004a), succinic acid (Yuzbashev et al. 2010; Jost et al. 2014), α-ketoglutaric acid (Stottmeister et al. 2005; Yin et al. 2012; Otto et al. 2012), and citric acid (Rymowicz et al. 2006; Förster et al. 2007a; Levinson et al. 2007; Rymowicz et al. 2006, 2010; Holz et al. 2009; Kamzolova et al. 2011a; Rywińska et al. 2009, 2013; Morgunov et al. 2013). Citric acid (CA) is considered to be the most economically feasible product with the annual world production of 1.4 million ton (Anastassiadis et al. 2008).

Yeast *Yarrowia lipolytica* is able to produce CA from various substrates, such as glucose, glucose syrups, ethanol, methanol, *n*-alkanes, mixtures of saturated free fatty acids of animal origin, glycerol, and glycerol-containing waste of biodiesel industry (Barth and Gaillardin 1996; Finogenova et al. 2005; Groenewald et al. 2014; Aurich et al. 2012). It had been reported that yeast *Y. lipolytica* is able to grow on GW and produce CA and isocitric acid (ICA) in a proportion which depended on the strain, the waste composition, and the cultivation conditions. The physiological bases of microbial production of citric acids from GW are well studied (Rymowicz et al. 2006, 2010; Förster et al. 2007a; Levinson et al. 2007;

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Holz et al. 2009; Kamzolova et al. 2011a; Rywińska et al. 2009, 2013; Morgunov et al. 2013). The main condition of CA and ICA overproduction by yeast *Y. lipolytica* is the limitation of cell growth by inorganic nutrients (especially nitrogen) under excess of GW. No intense synthesis of CA was revealed in complete media containing all the nutrients in abundance. However, up to now, a little is known on the biochemical mechanisms of GW assimilation and oversynthesis of CA and ICA. GW consists of two substrates: glycerol and fatty acids, and theoretically, glycerol and fatty acids can be consumed either simultaneously or successively.

The aim of this work was to elucidate metabolic pathways of GW utilization by investigating the growth dynamics and the consumption of glycerol and fatty acids as well as by measuring the activity of the enzymes involved in the glycerol and fatty acid metabolism in the wild and mutant strains of yeast *Y. lipolytica* in order to develop an efficient process of CA production from wastes of biodiesel industry.

Materials and methods

Microorganisms

The study was carried out with the earlier selected citric acid-producing *Y. lipolytica* strains: the wild strain *Y. lipolytica* VKM Y-2373 obtained from the All-Russian Collection of Microorganisms (VKM) and the mutant strain *Y. lipolytica* NG40/UV7 from the Culture Collection of the Laboratory of Aerobic Metabolism of Microorganisms, Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences (Pushchino, Russia). The mutant strain *Y. lipolytica* NG40/UV7 was obtained as follows: initially, the strain *Y. lipolytica* VKM Y-2373 was exposed to 40 μg ml^{-1} of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; from 200 grown colonies, the strain named *Y. lipolytica* NG40, which displayed in the medium with glucose higher (up to 20 %) CA production as compared with the parent strain *Y. lipolytica* VKM Y-2373, was selected. Then, 1 ml of the selected strain *Y. lipolytica* NG40 suspension was irradiated with a Bio 2-W 254-nm lamp (Fimet, Moscow, Russia) for 7 min at a distance of 10 cm. From 800 grown colonies, the strain named *Y. lipolytica* NG40/UV7 with impaired ability to grow on acetate probably due to defects in the tricarboxylic acid cycle (TCA) was selected; its CA production exceeded that of the parent strain by 40 % in the medium with glucose. This strain was able to produce a significant amount of CA from raw glycerol (Morgunov et al. 2013).

