ISSN 1330-9862
(FTB-2466)

original scientific paper

Citric Acid Production by Yeast Grown on Glycerol-Containing Waste from Biodiesel Industry

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Received: March 3, 2010
Accepted: August 16, 2010

Summary

The possibility of using glycerol and glycerol-containing waste from biodiesel manufacture as a carbon and energy source for microbiological production of citric acid has been studied. Acid formation on the selective media had previously been tested in 66 yeast strains of different genera (*Candida, Pichia, Saccharomyces, Torulopsis* and *Yarrowia*). Under growth limitation by nitrogen, 41 strains (belonging mainly to species *Yarrowia lipolytica*) produced acids; unlike 25 strains of the genera *Debaryomyces, Candida, Pichia, Saccharomyces* and *Torulopsis*. Among the 41 acid-producing strains, mutant strain *Yarrowia lipolytica* N15 was selected since it was able to produce citric acid presumably in high amounts. The citric acid production by the selected strain was studied in dependence on the medium pH, aeration and concentration of glycerol. Under optimal conditions, the mutant *Y. lipolytica* N15 produced up to 98 g/L of citric acid when grown in a fermentor with the medium containing pure glycerol, and 71 g/L of citric acid when grown on glycerol-containing waste. The effect of growth phases on physiological peculiarities of the citric acid producer was discussed.

Key words: Yarrowia lipolytica, citric acid production, biodiesel production, glycerol, glycerol-containing waste

Introduction

In the 20th century, oil hydrocarbons were considered to be the main source of energy and the cheapest and readily available raw material for biotechnology. However, the ever-increasing rise in the cost of oil starting with the oil crisis of the seventies and the deterioration of the global ecological situation in the last years have made us turn to alternative energy sources, such as biodiesel produced from renewable plant raw materials. In 2010, the European Community (EU directive 2003/30/EC) planned to raise the percent of biodiesel to 5.75 % of the total fuel. Biodiesel can be produced from various vegetable oils and animal fats. The technology consists in that oil triglycerides are hydrolyzed and then methylated with the formation of methylated fatty acids, which are used just as biodiesel. One of the major wastes from this technological process is glycerol, which is formed in an amount of more than 1 kg per 10 kg of the biodiesel produced. In 2007, the amount of glycerol-containing waste in Europe reached 600 000 tonnes (*1*), which poses the problem of its utilization.

Biodiesel waste, which contains glycerol (up to 80 %), oil residue, free fatty acids, sodium and potassium salts, and water, may serve as a raw material for various biotechnological processes. Several efforts were made for microbiological conversion of technological glycerol (crude glycerol) into valuable products: 1,3-propanediol

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(*1,2*), microbial biomass and lipids (*3–6*), food-grade pigments (*7*), erythritol (*8*), mannitol (*9*), L-lysine (*10*), organic acids, in particular succinic acid (*11*) and citric acid (*1,2,8,12–16*).

Citric acid (CA) has attracted increased interest due to its distinctive properties as an acidulate, flavouring agent and antioxidant, and it is used mainly in food and beverage industry (70 % of the total CA production). In recent years, the consumption of CA and its salt, trisodium citrate, has reached 1 400 000 tonnes with growth at 5 % per year (*17*).

CA is an intermediate of tricarboxylic acid cycle and holds a key position in the metabolism of each microbial cell. However, under certain conditions of fermentation, fungi, bacteria and yeasts produce CA in excessive amounts. Traditionally, different strains of fungi, mostly belonging to *Aspergillus niger*, have been used in the commercial production of CA from molasses, sucrose or glucose. Alternatively, there is a great interest in various yeasts belonging to *Candida* (*Yarrowia*) *lipolytica*, which is capable of CA production from various carbon sources, such as *n*-alkanes (*18–20*), glucose (*18,19,21–25*), ethanol (*26–28*) and plant oils (*29–33*). The relevant literature data on attempts to use glycerol as carbon source for CA production are rare (*1,4,8,12–14,32,34*).

The goal of the present work is: (*i*) to study CA production from pure glycerol and glycerol-containing waste from biodiesel industry as carbon sources by different yeast genera (*Debaromyces*, *Candida*, *Pichia*, *Saccharomyces*, *Yarrowia* and *Torulopsis*); (*ii*) to estimate the effect of cultivation conditions (the medium pH, oxygen supply, and concentration of carbon substrate) on CA production by the selected strain; and (*iii*) to develop a method for CA production.

Materials and Methods

Yeast strains

Screening for CA producer was carried out among 59 natural yeast strains belonging to the genera *Debaryomyces*, *Candida*, *Pichia*, *Saccharomyces*, *Yarrowia* and *Torulopsis* and 7 mutant strains with impaired ability to grow on acetate probably due to defects in the tricarboxylic acid cycle (*35*). Strains were obtained from the All-Russian Collection of Microorganisms (VKM) and from the collection of the Laboratory of Aerobic Metabolism of Microorganisms of the Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences (Pushchino, Russia). The strains were maintained at 4 °C on agar slants with *n*-alkanes as the carbon source.

Chemicals

All chemicals and enzymes were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glycerol-containing waste was provided from the Pythia Institute of Biotechnology (Thessaloniki, Greece). This is the product of transesterification reaction of rapeseed oil, methanol and NaOH at a molar ratio of 1:3:0.5, respectively. It contained (in %, by mass): glycerol 80, sodium salts 1, heavy metals 1, impurities of organic nature (oil residue, fatty acids) 5, methanol as a minor component, water 12.7. Crude glycerol was analysed enzymatically using biochemical kit (Roche Diagnostics GmbH, Mannheim, Germany). Additionally, it was analysed for oil and fatty acid content. It was washed twice with *n*-hexane, the mixture was divided into two layers, of which the upper phase contained *n*-hexane and lipids. Hexane extract was collected into a glass flask with precision. The lipid extract was dried by passing it through a glass filter with anhydrous sodium sulphate; solvent was evaporated to constant mass of lipids.

Assessment of acid formation on solid media

The acid formation was assessed under nitrogen limitation of yeast growth by measuring the zones of $CaCO_3$ dissolution in Petri dishes with the agar medium containing (in g/L): glycerol 20, $MgSO_4 \cdot 7H_2O$ 0.7, $Ca(NO_3)_2$ 0.4, NaCl 0.5, KH_2PO_4 1.0, K_2HPO_4 0.1, and trace elements as described by Burkholder *et al.* (*36*) with slight modifications (in mg/L): I^- 0.1, B^{3+} 0.01, Fe^{2+} 0.05, Zn^{2+} 0.04, Mn^{2+} 0.01, Cu^{2+} 0.01, Mo^{2+} 0.01, yeast autolysate 8 mL/L (as a source of nitrogen and vitamins), and Bacto agar 20.0 g/L. Chemically pure powdered $CaCO_3$ (6 g/L) was added into the heated medium directly before it was poured into the dishes. The cultures were plated onto cooled agar medium and incubated at (28±1) °C for 7 days.

