Strain and process development for citric acid production from glycerol-containing waste of biodiesel manufacture

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In the present work, the possibility of using glycerol and glycerol-containing waste from biodiesel manufacture as a carbon and energy source for microbiological production of citric acid was studied. Acid-formation ability on selective media was preliminary tested for 66 yeast strains of different genera (*Candida, Pichia*, *Saccharomyces*, *Torulopsis,* and *Yarrowia*). Under growth limitation by nitrogen source, 41 strains belonging mainly to the species *Yarrowia lipolytica* were found to excrete organic acids to the culture broth. In contrast, 25 strains of the genera *Debaryomyces*, *Candida, Pichia*, *Saccharomyces* and *Torulopsis* did not show acid excretion. Among the 41 acid-producing strains, the wild strain *Y. lipolytica* VKM Y-2373 and the mutant strain *Y. lipolytica* N 15 exhibited the highest ability to accumulate citric acid in the culture broth. The production of citric acid by these two strains was studied as a function of the medium pH, degree of aeration and the concentration of glycerol in the medium. Under optimal conditions, the best acid producer *Y. lipolytica* N 15 accumulated up to 98 and 71 g/L citric acid in the culture broth when grown in the media supplemented with glycerol and glycerol-containing waste, respectively.

Keywords *Yarrowia lipolytica*; citric acid production; biodiesel production; glycerol; glycerol-containing waste

1. Background

Citric acid (CA) has attracted increased interest in recent years. The consumption of CA and its salt, trisodium citrate, reached 1 400,000 tons per year, with an annual increment of about 5% [1]. CA is widely used in the food and beverage industries as a flavoring and preservative (~70%), in the production of washing powders (~20%), in the chemical and pharmaceutical industries (~10%). As a food additive, CA is denoted as E330. Citrate salts of various metal ions are used to make those ions biologically available in many dietary supplements. The buffering properties of citrates are used to control pH in household cleaners and pharmaceuticals. The ability of CA to chelate metals makes it useful in soaps and laundry detergents. By chelating bivalent metals in hard water, CA improves foam formation and makes unnecessary water softening. CA is also used for the regeneration of ion exchange materials used in water softeners due to the stripping off of accumulated metal ions as citrate complexes.

Usually, CA is produced on a commercial scale from molasses, sucrose or glucose by using various strains of fungi, mostly belonging to the species *Aspergillus niger*. It should be noted that the production of CA by fungi is associated with the accumulation of solid and liquid wastes in significant amounts.

Alternatively, CA can be produced by some yeast species. It is known that the species *Yarrowia* (syn. *Candida*, *Saccharomycopsis*, *Endomycopsis*) *lipolytica*, *C. guilliermondii* and *C. oleophila* are able to accumulate in the medium some tricarboxylic acid cycle (TCA) intermediates, such as CA and *threo*-Ds-isocitric acid (ICA). The range of substrates suitable for the formation of organic acids by the yeast species is wider than in the case of fungi. Namely, the aforementioned yeasts can utilize *n*-alkanes, glucose, ethanol and vegetable oils [1]. The transformation of these substrates was studied in batch and continuous cultures and successfully simulated in terms of numerical models. These studies showed that the yeasts are more tolerant to high substrate concentrations and metal ions than the fungi, thus allowing the use of relatively crude substrates for process cultivation.

The biochemical pathways involved in the production of CA and ICA by the yeasts is rather well studied. It is known that CA and ICA usually accumulate in the culture broth under the limitation of yeast growth by nitrogen or some other elements, such as sulphur, phosphorus, and magnesium [1]. It has been reported that the ratio of the accumulated CA to its by-product, ICA, depends on the carbon source used. For example, in the case of glucose-containing media, this ratio is very high. At the same time, CA and ICA accumulate in equal amounts in the medium with n-alkanes as the carbon source.

Recently, glycerol and glycerol-containing materials became of great practical interest for various microbial transformations. Why? In the 20th century, petroleum hydrocarbons were considered to be the main source of carbon and energy for microbial biotechnology due to their relatively low cost. However, the ever-increasing cost of crude oil beginning with the 1973 oil crisis and the deterioration of global ecological situation in recent years forced researchers to pay more attention to alternative energy sources, such as biodiesel. From 2002 to 2006, the production of biodiesel increased by approximately 40% every year. In 2006, the world biodiesel production reached 5-6 million tonnes (4.9 million tonnes in Europe and the rest mainly in the USA). In 2007 and 2008, the production of biodiesel in Europe

reached 5.7 and 16 million tonnes, respectively. For comparison, the total demand of diesel fuel in the USA and Europe is approximately 490 million tonnes. In 2010, the European Community issued a directive (EU directive 2003/30/EC) 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 triglycerides of oils and fats are first hydrolyzed and then methylated with the formation of methyl fatty acids, which are just used as biodiesel. The major waste of this technological process is glycerol; for every 1 tonne of biodiesel manufactured, 100 kg of glycerol is produced as the by-product. In the past, there was a good market for this glycerol. However, with the increase in the global biodiesel production, the market price for waste glycerol, which contains 20% water and catalyst residues, dramatically decreased. For this reason, researches have been initiated in order to use waste glycerol as raw material for chemical synthesis.