Chemicals

All chemicals were of analytical grade (Sigma-Aldrich, St. Louis, MO, USA). The sample of GW was provided by the Pythia Institute of Biotechnology (Thessaloniki, Greece). The sample of GW is the product of the transesterification reaction of rapeseed oil, methanol, and NaOH. The sample of GW contained (in percent, by mass) the following: glycerol, 65; fatty acids and oil residue, 20; sodium salts, 1; heavy metals, 1.5; methanol as a trace component, 0.1; and water, 12.4. Composition of fatty acids (% of the sum) is as follows: tetradecanoic, 0.2; palmitic, 12.7; stearic, 18.0; oleic, 11.3; linoleic, 49.2; alpha-linolenic, 8.3; and eicosanoic, 0.3.

Cultivation conditions of media

In growth experiments, *Y. lipolytica* VKM Y-2373 was cultivated in a 10-l ANKUM-2M fermentor (Russia) with an initial volume of 5 l on the complete nutrient medium containing the following (g l^{-1}): GW in concentrations as indicated in the text; $(NH_4)_2SO_4$, 6.0; $MgSO_4 \cdot 7H_2O$, 1.4; NaCl, 0.5; $Ca(NO_3)_2$, 0.8; KH_2PO_4, 2.0; K_2HPO_4, 0.2; yeast autolysate; and Burkholder's trace element solution (Burkholder et al. 1944). The incubation time was 18 h.

In the CA production experiments, *Y. lipolytica* VKM Y-2373 and *Y. lipolytica* NG40/UV7 were cultivated in the same medium during 192 h. The fermentation conditions in the growth and CA production experiments were maintained automatically at the constant level: temperature, 28±0.5 °C; pH, 5.0±0.1; pO_2, 50 % (of air saturation); and agitation rate, 800 rpm. At regular intervals, the culture was sampled for analyses.

Assays

The biomass, the concentration of ammonium, the glycerol content, and citric and isocitric acids were assayed as described earlier (Morgunov et al. 2013). The fatty acids were analyzed as described earlier (Kamzolova et al. 2011a, b).

In order to measure the enzyme activity, the yeast cells were collected by centrifugation at 3000*g* for 10 min (4 °C) and washed with an ice-cold 0.9 % NaCl solution. The cell pellet was suspended in a proportion of 1:10 in 100 mM phosphate buffer (pH 7.4) supplemented with 1 mM EDTA. Cells in the suspension were disrupted with glass beads (d=150 −250 μ; BDH Chemicals Ltd., Poole, England) on a planetary mixer for 3 min at 1000 rpm (0 °C). The cell homogenate was centrifuged at 5000*g* for 30 min (4 °C), and the supernatant was used for the assay of cytoplasmic, mitochondrial, and peroxisomal enzymes. The activity of enzymes [glycerol kinase, NAD-dependent glycerol dehydrogenase (NAD-GDH), and FAD-dependent glycerol dehydrogenase (FAD-GDH), citrate synthase, aconitate hydratase, NAD- and NADP-dependent isocitrate dehydrogenases, isocitrate lyase, and malate synthase] was measured using standard methods with slight modifications as described earlier (Morgunov et al. 2013). The activity of glyceraldehyde-3-phosphate

dehydrogenase was determined as described by Krebs (1955). The activities of pyruvate kinase and pyruvate dehydrogenase were determined as described by Gancedo et al. (1968). The activity of oxoglutarate dehydrogenase was determined as described by Sofronova et al. (1976). The activity of fumarate hydratase was assayed using the method of Massey (1955). The activity of malate dehydrogenase was assayed using the method of Kataeva and Finogenova (1987). The specific enzyme activities were expressed as per nanomole (min mg protein). The amount of protein in the cell-free extracts was determined using the Bradford method.