Assessment of the acid formation by yeast in liquid cultures

The acid formation in liquid medium was assessed after a 6-day cultivation of the studied strains under nitrogen deficiency in 750-mL flasks with 50 mL of the medium containing (in g/L): $(NH_4)_2SO_4$ 0.3, $MgSO_4 \cdot 7H_2O$ 0.7, $Ca(NO_3)_2$ 0.4, NaCl 0.5, KH_2PO_4 1.0, K_2HPO_4 0.1, Burkholder trace elements, Difco yeast extract 0.5, and glycerol 30. Since growth was followed by a decrease in the pH of the medium, in order to maintain the medium at pH=4.5–5.5, 10 % NaOH was periodically added using pH paper strips.

Yeast cultivation in a fermentor

The yeasts were cultivated in a 10-litre ANKUM-2M (SKB, Pushchino, Russia) with an initial working volume of 5.0 L. The medium contained (in g/L): $(NH_4)_2SO_4$ 3.0, $MgSO_4 \cdot 7H_2O$ 1.4, NaCl 0.5, $Ca(NO_3)_2$ 0.8, KH_2PO_4 2.0, K_2HPO_4 0.2, Burkholder trace elements, Difco yeast extract 1.0 and thiamine 0.02. Glycerol (170 g/L) or glycerol-containing waste (100 g/L) were used as the sole carbon and energy source. The fermentation conditions were maintained automatically at the constant level: temperature was (28±0.5) °C; pH=4.5±0.1 was adjusted with 20 % NaOH; dissolved oxygen concentration (pO_2) was 60 % (from air saturation); and agitation was 800 rpm. Pulsed addition of glycerol-containing materials was performed as the pO_2 value increased by 5 %, indicating a decrease in respiratory activity of the cells due to the total consumption of carbon sources. Cultivation was performed as indicated in the text.

Measurement techniques

Yeast growth was followed by measuring the absorbance of the culture at 540 nm with a Spekol 221 spectrophotometer (Carl Zeiss, Jena, Germany). The dry bio-

mass was estimated from the absorbance of the cell suspension using a calibration curve.

Concentration of ammonium was determined potentiometrically with an Ecotest-120 ionometer using an Ekom-NH_4 electrode (Econix, Moscow, Russia).

Glycerol was analysed enzymatically using biochemical kit (Roche Diagnostics GmbH). The determination of glycerol was based on the measurement of NADH produced during the conversion of glycerol to L-lactate in coupled reactions; reactions were catalyzed by glycerol kinase, pyruvate kinase and L-lactate dehydrogenase.

Concentration of organic acids was determined using high-performance liquid chromatograph (Pharmacia LKB, Uppsala, Sweden) on an Inertsil ODS-3 reversed-phase column (250×4 mm, Elsiko, Rostov-On-Don, Russia) at 210 nm; 20 mM phosphoric acid was used as a mobile phase with the flow rate of 1.0 mL/min; the column temperature was maintained at 35 °C. CA and, *threo*-D(*S*)-(+)-isocitric acid (ICA) was identified using the standard solutions (Roche Diagnostics GmbH). Moreover, diagnostic kits (Roche Diagnostics GmbH) were used for the assay of CA and ICA. The determination of CA was based on the measurement of the NADH produced during the conversion of CA to oxaloacetate and its decarboxylation product pyruvate, and following the conversion to L-malate and L-lactate. Reactions are catalysed by citrate lyase, malate dehydrogenase and L-lactate dehydrogenase. The determination of ICA was based on the measurement of the NADPH produced during the conversion of ICA to α-ketoglutarate, a reaction catalysed by isocitrate dehydrogenase.

Protein was determined by the Lowry method, while carbon, hydrogen, and nitrogen were measured on a C/H/N element analyzer (Carlo Erba Instruments, Milan, Italy), and the ash content was determined by burning the sample in a muffle furnace. The oxygen content (O) was calculated from:

$$\text{O (in \%)} = 100 - (\text{C} + \text{H} + \text{N} + \text{ash}) \qquad /1/$$

where C, H and N are the values of carbon, hydrogen, and nitrogen content (in %).

Methyl esters of fatty acids were obtained by the method of Sultanovich *et al.* (*37*) and analysed by gas-liquid chromatography on a Chrom-5 gas chromatograph (Laboratorni pristroje, Prague, Czech Republic) with a flame-ionization detector. The column (2 m×3 mm) was packed with 15 % Reoplex 400 applied to Chromaton N-AW (0.16–0.20 mm). The temperature of the column was 200 ºC. The lipid content in the biomass was determined from the total fatty acid content by using docosane ($C_{22}H_{46}$) as internal standard.

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 was used for calculations of the mass yield of CA (Y_{CA}), volumetric citric acid productivity (Q_{CA}) and specific citric acid production rate (q_{CA}).

The mass yield of CA production (Y_{CA}), expressed in g of CA per g of glycerol, was calculated from:

$$Y_{CA} = \frac{P}{S} \qquad /2/$$

while the volumetric citric acid productivity (Q_{CA}), expressed in g/(L·h), was calculated from:

$$Q_{CA} = \frac{P}{V \cdot t} \qquad /3/$$

and the specific citric acid production rate (q_{CA}), expressed in g per g of cell per h, was calculated from:

$$q_{CA} = \frac{P}{X \cdot t} \qquad /4/$$

where P is the total amount of CA in the culture liquid at the end of cultivation (g), S is the total amount of glycerol/crude glycerol consumed (g), V is the initial volume of culture liquid (L), t is the fermentation duration (h), and X is the average working biomass in the fermentor (g).

Energy yield of CA from glycerol (η_{CA}) is estimated as a fraction of energy content of the substrate (glycerol) which is incorporated into CA. It was calculated on the basis of mass and energy balance theory (*38–40*).

The energy content in the biomass was calculated on the basis of mass and energy balance theory (*38–40*).

Statistical analysis

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

Results and Discussion

Selection of citric acid-producing yeast strains

Screening of citric acid-producing yeasts was performed among 59 natural yeast strains belonging to the genera *Debaryomyces*, *Candida*, *Pichia*, *Saccharomyces*, *Torulopsis* and *Yarrowia*, and 7 mutant strains of *Y. lipolytica*. The criterion for selecting the yeast strains was good growth on glycerol. In this experiment, pure glycerol was used. All the 66 strains were cultivated on the selective medium with powdered $CaCO_3$.

It has been accepted that the necessary conditions for intense citric acid production by yeasts usually include: (*i*) excess of carbon source in the medium, and (*ii*) growth limitation by mineral components, such as nitrogen, phosphorus, sulphur, and magnesium (*1*,*8*,*12*,*14–16*). Our experiments indicate that when yeast growth was limited with phosphorus or sulphur, a significant amount of the co-product of the metabolism, ICA, was produced in addition to CA in all the tested strains (Table 1). For this reason, further experiments were carried out under growth limitation by nitrogen.