One of the initiatives is The Glycerol Challenge in the UK. Crude glycerol is routinely purified by vacuum distillation. The refined glycerol (98% pure) can be utilized either directly or after conversion to other products. The following announcements were made in 2007: A joint venture of Ashland Inc. and Cargill made announcement to manufacture propylene glycol from glycerol in Europe; Dow Chemical made a similar announcement to produce propylene glycol from glycerol in North America and epichlorhydrin in China (epichlorhydrin is a raw material for the manufacture of epoxy resins).

Some efforts were made for the microbiological conversion of technological glycerol (crude glycerol) into valuable products: 1,3-propanediol, microbial biomass and lipids [2], food pigments [3], erythritol, mannitol [4], L-lysine [5], organic acids, in particular, succinic acid [6] and citric acid [2,4,7,8].

The goal of the present work was (1) to study the production of citric acid from pure glycerol and the glycerol-containing waste of biodiesel industry by representatives of different yeast genera (*Debaromyces*, *Candida*, *Pichia*, *Saccharomyces, Yarrowia* and *Torulopsis*); (2) to estimate the effect of cultivation conditions (the medium pH, oxygen supply, and the concentration of carbon substrate) on the production of CA by the selected strain; and (3) to develop a suitable process for the CA production.

2. Selection of citric-acid-producing yeast strains

CA producers were selected by screening 59 naturally occurring yeast strains from the genera *Debaryomyces, Candida*, *Pichia, Saccharomyces, Yarrowia* and *Torulopsis*, as well as 7 mutant strains with impaired ability to grow on acetate probably due to defects in the tricarboxylic acid cycle. The strains were obtained from the All-Russia 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). Based on the finding that nitrogen deficiency stimulates the formation of CA from glycerol by yeast strains [2,4,7,8], we used for screening the following medium and cultivation conditions: the medium contained glycerol in an excessive amount and nitrogen in a growth-limiting concentration; nitrogen was added to the medium as yeast autolysate (that is, in an organic form). The medium contained glycerol 20 g/L, $MgSO_4 \cdot 7H_2O$ 0.7 g/L, $Ca(NO_3)_2$ 0.4 g/L, NaCl 0.5 g/L, KH_2PO_4 1.0 g/L, K_2HPO_4 0.1 g/L, Burkholder's trace element solution with slight modifications (mg/L): I^{-1} 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 мл/L (as a source of nitrogen and vitamins), and 20.0 g/L Bacto agar. Powdered calcium carbonate ($CaCO_3$) was added in an amount of 6 g/L to the liquid agar immediately before its pouring into the dishes. The yeast strains were plated onto solidified agar media and incubated at $(28\pm1)^oC$ for 7 days.

The production of acids was evaluated by measuring the diameter of the $CaCO_3$ dissolution zones appeared around the yeast colonies grown on the $CaCO_3$-containing agar at 28^oC for 6 days. The results are listed in Table 1. All the strains under study were found to be able to grow on glycerol. Under the condition of growth limitation by nitrogen, acid production was observed for 41 strains (belonging mainly to the 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 $CaCO_3$ 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); 7 strains formed zones greater than 8 mm.

It should be noted that our test for acid formation on agar media cannot distinguish CA and other metabolite - *threo*-Ds-isocitric acid (ICA). To identify the excreted acids and verify the results of producer selection on agar media, the most active mutant strains *Y. lipolytica* UV-5, *Y. lipolytica* NG-40, *Y. lipolytica* NG-80, *Y. lipolytica* N 11, *Y. lipolytica* N 13, and *Y. lipolytica* N 15, which formed the largest zones of $CaCO_3$ dissolution, were further tested for their acid-producing ability in 750-mL flasks with 50 mL of the nitrogen-deficient liquid medium containing $(NH_4)_2SO_4$ 0.3 g/L, $MgSO_4 \cdot 7H_2O$ 0.7 g/L, $Ca(NO_3)_2$ 0.4 g/L, NaCl 0.5 g/L, KH_2PO_4 1.0 g/L, K_2HPO_4 0.1 g/L, Burkholder's trace element solution, "Difco" yeast extract 0.5 g/L, and glycerol 30 g/L. Taking into account the fact that yeast growth decreases the pH of the medium, it was maintained at a level of 4.5-5.5 by the periodical addition of 10% NaOH.