Calculation of fermentation parameters

To take into account the medium dilution due to the addition of NaOH solution for maintaining the constant pH value, the total amount of CA in the culture broth (the final volumes were 7.84 l with *Y. lipolytica* VKM Y-2373 and 7.77 l with *Y. lipolytica* NG40/UV7) was used for calculations of the mass yield of CA (Y_{CA}) and volumetric citric acid productivity (Q_{CA}). The biomass yield ($Y_{x/s}$), the specific growth rate (μ), the mass yield coefficient of CA production (Y_p), and the volumetric productivity (Q_p) were calculated as described earlier (Morgunov et al. 2013).

Statistical analysis

All the data presented are the mean values of the three experiments and two measurements for each experiment; standard deviations were calculated (SD<10 %).

Results

The growth of *Y. lipolytica* on GW

The wild-type strain *Y. lipolytica* VKM Y-2373 was grown in the complete cultivation medium with GW (20 g l^{-1}) containing 13 g l^{-1} glycerol and 4 g l^{-1} fatty acids. The time course of *Y. lipolytica* growth is shown in Fig. 1. As seen from Fig. 1a, immediately after the inoculation, the yeast culture began to grow and consume GW; the amounts of glycerol and the fatty acid fractions from GW started to decrease from the first hours of cultivation; glycerol and fatty acids were exhausted by 18 and 14 h of cultivation, respectively. At the end of cultivation, the biomass comprised 21.0 g l^{-1} and the biomass yield ($Y_{x/s}$) was 1.05. The maximum specific growth rate (μ_{max}) calculated from the linear segment of the semilogarithmic plot of the growth curve amounted to ≈0.352 h^{-1}. Glycerol and fatty acids were utilized with the constant volumetric uptake rates of 0.70−0.78 and 0.2−0.25 g l^{-1} h^{-1}, respectively, during the whole cultivation period. No synthesis of CA was revealed in

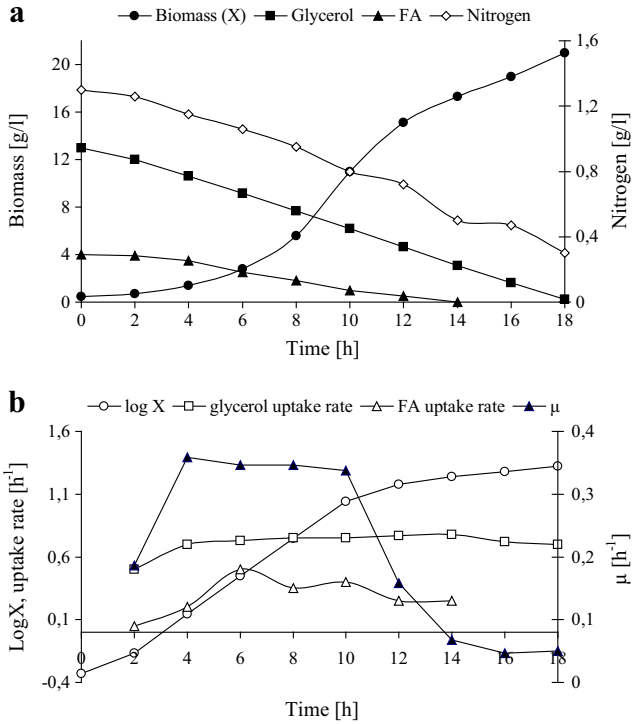

Fig. 1 Time courses of growth and consumption of glycerol, fatty acids, and nitrogen by *Y. lipolytica* VKM Y-2373 (**a**) and the calculated parameters of the process (**b**)

complete media containing all the nutrients in abundance, and the level of nitrogen was 0.3 g l^{-1}.