We used the medium which meets the following conditions: it contained glycerol in an excessive amount and nitrogen in a growth-limiting concentration (50 mg/L); nitrogen was added into the medium in an organic form of yeast autolysate but not as ammonium sulphate or other inorganic compounds. The production of acids was evaluated by measuring the diameter of the dissolution zones that appeared around the yeast colonies grown on the $CaCO_3$-containing agar at 28 °C for 7 days when the

Table 1. The effect of limiting component on CA production by yeast *Y. lipolytica* grown in liquid medium with pure glycerol

Strain	Limiting component	γ(biomass) g/L	γ(CA) g/L	γ(ICA) g/L	γ(CA)/γ(ICA)	Y_{CA} g/g
Y. lipolytica N15	nitrogen	3.39±0.42	19.08±0.60	1.70±0.20	11.2:1	0.55
	phosphorus	2.81±0.52	15.34±0.46	2.69±0.30	5.7:1	0.50
	sulphur	3.18±0.42	18.76±0.36	3.17±0.40	5.9:1	0.49
Y. lipolytica VKM Y-2373	nitrogen	2.70±0.62	13.40±0.36	1.69±0.20	7.9:1	0.48
	phosphorus	2.91±0.52	12.34±0.56	5.29±0.50	2.1:1	0.50
	sulphur	2.88±0.32	14.30±0.50	3.69±0.30	3.9:1	0.45
Y. lipolytica 212	nitrogen	2.90±0.52	12.40±0.26	2.69±0.30	4.6:1	0.49
	phosphorus	3.01±0.52	13.34±0.46	3.91±0.41	3.4:1	0.50
	sulphur	3.12±0.52	14.14±0.55	4.12±0.60	3.4:1	0.45

Note: When changing the medium with the aim of growth limitation, we took into account two requirements: (*i*) glycerol should be in excess during the whole cultivation time, and (*ii*) yeast growth should be limited by a deficiency of one of the mineral components of the cultivation medium. Preliminary experiments showed that the growth of *Y. lipolytica* VKM Y-2373 was limited when the medium for cultivation contained 300 mg/L of $(NH_4)_2SO_4$ (nitrogen limitation), 0.75 mg/L of KH_2PO_4 (phosphorus limitation), 1.75 mg/L of $MgSO_4{\cdot}7H_2O$ (sulphur limitation), or 0.7 mg/L of $MgSO_4{\cdot}7H_2O$ (magnesium limitation)
The values are the means of three experiments and two measurements for each experiment

diameter of the zones of dissolution due to acid production was maximal. The results are listed in Table 2. Under the growth limitation by nitrogen, acid production was observed in 41 strains (belonging mainly to species *Yarrowia lipolytica*), while 25 strains of the genera *Debaryomyces*, *Candida*, *Pichia*, *Saccharomyces* and *Torulopsis* did not excrete acids. The distribution of strains with respect to the diameter of the formed $CaCO_3$ dissolution zones was as follows: 16 strains (0.5–2 mm), 10 strains (2.5–4 mm), 3 strains (4.5–6 mm), 5 strains (6–8 mm) and 7 strains formed zones greater than 8 mm. Note that the test of the acid formation on solid media cannot indicate which acid was produced (CA or ICA).

To identify the acids and verify the results of the producer selection on solid media, mutant strains *Y. lipolytica* UV 5, *Y. lipolytica* NG 40, *Y. lipolytica* NG 80, *Y. lipolytica* N11, *Y. lipolytica* N13, and *Y. lipolytica* N15, which formed the largest zones of chalk dissolution, were further tested for their acid-producing capacity in a liquid

Table 2. Acid production by yeast on agar medium with powdered $CaCO_3$

Strain	*d*/mm	Strain	*d*/mm	Strain	*d*/mm
Debaromyces hansenii	0	*Y. lipolytica* 69	3	*Y. lipolytica* 655	0
Candida catenulata VKM Y-5	0	*Y. lipolytica* 76	1	*Y. lipolytica* 666	0
C. catenulata VKM Y-36	0	*Y. lipolytica* 79	2	*Y. lipolytica* 667	8
C. rugosa VKM Y-67	0	*Y. lipolytica* 86	8	*Y. lipolytica* 668	1.5
C. paludigena VKM Y-2443	2.0	*Y. lipolytica* 212	8	*Y. lipolytica* 670	0.5
C. zeylanoides VKM Y-6	0	*Y. lipolytica* 214	4	*Y. lipolytica* 672	0
C. zeylanoides VKM Y-14	0	*Y. lipolytica* 281	4	*Y. lipolytica* 681	4.5
C. zeylanoides VKM Y-2324	0	*Y. lipolytica* 374/1	3	*Y. lipolytica* 683	7
C. zeylanoides VKM Y-2595	0	*Y. lipolytica* 374/3	1.5	*Y. lipolytica* 694	5
Pichia anomala VKM Y-118	0	*Y. lipolytica* 374/4	0.5	*Y. lipolytica* 695	3
P. guilliermondii H-P-4	0	*Y. lipolytica* 374/5	2	*Y. lipolytica* VKM Y-2373	8.5
P. besseyi VKM Y-2084	0	*Y. lipolytica* 374/6	0	*Y. lipolytica* 706	7
P. media VKM Y-1381	0	*Y. lipolytica* 374/8	1	*Y. lipolytica* 709	4
P. inositovora VKM Y-2494	0	*Y. lipolytica* 387	2	*Y. lipolytica* 710	5
Saccharomyces cerevisiae VKM Y-381	0	*Y. lipolytica* 571	2	*Y. lipolytica* 716	2
Torulopsis candida 127	0	*Y. lipolytica* 581	1	*Y. lipolytica* UV 4	3
T. candida 420	0	*Y. lipolytica* 582	2	*Y. lipolytica* UV 5	9
Yarrowia lipolytica VKM Y-57	0	*Y. lipolytica* 585	1	*Y. lipolytica* NG 40	9
Y. lipolytica 12a	0	*Y. lipolytica* 591	0	*Y. lipolytica* NG 80	9
Y. lipolytica 9b	3	*Y. lipolytica* 607	3.5	*Y. lipolytica* N11	9
Y. lipolytica VKM Y-47	0	*Y. lipolytica* 645	4	*Y. lipolytica* N13	10
Y. lipolytica 68	0	*Y. lipolytica* 646	1	*Y. lipolytica* N15	11

d=diameter of the $CaCO_3$ dissociation zone

medium containing nitrogen in the limiting concentration. Biomass, CA, ICA and residual glycerol contents were determined after 6 days of cultivation when the specific rate of CA production reached the maximum. If the incubation time increased, the production rate decreased.

As seen from Table 3, all the tested strains synthesized predominantly CA (a ratio between CA and ICA varied from 2.6:1 to 14.8:1). However, the absolute amounts of CA or ICA accumulated in the medium and their ratios were strain dependent. The highest ratio of CA to ICA (11.2:1) and the largest mass yield of 0.55 g/g were observed in mutant strain *Y. lipolytica* N15. As seen from Table 1, *Y. lipolytica* N15 formed the largest zones of chalk dissolution around the colonies. That is why the mutant strain *Y. lipolytica* N15 was selected for further studies as a promising producer of CA from glycerol.

Determination of optimal conditions for citric acid production from glycerol

In further experiments, the citric acid-producing ability of *Y. lipolytica* N15 was studied in dependence on pH, concentration of dissolved oxygen, and glycerol concentration (Fig. 1).

The strain was grown in a fermentor under nitrogen limitation to the phase of active acid formation (biomass of 10 g/L). Yeast cells were separated from the culture liquid by centrifugation, washed twice with 0.9 % NaCl, and suspended in 50 mM phosphate buffer (pH=7.0). The cell suspension was placed in 750-mL Erlenmeyer flasks with 50 mL of the medium free of nitrogen and vitamins but containing 20 g/L of glycerol and incubated on a shaker (180–200 rpm) at 28 °C for 22 h. By the end of the experiment, pH of the medium slightly decreased (by 0.5–0.3 units), biomass remained at a constant level of (2.5±0.3) g/L, and the cell viability remained virtually unchanged. Microscopic examination of the cells and the measurement of extracellular protein in the incubation medium showed that the cells were not disrupted in all experiments.