Biomass, CA, ICA and residual glycerol contents were determined after 144 h of cultivation.

Yeast growth was monitored by measuring the absorbance of the culture at 540 nm with a Spekol 221 spectrophotometer (Carl Zeiss, Jena, Germany). The cell biomass was estimated from the absorbance of the cell suspension using a calibration curve. The concentration of ammonium was determined potentiometrically with an

Ecotest-120 ionometer (Econix, Russia) using an Ekom-NH_4 electrode (Econix, Russia). Glycerol was analysed enzymatically using a biochemical kit purchased from Boehringer Mannheim/R-Biopharm, Germany. The determination of glycerol was based on the measurement of NADH produced during the conversion of glycerol to l-lactate in the coupled reactions catalyzed by glycerol kinase, pyruvate kinase and l-lactate dehydrogenase. The concentration of organic acids was determined using an HPLC chromatograph (LKB, Sweden) equipped with an Inertsil ODS-3 reversed-phase column (250 x 4 mm, Elsiko, Russia) at 210 nm. 20 mM phosphoric acid was used as the mobile phase with a flow rate of 1.0 mL/min; the column temperature was maintained at 35^{o}C. CA and ICA were identified using standard solutions purchased from Boehringer Mannheim, Germany. In addition, the diagnostic kits from Roche Diagnostics GmbH, Germany, were used for the assay of CA and ICA. The determination of CA was based on the measurement of the amount of NADH produced during the conversion of CA to oxaloacetate and its decarboxylation product pyruvate, and the following conversion to l-malate and l-lactate. The reactions are catalysed by citrate lyase, malate dehydrogenase and l-lactate dehydrogenase. The determination of ICA was based on the measurement of NADPH produced during the conversion of ICA to α-ketoglutarate catalysed by isocitrate dehydrogenase.

As seen from Table 2, all the strains under study excreted predominantly CA (the CA-to-ICA ratio varied from 2.6 to 14.8). The absolute amounts of CA or ICA accumulated in the medium and their ratios were strain-dependent. The highest proportion of CA to ICA (11.2:1) and the largest acid yield (0.55 g/g) were observed in the mutant strain *Y. lipolytica* N 15. As evident from Table 1, *Y. lipolytica* N 15 formed the largest zone of $CaCO_3$ dissolution around the colonies. For this reason, the mutant strain *Y. lipolytica* N 15 was selected for further studies as the promising producer of CA from glycerol.

Table 1 Acid-producing ability of various yeast strains on agar medium supplemented with powdered CaCO3

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. rugoza 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* N 11	9
Y. lipolytica VKM Y-47	0	*Y. lipolytica* 645	4	*Y. lipolytica* N 13	10
Y. lipolytica 68	0	*Y. lipolytica* 646	1	*Y. lipolytica* N 15	11

Note: "D (mm)" stands for the diameter of the $CaCO_3$ dissolution zone

Table 2 The production of CA and ICA by the mutant Yarrowia lipolytica strains grown on glycerol

Strain	Biomass (g/L)	CA (g/l)	ICA (g/l)	CA:ICA	Y_{CA} (%)
Y. lipolytica UV-5	3.59	19.73	2.14	9.2:1	49
Y. lipolytica NG-40	3.94	16.60	3.49	4.8:1	42
Y. lipolytica NG-80	3.78	15.44	3.84	4.0:1	39
Y. lipolytica N 11	3.10	16.64	2.65	6.2:1	42
Y. lipolytica N 13	3.45	16.04	3.32	4.8:1	40
Y. lipolytica N 15	3.39	19.08	1.70	11.2:1	55

3. Determination of optimal conditions for the production of CA from glycerol

In further experiments, the citric acid-producing ability of *Y. lipolytica* N 15 was studied as a function of the medium pH, the concentration of dissolved oxygen, and the concentration of glycerol in the cultivation medium.

The strain was grown in a fermentor under nitrogen limitation to the phase of active acid formation (the biomass at that moment was 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 containing 20 g/L glycerol without the source of nitrogen and vitamins. The suspension was incubated on a shaker (180–200 rpm) at 28°C for 22 h. By the end of the experiment, the pH of the medium slightly decreased (by 0.5–0.3 units), the biomass remained at a constant level of 2.5±0.3 g/L, and the cell viability almost did not change. The microscopic examination of cells and the measurement of the extracellular protein content in the incubation medium showed that the cells were not disrupted in all experimental variants.