The key enzymes involved in glycerol metabolism (glycerol kinase, NAD-dependent glycerol dehydrogenase, and FAD-dependent glycerol dehydrogenase), fatty acid metabolism (isocitrate lyase and malate synthase), and the tricarboxylic acid cycle (citrate synthase, aconitate hydratase, NAD-dependent isocitrate dehydrogenase, and NADP-dependent isocitrate dehydrogenase) were examined in the crude cell extracts taken at different hours of the *Y. lipolytica* VKM Y-2373 growth. Table 1 shows typical data on enzyme activities of *Y. lipolytica* VKM Y-2373. It can be seen that glycerol kinase was induced from the start of yeast growth and remained at a high level (130−170 nmol (min mg protein)$^{-1}$) during the whole cultivation period. NAD- and FAD-dependent glycerol-3-phosphate dehydrogenases also rose at 4 h of cell growth. However, the activity of NAD-dependent glycerol-3-phosphate dehydrogenase was reduced by twofold at the end of cell growth, while the activity of FAD-dependent glycerol-3-phosphate dehydrogenase was increased by 1.5 times due to the key role of this enzyme in the oxidation of glycerol-3-phosphate. The activities of enzymes involved in the conversion of dioxyacetone phosphate into pyruvate, glyceraldehyde-3-phosphate dehydrogenase, and pyruvate kinase were maintained at the similar high level throughout the cultivation and varied from 120 to 130 nmol (min mg protein)$^{-1}$ and from 305 to 270 nmol (min mg protein)$^{-1}$, respectively. The

Table 1 Activities of enzymes of *Y. lipolytica* VKM Y-2373, grown on BDW under the excess of nutrition components in the medium

Enzymes (nmol (min mg protein)$^{-1}$)	Time (h)		
	4	12	18
Glycerol kinase (EC 2.7.1.30)	105	130	170
NAD-dependent glycerol-3-phosphate dehydrogenase (EC 1.1.1.8)	68	45	34
FAD-dependent glycerol-3-phosphate dehydrogenase (EC 1.1.99.5)	55	66	82
Glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) (EC 1.2.1.12)	120	125	130
Pyruvate kinase (EC 2.7.1.40)	305	300	270
Pyruvate dehydrogenase (acetyl-transferring) (EC 1.2.4.1.)	83	130	110
Citrate synthase (EC 4.1.3.7)	1460	2000	1760
Aconitate hydratase (К.Ф. 4.2.1.3)	330	400	395
NAD-dependent isocitrate dehydrogenase (EC 1.1.1.41)	80	84	100
NADP-dependent isocitrate dehydrogenase (EC 1.1.1.42)	300	280	250
Oxoglutarate dehydrogenase (succinyl transferring) (EC 1.2.4.2)	30	40	20
Succinate dehydrogenase (EC 1.3.5.1)	220	260	180
Fumarate hydratase	280	300	290
Malate dehydrogenase (EC 1.1.1.37)	2000	1800	2100
Malate synthase (EC 4.1.3.2)	60	70	57
Isocitrate lyase (EC 4.1.3.1.)	110	135	120

The values are the means of the three experiments and two measurements for each experiment which varied by no more than 10 %

activity of pyruvate dehydrogenase remained at a level of 83 −130 nmol (min mg protein)$^{-1}$ during the whole cultivation period. The key enzymes of the glyoxylate cycle, isocitrate lyase, and malate synthase, which are involved in the metabolism of fatty acids, were also induced from the start of yeast growth on the GW and maintained at the approximately similar level throughout the cultivation; the activity of isocitrate lyase comprised 110−135 nmol (min mg protein)$^{-1}$, and the activity of malate synthase was 57−70 nmol (min mg protein)$^{-1}$. The activity of citrate synthase, a key enzyme of tricarboxylic acid cycle, remained at a high level of 1460−2000 nmol (min mg protein)$^{-1}$ during the whole cultivation period. The activity of aconitate hydratase performing the interconversion of a pair of citrate/isocitrate was maintained at a similar level, although the activity of this enzyme was about four times lower than the activity of citrate synthase. The activities of enzymes metabolizing isocitrate—NAD- and NADP-dependent isocitrate dehydrogenases and succinate dehydrogenase—remained approximately at the similar level throughout the cultivation; the activity of NAD-dependent isocitrate dehydrogenase varied from 80 to 100 nmol (min mg protein)$^{-1}$, the activity of NADP-dependent isocitrate dehydrogenases was from 250 to 300 nmol (min mg protein)$^{-1}$, and the activity of succinate dehydrogenase was in a range of 180−260 nmol (min mg protein)$^{-1}$. It should be noted that very high activity of succinate dehydrogenase, which exceeded by 2.6 times the activity of NAD-dependent isocitrate dehydrogenase, can be explained by the operation of the glyoxylate cycle. The enzyme activities of the left branch of TCA cycle—fumarate hydratase and malate dehydrogenase—were induced at the early growth phase and remained at a level of 280−300 and 1800−2100 nmol (min mg protein)$^{-1}$, respectively, during the whole cultivation period.