CA was produced at all the pH values studied (3.0–8.0) and reached the maximum (6.10–6.17 g/L) at pH= 4.5–6.0 (Fig. 1a). At lower and higher pH values, the production of CA decreased. The lowest CA production was observed at pH=3.0. The optimum pH range for acid production (4.5–6.0) corresponds to the data on the optimum pH values (4.5–6.0) for the growth of *Y. lipolytica* and biosynthesis of CA in the medium containing ethanol as a carbon source (*27*), glucose (*41*) and glycerol (*1,8,12,14–16*).

The effect of the dissolved oxygen concentration on CA production was studied at pH=4.5. Different rates of aeration were obtained by varying the volume of the medium (from 50 to 500 mL) in 750-mL flasks. CA was excreted into the medium at all the concentrations of dissolved oxygen tested. At the aeration rates of (amount of O_2) 0.30 and 0.56 mmol/(L·min), the biosynthesis of CA reached the maximum ((6.97–7.77) g/L); at the aeration rates of (amount of O_2) 0.20 and 0.24 mmol/(L·min), the biosynthesis of CA was 1.5 times lower; and at the

Table 3. CA production by mutant strains of *Y. lipolytica* grown in liquid medium with pure glycerol

Strain	γ(biomass) / g/L	γ(CA) / g/L	γ(ICA) / g/L	γ(CA)/γ(ICA)	Y_{CA} / g/g
Y. lipolytica UV 5	3.59±0.33	19.73±0.53	2.14±0.20	9.2:1	0.49
Y. lipolytica NG 40	3.94±0.52	16.60±0.46	3.49±0.30	4.8:1	0.42
Y. lipolytica NG 80	3.78±0.42	15.44±0.36	3.84±0.40	4.0:1	0.39
Y. lipolytica N11	3.10±0.32	16.64±0.46	2.65±0.20	6.2:1	0.42
Y. lipolytica N13	3.45±0.44	16.04±0.40	3.32±0.30	4.8:1	0.40
Y. lipolytica N15	3.39±0.42	19.08±0.60	1.70±0.20	11.2:1	0.55

The values are the means of three experiments and two measurements for each experiment

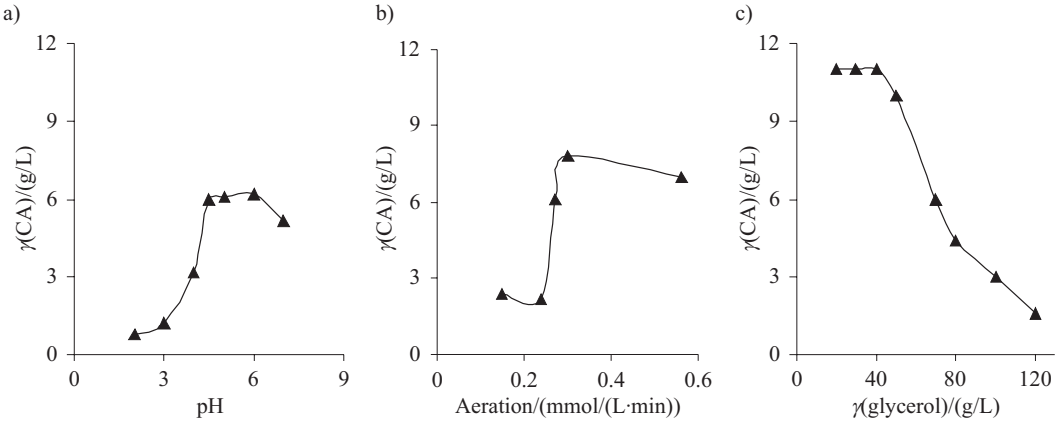

Fig. 1. The effect of (a) pH medium, (b) aeration and (c) glycerol concentration on CA production by *Y. lipolytica* N15 grown on pure glycerol in flasks

aeration rates below (amount of O_2) 0.2 mmol/(L·min), virtually none CA was excreted into the medium (Fig. 1b). Thus, CA was actively produced by the *Y. lipolytica* cells grown on glycerol only under a sufficiently high aeration of the medium. At low aeration rates, CA was not accumulated. Similar trends had been found for *Y. lipolytica* grown on *n*-alkane (*42*) and ethanol (*28*,*43*). It seems that oxygen deficiency causes a decrease in the activity of some mitochondrial enzymes (citrate synthase, aconitase, malate dehydrogenase, and NADP-dependent isocitrate dehydrogenase) involved in the synthesis of CA.

The effect of glycerol on CA synthesis by *Y. lipolytica* N15 was studied at pH=4.5; the volume of the medium in the cultivation flasks was 50 mL; glycerol concentration ranged from 20 to 120 g/L. CA production was observed at all the glycerol concentrations studied. The synthesis of CA reached the maximum (11 g/L) at glycerol concentrations from 20 to 40 g/L and decreased for 2.5 times at glycerol concentrations above 80 g/L (Fig. 1c). Similar trends had been found for pyruvate-producing strain grown on pure glycerol (*44*) and poly(3-hydroxybutyrate) production from crude glycerol (*45*). Rymowicz *et al.* (*46*) indicated that a high initial concentration of glycerol up to 150 g/L favours erythritol production by strain *Y. lipolytica* Wratislavia Ki, which compensates for the difference between the extracellular and intracellular water potential.

Thus, the highest CA production by *Y. lipolytica* N15 grown on glycerol occurs at pH=4.5, high concentration of dissolved oxygen, and at glycerol concentrations from 20 to 40 g/L. The optimization of cultivation conditions (pH, concentrations of dissolved oxygen and glycerol) resulted in the improvement of productivity of CA biosynthesis by 45 %.

Citric acid production by Y. lipolytica, grown on pure glycerol in a fermentor

Further experiments were carried out with *Y. lipolytica* N15 grown in a 10-litre fermentor at pH=4.5 under nitrogen limitation; concentration of dissolved oxygen was maintained at 60 % of saturation, while the initial concentration of glycerol was 20 g/L. Pulsed addition of glycerol was performed as the pO_2 value increased by 5 %, indicating a decrease in respiratory activity of the cells due to the total consumption of carbon sources.