CA was produced at all the pH values used (from 3.0 to 8.0) and reached a maximum (6.10–6.17 g/L) at pH 4.5–6.0. Beyond this pH interval, the production of CA was lower, being minimal at pH 3.0.

The effect of dissolved oxygen on the CA production was studied at pH 4.5. The degree of aeration was varied by changing the volume of the medium (from 50 to 500 ml) in the 750-mL incubation flasks. CA was excreted to the medium at all the concentrations of dissolved oxygen studied. At the aeration rates of 0.30 and 0.56 mmol O_2/L min, the concentration of excreted CA reached a maximum (6.97 – 7.77 g/L); at the aeration rates of 0.20 and 0.24 mmol O_2/L min, the concentration of CA was 1.5 times lower; and at the aeration rates below 0.2 mmol O_2/L min, CA was almost absent in the medium. Thus, CA was actively produced by the glycerol-grown *Y. lipolytica* cells only under sufficiently high aeration rates of the medium. At low aeration rates, CA did not accumulate. Presumably, oxygen deficiency decreases the activity of some mitochondrial enzymes (citrate synthase, aconitase, malate dehydrogenase, and NADP-dependent isocitrate dehydrogenase) involved in the formation of CA.

The effect of glycerol on the CA synthesis by *Y. lipolytica* N 15 was studied at pH 4.5; the volume of the medium in the cultivation flasks was 50 mL; the glycerol concentration ranged from 20 to 120 g/L. CA accumulated in the medium at all the glycerol concentrations studied. The amount of CA reached a maximum (11 g/L) at glycerol concentrations from 20 to 40 g/L and decreased 2.5 times at glycerol concentrations above 80 g/L.

Thus, the highest CA production by the strain *Y. lipolytica* N 15 grown on glycerol occurs at pH 4.5, high concentrations of dissolved oxygen, and glycerol concentration in the interval of 20 to 40 g/L.

The optimization of cultivation conditions (pH, degree of aeration and the concentration of glycerol) resulted in the improvement of CA production by 45%.

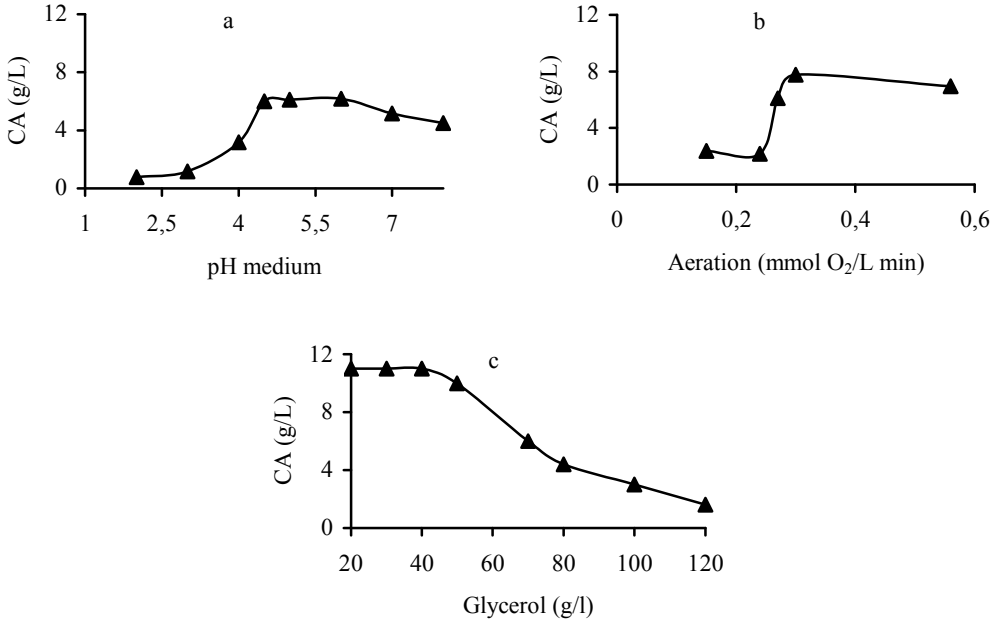

Fig. 1 The effect of the cultivation parameters, pH (a), degree of aeration (b), and glycerol concentration (c), on the CA production by the strain *Y. lipolytica* N 15 grown on glycerol in Erlenmeyer flasks.