The citric acid production by *Y. lipolytica*

The data on the efficiency of growth and CA and ICA synthesis in the course of the growth of a wild strain *Y. lipolytica* VKM Y-2373 and a mutant strain *Y. lipolytica* NG40/UV7 under the developed regime of GW feeding are presented in Table 2. The regime of GW supplement included the initial addition of GW (by 20 g l^{-1}) and then by 100 g per entire fermenter volume. The substrate feeding was carried out synchronously with the sharp decrease in a respiratory activity of cells. A decrease in a respiratory activity of cells was accompanied by an increase in the level of pO_2 by 5–10 % from the constant level during the process. Such increase in pO_2 indicated the complete consumption of GW by cells. The total amount of GW added during cultivation reached 0.9 and 1 kg per fermentor for the processes of CA production by *Y. lipolytica* VKM Y-2373 and *Y. lipolytica* NG40/UV7, respectively. The biosynthesis of CA was initiated by the exhaustion of nitrogen in the medium after 24 h, when the culture was transferred to the phase of growth retardation and the stationary phase. CA and ICA were not excreted into the medium until the yeast growth was retarded by nitrogen limitation. The excretion of CA and ICA occurred in the phase of growth retardation and in the stationary phase and continued all the time when GW was present in the cultivation medium.

Table 2 Parameters of growth and CA production by different strains of *Y. lipolytica*

Parameters	VKM Y-2373	NG40/UV7
Biomass (g l^{-1})	21.0	18.8
Citric acid (CA) (g l^{-1})	67.7	122.2
Isocitric acid (ICA) (g l^{-1})	40.4	2.3
CA:ICA ratio	1.7:1	53:1
The mass citric acid yield (Y_p) (g g^{-1})	0.59	0.95
The volumetric productivity of CA biosynthesis (Q_p) (g l^{-1} h^{-1})	0.55	0.99

The values are the means of the three experiments and two measurements for each experiment which varied by no more than 10 %

As seen from Table 2, the developed regime of the GW feeding provided good growth and high acid-producing activity of both strains. The biomass reached 21 g l^{-1} for *Y. lipolytica* VKM Y-2373 and 18.8 g l^{-1} for *Y. lipolytica* NG40/UV7. At the end of cultivation (192 h), the wild strain *Y. lipolytica* VKM Y-2373 produced 67.7 g l^{-1} of CA and 40.4 g l^{-1} of ICA; the ratio between CA and ICA was 1.7:1. The mutant strain *Y. lipolytica* NG40/UV7 produced 122.2 g l^{-1} of CA and 2.3 g l^{-1} of ICA, so that the CA:ICA ratio was equal to 53:1. The citric acid mass yield (Y_p) reached 0.59 and 0.95 g g^{-1} for *Y. lipolytica* VKM Y-2373 and *Y. lipolytica* NG40/UV7, respectively. The volumetric productivity of CA biosynthesis (Q_p) reached 0.55 and 0.99 g l^{-1} h^{-1} for *Y. lipolytica* VKM Y-2373 and *Y. lipolytica* NG40/UV7, respectively.