Fig. 2 shows the curves of growth of *Y. lipolytica* N15 and the parental strain *Y. lipolytica* VKM Y-2373 (for comparison), ammonium consumption, CA and ICA synthesis with pure glycerol. The nitrogen supply was exhausted after 24 h of cultivation by both strains. The biomass growth continued for 36 h and reached 20 g/L for *Y. lipolytica* N15 and 21 g/L for *Y. lipolytica* VKM Y-2373; then the yeast culture entered the stationary phase because of the nitrogen exhaustion from the medium. A bulk of CA was accumulated in the medium just during the stationary growth phase. By the end of the cultivation period (144 h), *Y. lipolytica* N15 produced 98 g/L of CA with insignificant amount of ICA (3.3 g/L); a CA/ICA ratio was 30:1. Also, CA was produced principally during the stationary growth phase by *Y. lipolytica* VKM Y-2373 (94 g/L of CA by the end of the cultivation period), but the disadvantage of the parental strain lies in

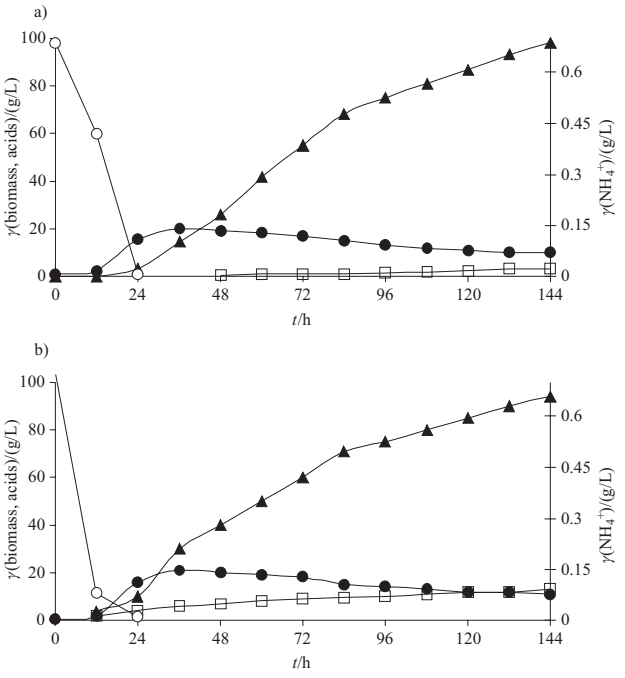

Fig. 2. Time courses of (●) growth, (○) ammonium consumption, (▲) CA and (□) ICA production by (a) *Y. lipolytica* N15 and (b) *Y. lipolytica* VKM Y-2373, grown on pure glycerol

the enough high production of ICA (13.1 g/L) as a by-product of fermentation (CA/ICA ratio was 7.5:1).

The volumetric citric acid productivity (Q_{CA}) and the specific citric acid production rate (q_{CA}) using *Y. lipolytica* N15 reached 1.14 g/(L·h) and 0.076 g per g of cell per h, respectively, which corresponded to the best values reported previously for citrate-producing strains grown on various carbon sources (*8*,*15*,*16*,*22*,*23*,*25*,*29*,*33*,*41*,*47*,*48*).

The mass yield (Y_{CA}) of *Y. lipolytica* N15 was 0.7 g of CA per g of glycerol, which is comparable to those reported in the literature for citrate-producing strains, found within the range from 0.41 to 0.77 g of CA per g of carbon source (*23*,*47*,*49*). However, higher values of CA yield were obtained from *n*-hexadecane (1.44 g of CA per g of *n*-hexadecane) (*47*), sucrose (0.82 g of CA per g of sucrose) (*34*), ethanol (0.87 g of CA per g of ethanol) (*28*) and rapeseed oil (1.55 g of CA per g of oil) (*33*).

However, since carbon substrates are characterized by different energy capacities, it is inappropriate to compare the mass yields from different substrates. It is more correct to compare the energy yields of citric acid (η_{CA}) in different strains.

The value of η_{CA} indicates a fraction of energy content of the substrate (glycerol), which is incorporated into CA; it was calculated on the basis of mass and energy balance theory (*38–40*). Quantities that characterize mass and energy balance of cell metabolism are based on the universal unit of reductivity, redoxon; it represents an electron that can be transferred to oxygen; a former variant of this unit was known as available electron (*40*). By definition, energy yield of the product is a fraction of the total amount of substrate redoxons (available electrons) incorporated into the product.

The value of η_{CA} was calculated using elementary composition of CA and glycerol:

$$\eta_{CA}=(\gamma_{CA}\delta_{CA})/(\gamma_S\delta_S)Y_{CA} \qquad /5/$$

where δ_S and δ_{CA} are mass fractions of carbon in glycerol (S) and citric acid (CA), respectively; γ_S and γ_{CA} are reductance degree, *i.e.* the number of redoxons per 1 carbon atom of glycerol (S) and citric acid (CA), respectively.

For the substance (individual compound or a mixture) having the elementary composition of $CH_pO_nN_q$, the reductance degree (γ) was calculated as follows:

$$\gamma=4+p-2n-3q \qquad /6/$$

where 4 and p are the numbers of redoxons of the carbon and hydrogen atoms, respectively; n and q are the numbers of redoxons that lost their energy when they were bound up with oxygen and nitrogen atoms, respectively (*40*). The elementary composition of CA is $C_6H_8O_7$ or $CH_{8/6}O_{7/6}$ after the calculation per 1 carbon atom, from which the reductance degree is:

$$\gamma_{CA}=4+1.333-(2\cdot1.167)=2.999 \qquad /7/$$

The mass fraction of carbon in the molecule of CA (δ_{CA}) is 0.375. Therefore, the value of $\gamma_{CA}\delta_{CA}$ is 1.125. Correspondingly, the value of $\gamma_S\delta_S$ for glycerol is 1.822. Thus, the energy yield of citric acid from glycerol can be calculated as follows:

$$\eta_{CA}=(1.125/1.822)\cdot Y_{CA} \qquad /8/$$

The value of η_{CA} was 0. 432 for *Y. lipolytica* N15. To compare, the maximum η_{CA} value calculated on the basis of the data published in literature was 0.44 for glucose-grown yeasts (*25*) and 0.41 for the rapeseed oil-grown yeasts (*33*).

Energy capacity of the compounds (Q) can be calculated from their elemental composition, since the heat evolved per equivalent of available electrons transferred to oxygen was shown to be about 112.97 kJ (27 kcal) (*39*). The calculated energy capacities of glucose, glycerol, ethanol, and hydrocarbons were about 15, 17, 29 and 48 kJ/g, respectively. Thus, it can be stated that the maximum theoretically possible mass yield of CA (Y_{CA}) from glycerol should be almost similar to that from glucose and lower than that from ethanol and rapeseed oil by 0.6- and 2.8-fold, respectively.

Citric acid production by Y. lipolytica grown on glycerol-containing waste from biodiesel industry

Both strains showed good growth on the glycerol-containing waste from biodiesel industry, which indicates that this substrate contains easily available nutrients (Fig. 3). However, the CA production on crude glycerol differed considerably from that on pure glycerol. In 144 h, *Y. lipolytica* N15 produced only 71 g/L of CA, and 5.6 g/L of ICA, so that the CA/ICA ratio of 12.7:1 was lower than with pure glycerol. Parental strain, *Y. lipolytica* VKM Y-2373, produced rather high concentration of ICA (46.6 g/L), which was likely due to the contamination of crude glycerol by oil residues and fatty acids. Wild-type strains secrete approx. 35–50 % ICA on triglycerides, ethanol or acetate (*19*,*20*,*26*,*30–34*), the concentrations of ICA in the