Enzyme activities of the citrate-producing *Y. lipolytica* cells

Table 3 shows typical data on enzyme activities examined in homogenates of wild strain *Y. lipolytica* VKM Y-2373 and mutant strain *Y. lipolytica* NG40/UV7 taken from the exponential growth phase at 12 h (phase I, no acid production), the growth retardation at 30 h (phase II, the beginning of the CA and ICA production), and the stationary phase at 96 h, when the active acid production occurred (phase III). It can be seen that the exponential-phase cells (growth phase I) of both strains exhibited high activities of all enzymes studied (glycerol kinase, isocitrate lyase, citrate synthase, aconitate hydratase, NAD- and NADP-dependent isocitrate dehydrogenases). However, the activities of aconitate hydratase and NAD-dependent isocitrate dehydrogenase were lower than activity of citrate synthase by 4.4 and 18.25 times, respectively, in wild strain *Y. lipolytica* VKM Y-2373 and by 14.5 and 19.3 times, respectively, in mutant *Y. lipolytica* NG40/UV7. The transition of both strains from the growth phase to growth retardation phase II caused by the exhaustion of nitrogen in the medium resulted in two- and threefold decrease in activities of aconitate hydratase and NAD-dependent isocitrate dehydrogenase, respectively, in both strains. During the stationary phase (period of active acid production, phase III), a further decrease in the activities of aconitate hydratase and NAD-dependent isocitrate dehydrogenase was observed: the activity of aconitate hydratase was lower by 3.5 times in a wild strain of *Y. lipolytica* VKM Y-2373 and by 6 times in a mutant strain of *Y. lipolytica* NG40/UV7 as compared with that in the exponential-phase cells. The activity of NAD-isocitrate dehydrogenase was lower by four times in a wild strain of *Y. lipolytica* VKM Y-2373 and by nine times in a mutant strain of *Y. lipolytica* NG40/UV7 than that in the exponential-phase cells. It should be noted that the transition of both strains from the growth phase to growth retardation phase II and stationary phase III had no remarkable effect on the activities of glycerol kinase, isocitrate lyase, citrate synthase, and NADP-dependent isocitrate dehydrogenase.

Discussion

The aim of this work was to study the metabolic pathways of the GW utilization in the wild and mutant strains of yeast *Y. lipolytica*—producers of CA and ICA—in order to develop a high-effective process of CA production.

GW consisted of two substrates: glycerol (65 %) and fatty acids (20 %). It is well known that glycerol is involved in yeast

Table 3 Activities of enzymes of different *Y. lipolytica* strains grown on GW under growth limitation by nitrogen

Enzymes (nmol (min mg protein)$^{-1}$)	VKM Y-2373			NG40/UV7		
	I	II	III	I	II	III
Glycerol kinase	105	95	120	112	107	91
Citrate synthase	1460	1500	1530	1740	1600	1600
Aconitate hydratase	330	165	94	120	60	20
NAD-dependent isocitrate dehydrogenase	80	40	20	90	30	10
NADP-dependent isocitrate dehydrogenase	300	250	220	80	90	90
Isocitrate lyase	110	115	105	89	90	100

The values are the means of the three experiments and two measurements for each experiment, which varied by no more than 10 %

I, the exponential growth phase; II, the retardation phase, the start of acid synthesis; III, the stationary phase, active acid production

metabolism by means of its phosphorylation by glycerol kinase (Ermakova and Morgunov 1987; Makri et al. 2010; Workman et al. 2013; Tomaszewska et al. 2014), while fatty acids are metabolized via the glyoxylate cycle (Ermakova et al. 1986; Holdsworth et al. 1988; Kamzolova et al. 2011b). It should be noted that in glycerol-grown cells, the glyoxylate cycle is inactive (Morgunov et al. 2004a; Kamzolova et al. 2011b), as in microbial cells grown on glucose (Rohr and Kubicek 1981; Ermakova et al. 1986; Holdsworth et al. 1988).

As seen from Fig. 1, glycerol and fatty acid fractions started to decrease from the first hours of cultivation. Moreover, the utilization of glycerol and fatty acids occurred concurrently, although glycerol was utilized at a higher rate than fatty acids. Thus, the glycerol and fatty acids do not suppress the metabolism of each other. The simultaneous utilization of glycerol and fatty acids is confirmed by the results of enzyme assay in *Y. lipolytica*. As seen from Table 1, at 4 h of yeast cultivation on the GW, the key enzyme of glycerol metabolism, glycerol kinase, was induced and the key enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase, which are involved in the metabolism of fatty acids, were also induced. In older yeast cells (12 and 18 h old) grown on GW, the activity of glycerol kinase, isocitrate lyase, and malate synthase was also high, due to the fact that glycerol and fatty acids were actively metabolized during the whole cultivation period. The active functioning of the glyoxylate cycle during the assimilation of GW is confirmed by high activities of citrate synthase and aconitate hydratase, which operate both in the glyoxylate cycle and in tricarboxylic acid cycle.

It should be noted that the phenomenon of utilization of two different substrates in *Y. lipolytica* is poorly studied. The hexokinase from *Y. lipolytica* substituted effectively for hexokinase II from *Saccharomyces cerevisiae* in catabolite repression of invertase (Petit and Gancedo 1999). The hexokinases from *Schizosaccharomyces pombe* or *Kluyveromyces lactis* were much less effective in this role. Other authors shown that hexokinase is involved in the glucose catabolite repression of extracellular LIP2 in *Y. lipolytica* (Fickers et al. 2005). Mutants with increased capability of extracellular lipase production were obtained by chemical mutagenesis using *Y. lipolytica* (Fickers et al. 2003). A high level of lipase production in the glucose-grown mutant *Y. lipolytica* LgX64.81 could be related to its phenotype; i.e., a lower sensibility to glucose catabolite repression is due to the modification in the level of HXK1 expression (Fickers et al. 2009). Iske et al. (1983) studied the mixed assimilation of the *n*-tetradecane-1-^{14}C-labeled paraffin and glucose by *Saccharomycopsis lipolytica* EH 59 and observed the incorporation of radioactive label from *n*-tetradecane only at the late hours of cultivation. We obtained the similar results confirming that glucose is a strong repressor of the hexadecane metabolism and a mild repressor of metabolism of oleic acid (and, probably, other fatty acids) (Kamzolova et al. 2011b). Workman et al. (2013) studied the growth of *Y. lipolytica* on a mixture of glycerol and glucose and observed preferential utilization of glycerol over glucose by *Y. lipolytica*. The authors explained the preference glycerol over glucose as a consequence of the genome containing only one gene encoding hexose transporter and at least three genes encoding proteins associated with glycerol transport. In contrast, glycerol does not suppress the metabolism of fatty acids in yeast *Y. lipolytica* grown on the mixture of the free saturated fatty acids and glycerol (Papanikolaou et al. 2006) or in yeast *Y. lipolytica* cultivated on rapeseed and sunflower oils (Kamzolova et al. 2005, 2011b).

The fact that glycerol and fatty acids can be consumed simultaneously is of special importance for the development of the efficient regime of GW feeding in the CA production process by *Y. lipolytica*. According to the auxiliary concept of Babel (2009), the simultaneous consumption of physiologically similar substrates could increase the yield of a target product. The simultaneous utilization of methanol and glucose by *Hansenula polymorpha* resulted in an increase in the biomass yield up to 25 % and in a higher growth rate as compared to those obtained on an alone substrate (Müller et al. 1983). Workman et al. (2013) reported that simultaneous utilization of glucose and glycerol resulted in the maximization of the metabolism of *Y. lipolytica* with regards to oxygen availability promoting ATP generation by oxidative phosphorylation and increased the specific growth rate and biomass yield. In our case, *Y. lipolytica* VKM Y-2373 grown on GW composed of two substrates (glycerol and fatty acids) showed the highest biomass yield ($Y_{x/s}$) of 1.05 g g^{-1} and the maximum specific growth rate (μ_{max}) of 0.352 ч$^{-1}$, while the cells cultivated on pure glycerol or fatty acids showed biomass yield ($Y_{x/s}$) of 0.47 and 0.87 g g^{-1}, respectively (Kamzolova et al. 2011b).