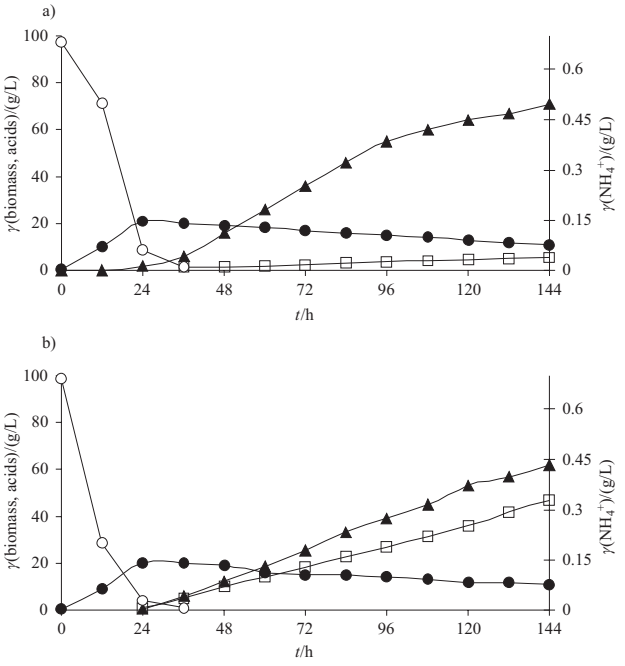

Fig. 3. Time courses of (●) growth, (○) ammonium consumption, (▲) CA and (□) ICA production by (a) *Y. lipolytica* N15 and (b) *Y. lipolytica* VKM Y-2373, grown on glycerol-containing waste from biodiesel industry

described reports were 70 g/L. Recently, Heretsch *et al.* (*49*) have described a large-scale ICA production (93 g/L) from sunflower oil by *Y. lipolytica* and demonstrated an application of ICA as a useful chiral building block for chemical synthesis. Also, monopotassium salt of ICA has been used in several biochemical analyses (assays of aconitate hydratase, NAD-isocitrate dehydrogenase, NADP-isocitrate dehydrogenase, isocitrate lyase); the perspectives of ICA as alimentary additive have also been considered (*32*,*49*). It should be noted that ICA is not widely used as compared to CA, because it is not easily isolated from plant tissue. Sigma Company produces isocitrate in small amounts at high price (250–280 €/g).

The volumetric citric acid productivity (Q_{CA}) of 0.89 g/(L·h) and the specific citric acid production rate (q_{CA}) of 0.058 g per g of cell per h for *Y. lipolytica* N15 were lower than on pure glycerol, but the mass yield (Y_{CA}) of 0.90 g of CA per g of glycerol and the energy yield coefficient of 0.56 were higher than on pure glycerol, probably due to the contamination of crude glycerol with oil residue and fatty acids.

The biomass composition of CA-producer cultivated on crude glycerol during the growth phase is given in Table 4. In the growth phase, crude glycerol was essentially converted into protein and lipids (30.4 and 29.9 % of dry biomass, respectively). The transition of the culture to the stationary phase initiated by nitrogen limitation of cell growth was accompanied by a decrease in the protein amount (to 21.9 %) and by a 2.9-fold reduction of lipid content; the intracellular content of carbon decreased from 55.7 to 45.6 % of dry biomass, the oxygen level increased from 22.6 to 30.1 % of dry biomass, whereas hydrogen content of biomass changed insignificantly during the cultivation period. A decrease in lipid

Table 4. Biomass composition of *Y. lipolytica* N15 grown on glycerol-containing waste from biodiesel industry

Parameters	Growth phase	CA production phase
Component (% of dry biomass)		
protein	30.4	21.9
lipid	29.9	10.3
carbon	55.7	45.6
hydrogen	8.2	7.9
nitrogen	6.2	3.6
oxygen	22.6	30.1
biomass energy (kJ/g)	25.5	21.0
Fatty acid composition (% of lipid)		
C16:0	7.3	4.6
C16:1	1.8	9.0
C17:0	0	0
C17:1	traces	traces
C18:0	1.2	0.3
C18:1	61.8	56.8
C18:2	24.8	24.3
C18:3	4.2	5.1
C20:0	traces	traces
C20:1	traces	traces
C16:1/C16:0	0.2	2.0
C18:1/C18:0	51.5	189.3
C18:2/C18:1	0.4	0.4

Analyses were performed in duplicate for each experiment which varied by no more than 10 %

content of biomass in the citric acid-production phase is not surprising since lipid synthesis and intensive CA production are processes competitive for acetyl-CoA.

It should be noted that the intracellular amount of nitrogen decreased from 6.2 % of dry biomass in the growth phase to 3.6 % of dry biomass in the acid-production phase (Table 4), showing the importance of both nitrogen limitation and a balance between nitrogen concentration and other nutrients for the optimum citrate excretion by the yeast. Similar data on the decrease of nitrogen content in *Saccharomycopsis lipolytica* D1805 biomass during yeast transition to the stationary phase (from 8.5 in the trophophase to 4 % at the end of exponential phase) were reported by Briffaud and Engasser (*23*). Moresi (*24*) revealed a reduction in intracellular nitrogen content from 7–8 to 2.3–4.4 % in *Y. lipolytica* ATCC 20346. There are also data on the importance of nitrogen limitation in *Candida oleophila* ATCC 20177 growth for CA production; the optimum concentration of NH_4^+ was found to be 1.2 mg/g (*21*).

Under cell growth limitation by nitrogen, the biomass energy decreased (from 25.5 to 21 kJ/g) (Table 4), which can be explained by a correlation between the biomass energy and the amount of lipids, the most energy-rich component of the cells, which essentially decreased in this case.

Lipids of *Y. lipolytica* N15 were represented mainly by oleic ($^{\Delta 9}$C18:1), linoleic ($^{\Delta 9,12}$C18:2), palmitic (C16:0) and palmitoleic ($^{\Delta 9}$C16:1) acids (Table 4). The transition of yeast to the citric acid-formation phase resulted in a significant alteration of the fatty acid composition: the content of oleic ($^{\Delta 9}$C18:1) and palmitic (C16:0) acids decreased from 61.8 to 56.8 % and from 7.23 to 4.57 %, respectively, whereas the amount of palmitoleic acid ($^{\Delta 9}$C16:1) increased from 1.8 to 9.03 %. The amount of linoleic acid ($^{\Delta 9,12}$C18:2) did not markedly change. The fatty acid desaturase activity during yeast cultivation was estimated by calculating the ratios between the desaturase product and substrate (C16:1/C16:0; C18:1/C18:0; C18:2/C18:1). High values of a C18:1/C18:0 ratio revealed in all variants are indicative of high activity of Δ9-desaturase, especially in the citric acid production phase.

Conclusions

To conclude, the results of the efficient CA production by glycerol-grown yeast *Y. lipolytica* obtained in the present work and the results by other authors listed in Table 5 confirm that the application of yeasts has a considerable promise for the industrial CA production. Traditional producer *A. niger* can produce CA from molas-

Table 5. Comparison of the present results with literature values regarding CA production by yeasts