Under optimal GW feeding (by 20 g l^{-1}), the CA production and the ratio between CA and a by-product—isocitric acid—depended on the strain used. It was revealed that wild strain *Y. lipolytica* VKM Y-2373 produced citrate and isocitrate with a ratio of 1.7:1, while the mutant strain *Y. lipolytica* NG40/UV7 synthesized presumably CA (122.2 g l^{-1}) with the citrate-to-isocitrate ratio of 53:1 and the yield of 0.95 g g^{-1}. Earlier, we indicated that this mutant produced 112 g l^{-1} CA under GW feeding by 20–80 g l^{-1} (Morgunov et al. 2013). Such high productivity is of considerable importance from an economic point of view. According to literature data, the wild strains of *Y. lipolytica* produced CA from 21.6 g l^{-1} (Levinson et al. 2007) to 62.5 g l^{-1} (Papanikolaou et al. 2008), while the mutant and recombinant strains were able to produce CA from 58 g l^{-1} (Celińska and Grajek 2013) to 131.5 g l^{-1} (Rywińska et al. 2009). The higher CA production has been reported for *S. lipolytica* (syn. *Y. lipolytica*) NTG9 grown on rapeseed oil; CA concentration reached 152.3 g l^{-1}, while ICA production was significant and a ratio of CA/ICA was equal to 5.34:1.00 (Good et al. 1985).

Aurich et al. (2003) obtained CA concentration of 198 g l^{-1}, which was achieved after a 300-h fed-batch cultivation of mutant strain *Y. lipolytica* H181. High CA production has been reported for mutant strain *Y. lipolytica* growing on glucose hydrols (Wojtatowicz et al. 1991), sucrose (Förster et al. 2007b), and *n*-paraffins (Stottmeister and Weissbrodt 1991; Crolla and Kennedy 2004).

To elucidate the mechanism of CA and ICA production from GW by *Y. lipolytica*, the activities of enzymes were determined. As seen from Table 3, the ratio between CA and ICA is defined by the activities of citrate synthase, aconitate hydratase, and NAD-isocitrate dehydrogenase. In the mutant *Y. lipolytica* NG40/UV7, high activity of citrate synthase (1600 nmol (min mg protein)$^{-1}$) and extremely low activity of aconitate hydratase (20 nmol (min mg protein)$^{-1}$) lead to presumable CA synthesis. At the same time, in wild strain *Y. lipolytica* VKM Y-2373, rather high activity of aconitate hydratase (94 nmol (min mg protein)$^{-1}$) and simultaneously low activity of NAD-isocitrate dehydrogenase (20 nmol (min mg protein)$^{-1}$) lead to the excretion of both CA and ICA. More refined hypotheses were expressed: in citrate- and isocitrate-producing yeast grown on fatty acids, the metabolism is divided between microsomes, peroxisomes, and mitochondria and the formation of acids and isoforms can occur in different compartments of the cells, which may be accompanied by a selective transport of acids from mitochondria (Marchal et al. 1980).

To conclude, the dynamics of growth of *Y. lipolytica* and the levels of relevant enzymes in the yeast cells show that glycerol does not suppress the metabolism of fatty acids; as a result, these both components of GW are concurrently utilized by the yeast *Y. lipolytica*. The efficient regime of GW feeding provided a high CA production (122.2 g l^{-1}) with a citrate-to-isocitrate ratio of 53:1 and the yield of 0.95 g g^{-1} using mutant strain *Y. lipolytica* NG40/UV7.

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