Strain	Substrate	Type of cultivation	Y_{CA} g/g	CA productivity Q_{CA} g/(L·h)	CA productivity q_{CA} g/(g·h)	γ(CA)/γ(ICA)	Reference
Y. lipolytica NRRL YB-423	pure glycerol	flask	0.54	–	–	11.3:1	*14*
Y. lipolytica ACA-DC 50109	crude glycerol	flask	0.56	–	0.014	n.d.	*28*
Y. lipolytica Wratislavia 1.31	crude glycerol	batch	0.62	–	0.05	n.d.	*15*
Y. lipolytica Wratislavia K1	pure glycerol	fed-batch	0.45	1.00	0.05	33:1	*8*
Y. lipolytica Wratislavia K1	crude glycerol	fed-batch	0.43	0.99	0.045	33:1	*8*
Y. lipolytica Wratislavia AWG7	pure glycerol	fed-batch	0.69	1.16	0.06	38:1	*8*
Y. lipolytica Wratislavia AWG7	crude glycerol	fed-batch	0.66	1.05	0.06	29:1	*8*
Y. lipolytica N15	pure glycerol	batch	0.70	1.14	0.076	30:1	present study
Y. lipolytica N15	crude glycerol	batch	0.90	0.89	0.058	12.7:1	present study

n.d.=not determined

ses with the volumetric productivity of the process of 0.8 g/(L·h) and Y_{CA} of 0.9 g of CA per g of molasses (*50,51*), while the mutant *Y. lipolytica* N15 produced CA from crude glycerol with productivity of 0.89 g/(L·h) and Y_{CA} of 0.9 g of CA per g of glycerol. The use of yeasts instead of moulds for CA production also represents a novel approach, since the traditional production of CA by using *A. niger* is associated with the accumulation of significant amounts of solid and liquid wastes. Moreover, yeasts are characterized by greater resistance to high substrate concentrations than fungi, with comparable conversion rates and have greater tolerance to metal ions, which allows the use of less refined substrates.

Acknowledgements

The authors are grateful to Dr A.M. Zyakun and Miss A.A. Odinokova (Institute of Biochemistry and Physiology of Microorganisms, Pushchino, Russia) for the determination of C, H and N content of biomass of citrate-producing strains. This work was supported by the Russian Academy of Sciences and Russian Foundation for Basic Research (grant No. 11-04-01593-a).

References

1. S. Papanikolaou, S. Fakas, M. Fick, I. Chevalot, M. Galiotou-Panayotou, M. Komaitis *et al.*, Biotechnological valorisation of raw glycerol discharged after bio-diesel (fatty acid methyl-esters) manufacturing process: Production of 1,3-propanediol, citric acid and single oil, *Biomass Bioeng. 32* (2008) 60–71.
2. S. Papanikolaou, G. Aggelis, Modelling aspects of the biotechnological valorization of raw glycerol: Production of citric acid by *Yarrowia lipolytica* and 1,3-propanediol by *Clostridium butyricum*, *J. Chem. Tech. Biotechnol. 78* (2003) 542–547.
3. P.A.E.P. Meesters, G.N.M. Huijberts, G. Eggink, High-cell-density cultivation of the lipid accumulating yeast *Cryptococcus curvatus* using glycerol as a carbon source, *Appl. Microbiol. Biotechnol. 45* (1996) 575–579.
4. S. Papanikolaou, G. Aggelis, Lipid production by *Yarrowia lipolytica* growing on industrial glycerol in a single-stage continuous culture, *Bioresour. Tech. 82* (2002) 43–49.
5. D.T. Johnson, K.A. Taconi, The glycerin glut: Options for the value-added conversion of crude glycerol resulting from biodiesel production, *Environ. Prog. 26* (2007) 338–348.
6. Z. Chi, D. Pyle, Z. Wen, C. Frear, S. Chen, A laboratory study of producing docosahexaenoic acid from biodiesel-waste glycerol by microalgal fermenation, *Process Biochem. 42* (2007) 1537–1545.
7. F. Mantzouridou, E. Naziri, M.Z. Tsimidou, Industrial glycerol as a supplementary carbon source in the production of β-carotene by *Blakeslea trispora*, *J. Agric. Food Chem. 56* (2008) 2668–2675.
8. A. Rywińska, W. Rymowicz, B. Zarowska, M. Wojtatowicz, Biosynthesis of citric acid from glycerol by acetate mutants of *Yarrowia lipolytica* in fed-batch fermentation, *Food Technol. Biotechnol. 47* (2009) 1–6.
9. A. André, A. Chatzifragkou, P. Diamantopoulou, D. Sarris, A. Philippoussis, M. Galiotou-Panayotou *et al.*, Biotechnological conversions of bio-diesel-derived crude glycerol by *Yarrowia lipolytica* strains, *Eng. Life Sci. 9* (2009) 468–478.
10. D. Rittmann, S.N. Lindner, V.F. Wendisch, Engineering of a glycerol utilization pathway for amino acid production by *Corynebacterium glutamicum*, *Appl. Environ. Microbiol. 74* (2008) 6216–6222.
11. P.C. Lee, W.G. Lee, S.Y. Lee, H.N. Chang, Succinic acid production with reduced by-product formation in the fermentation of *Anaerobiospirillum succiniciproducens* using glycerol as a carbon source, *Biotechnol. Bioeng. 72* (2000) 41–48.
12. S.B. Imandi, V.V.R. Bandaru, S.R. Solomanka, H.R. Garapati, Optimization of medium constituents for the production of citric acid from byproduct glycerol using Doehlert experimental design, *Enzyme Microb. Technol. 40* (2007) 1367–1372.
13. T.V. Finogenova, N.V. Shishkanova, I.T. Ermakova, I.A. Kataeva, Properties of *Candida lipolytica* mutants with the modified glyoxylate cycle and their ability to produce citric and isocitric acid. II. Synthesis of citric and isocitric acid by *C. lipolytica* mutants and peculiarities of their enzyme systems, *Appl. Microbiol. Biotechnol. 23* (1986) 378–383.
14. W.E. Levinson, C.P. Kurtzman, T.M. Kuo, Characterization of *Yarrowia lipolytica* and related species for citric acid production from glycerol, *Enzyme Microb. Technol. 41* (2007) 292–295.
15. W. Rymowicz, A. Rywińska, B. Żarowska, P. Juszczyk, Citric acid production from raw glycerol by acetate mutants of *Yarrowia lipolytica*, *Chem. Pap. 60* (2006) 391–395.
16. A. Rywińska, W. Rymowicz, High-yield production of citric acid by *Yarrowia lipolytica* on glycerol in repeated-batch bioreactors, *J. Ind. Microbiol. Biotechnol. 37* (2010) 431–435.
17. S. Anastassiadis, I.G. Morgunov, S.V. Kamzolova, T.V. Finogenova, Citric acid production patent review, *Recent Pat. Biotechnol. 2* (2008) 107–123.
18. A.B. Lozinov, L.M. Glazunova, I.T. Ermakova, The activity of citrate glyoxylate and pentosephosphate cycle enzymes during yeast growth on hexadecane and glucose, *Microbiology* (Moscow), *45* (1976) 33–40.
19. I.T. Ermakova, N.V. Shishkanova, O.F. Melnikova, T.V. Finogenova, Properties of *Candida lipolytica* mutants with the modified glyoxylate cycle and their ability to produce citric and isocitric acid. I. Physiological, biochemical and cytological characteristics of mutants grown on glucose or hexadecane, *Appl. Microbiol. Biotechnol. 23* (1986) 372–377.
20. U. Stottmeister, U. Behrens, E. Weissbrodt, G. Barth, D. Franke-Rinker, E. Schulze, Utilization of paraffins and other non-carbohydrate carbon sources for microbial citric acid synthesis, *Z. Allg. Mikrobiol. 22* (1982) 399–424 (in German).
21. S. Anastassiadis, A. Aivasidis, C. Wandrey, Citric acid production by *Candida* strains under intracellular nitrogen limitation, *Appl. Microbiol. Biotechnol. 60* (2002) 81–87.
22. U. Behrens, A. Thierrsch, E. Weissbrodt, U. Stottmeister, Particularities in the kinetics of growth and citric-acid accumulation by *Saccharomycopsis lipolytica*, *Acta Biotechnol. 7* (1987) 179–183.
23. J. Briffaud, M. Engasser, Citric acid production from glucose. I. Growth and excretion kinetics in a stirred fermentor, *Biotechnol. Bioeng. 21* (1979) 2083–2092.
24. M. Moresi, Effect of glucose concentration on citric acid production by *Yarrowia lipolytica*, *J. Chem. Tech. Biotechnol. 60* (1994) 387–395.
25. M. Wojtatowicz, W. Rymowicz, H. Kautola, Comparison of different strains of the yeast *Yarrowia lipolytica* for citric acid production from glucose hydrol, *Appl. Biochem. Biotechnol. 31* (1991) 165–174.
26. T.V. Finogenova, N.V. Shishkanova, E.A. Fausek, S.S. Eremina, Biosynthesis of isocitric acid from ethanol by yeasts, *Appl. Microbiol. Cell Phys. 36* (1991) 231–235.
27. T.V. Finogenova, S.V. Kamzolova, E.G. Dedyukhina, N.V. Shishkanova, A.P. Il'chenko, I.G. Morgunov *et al.*, Biosynthesis of citric and isocitric acids from ethanol by mutant *Yarrowia lipolytica* N 1 under continuous cultivation, *Appl. Microbiol. Biotechnol. 59* (2002) 493–500.

28. S.V. Kamzolova, N.V. Shishkanova, I.G. Morgunov, T.V. Finogenova, Oxygen requirements for growth and citric acid production of *Yarrowia lipolytica*, *FEMS Yeast Res. 3* (2003) 217–222.

29. D.W. Good, R. Droniuk, R.G. Lawford, J.E. Fein, Isolation and characterization of a *Saccharomycopsis lipolytica* mutant showing increased production of citric acid from canola oil, *Can. J. Microbiol. 31* (1985) 436–440.

30. P. Fickers, P.H. Benetti, Y. Waché, A. Marty, S. Mauersberger, M.S. Smit, J.M. Nicaud, Hydrophobic substrate utilisation by the yeast *Yarrowia lipolytica*, and its potential applications, *FEMS Yeast Res. 5* (2005) 527–543.

31. A. Förster, K. Jacobs, T. Juretzek, S. Mauersberger, G. Barth, Overexpression of the ICL1 gene changes the product ration of citric acid production by *Yarrowia lipolytica*, *Appl. Microbiol. Biotechnol. 77* (2007) 681–689.

32. M. Holz, A. Förster, S. Mauersberger, G. Barth, Aconitase overexpression changes the product ratio of citric acid production by *Yarrowia lipolytica*, *Appl. Microbiol. Biotechnol. 81* (2009) 1087–1096.

33. S.V. Kamzolova, I.G. Morgunov, A. Aurich, O.A. Perevoznikova, N.V. Shishkanova, U. Stottmeister, T.V. Finogenova, Lipase secretion and citric acid production in *Yarrowia lipolytica* yeast grown on animal and vegetable fat, *Food Technol. Biotechnol. 43* (2005) 113–122.

34. A. Förster, A. Aurich, S. Mauersberger, G. Barth, Citric acid production from sucrose using a recombinant strain of the yeast *Yarrowia lipolytica*, *Appl. Microbiol. Biotechnol. 75* (2007) 1409–1417.

35. T.V. Finogenova, I.F. Puntus, S.V. Kamzolova, I.N. Lunina, S.E. Monastyrskaia, I.G. Morgunov, A.M. Boronin, Obtaining of the mutant *Yarrowia lipolytica* strain producing citric acid from glucose, *Prikl. Biokhim. Mikrobiol. 44* (2008) 219–224 (in Russian).

36. P.R. Burkholder, J. McVeigh, D. Moyer, Studies on some growth factors of yeasts, *J. Bacteriol. 48* (1944) 385–391.

37. Y.A. Sultanovich, A.P. Nechaev, I.A. Barsukova, Method for the quantitative determination of fatty acid composition of microbial lipids. *USSR Inventor's Certificate No. 968072*, *Byull. Izobret. 39* (1982) 136.

38. I.G. Minkevich, Mass-energy balance for microbial product synthesis – Biochemical and cultural aspects, *Biotechnol. Bioeng. 25* (1983) 1267–1293.

39. I.G. Minkevich, V.K. Eroshin, Productivity and heat generation of fermentation under oxygen limitation, *Folia Microbiol. 18* (1973) 376–385.

40. L.E. Erickson, I.G. Minkevich, V.K. Eroshin, Application of mass and energy balance regularities in fermentation, *Biotechnol. Bioeng. 67* (2000) 748–774.

41. K.D. Rane, K.A. Sims, Citric acid production by *Candida lipolytica* Y-1095 in cell recycle and fed-batch fermentors, *Biotechnol. Bioeng. 46* (1995) 325–332.

42. U. Stottmeister, U. Behrens, W. Göhler, Effect of oxygen partial pressure on citric acid synthesis in *Saccharomycopsis lipolytica* using *n*-alkanes, *Z. Allg. Mikrobiol. 21* (1981) 677–687 (in German).

43. M. Matsumura, J. Kobayashi, Effect of high oxygen concentration on the growth of an ethanol assimilating yeast, *Candida* sp., *J. Ferment. Technol. 58* (1980) 525–532.

44. I.G. Morgunov, S.V. Kamzolova, O.A. Perevoznikova, N.V. Shishkanova, T.V. Finogenova, Pyruvic acid production by a thiamine auxotroph of *Yarrowia lipolytica*, *Process Biochem. 39* (2004) 1469–1474.

45. G. Mothes, C. Schnorpfeil, J.U. Ackermann, Production of PHB from crude glycerol, *Eng. Life Sci. 7* (2007) 475–479.

46. W. Rymowicz, A. Rywińska, M. Marcinkiewicz, High-yield production of erythritol from raw glycerol in fed-batch cultures of *Yarrowia lipolytica*, *Biotechnol. Lett. 31* (2009) 377–380.

47. M. Wojtatowicz, G.L. Marchin, L.E. Erickson, Attempts to improve strain A-101 of *Yarrowia lipolytica* for citric acid production from *n*-paraffins, *Process Biochem. 28* (1993) 453–460.

48. S.V. Kamzolova, T.V. Finogenova, I.G. Morgunov, Microbiological production of citric and isocitric acids from sunflower oil, *Food Technol. Biotechnol. 46* (2008) 51–59.

49. P. Heretsch, F. Thomas, A. Aurich, H. Krautscheid, D. Sicker, A. Giannis, Syntheses with a chiral building block from the citric acid cycle: (2*R*,3*S*)-Isocitric acid by fermentation of sunflower oil, *Angew. Chem. Int. Ed. Engl. 47* (2008) 1958–1960.

50. J.L. Meers, P.E. Milsom: Organic Acids and Amino Acids. In: *Basic Biotechnology*, J. Bu'Lock, B. Kristiansen (Eds.), Academic Press, Orlando, FL, USA (1987) pp. 359–383.

51. M. Röhr, C.P. Kubicek, Regulatory aspects of citric acid fermentation by *Aspergillus niger*, *Process Biochem. 16* (1981) 34–37.