4. Citric acid production by the *Y. lipolytica* cells grown on glycerol in fermentor

The yeast strains were cultivated in an ANKUM-2M fermentor (10 L) with 5.0 L of broth containing $(NH_4)_2SO_4$ 3.0 g/L, $MgSO_4$ $7H_2O$ 1.4 g/L, NaCl 0.5 g/L, $Ca(NO_3)_2$ 0.8 g/L, KH_2PO_4 2.0 g/L, K_2HPO_4 0.2 g/L, Burkholder's trace element solution, "Difco" yeast extract 1.0 g/L, thiamine 0.02 g/L, and glycerol 20 g/L. Cultivation parameters were maintained automatically at the following values: temperature - 28°C; pH was adjusted at a level of 4.5 by the addition of 20% NaOH; the concentration of dissolved oxygen (pO_2) was maintained at a level of 60% of the air saturation; the agitation rate was 800 rpm. Glycerol was added in portions when the pO_2 value increased by 5%, indicating a decrease in the respiratory activity of cells due to the total consumption of carbon source.

Figure 2 shows the growth curves of the mutant *Y. lipolytica* N 15 and the parental strain *Y. lipolytica* VKM Y-2373 (for comparison), as well as nitrogen consumption and the concentration of CA and ICA in the cultivation medium with glycerol. Nitrogen was exhausted in the medium after 24 h of cultivation of both strains. The biomass continued to increase for 36 h and reached 20 and 21 g/L for *Y. lipolytica* N 15 and *Y. lipolytica* VKM Y-2373, respectively. Then the yeast cultures went to the stationary phase because of nitrogen exhaustion in the medium. Most CA accumulated in the medium just during the stationary growth phase.

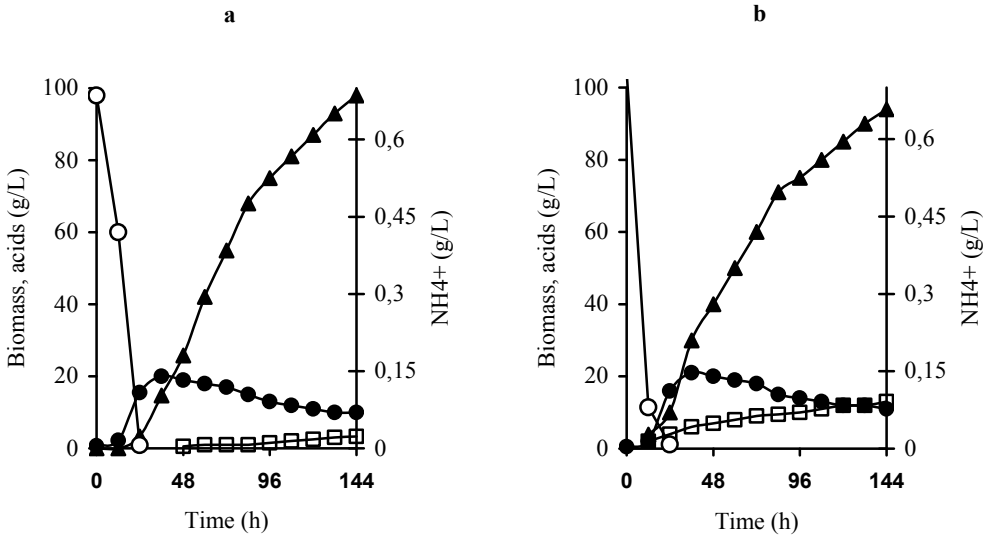

Fig. 2 Dynamics of (●) growth, (o) ammonium consumption, production of (▲) CA and (□) ICA by the *Y. lipolytica* N 15 (a) and *Y. lipolytica* VKM Y-2373 (b) strains grown on glycerol.

By the end of the cultivation period (144 h), *Y. lipolytica* N 15 accumulated 98 g/L CA with an insignificant amount of ICA (3.3 g/L); the CA:ICA ratio was equal to 30. In the case of *Y. lipolytica* VKM Y-2373, CA also mainly accumulated in the stationary growth phase (94 g/L CA by the end of the cultivation period). The disadvantage of the parental strain is that it accumulates a rather high amount of the by-product ICA (13.1 g/L with the CA:ICA ratio equal to 7.5).

To take into account the effect of medium dilution due to the addition of NaOH solution by the pH-controlling system, the total amount of CA in the culture broth was used for calculations of the mass yield of CA (Y_{CA}), the volumetric citric acid productivity (Q_{CA}) and the specific citric acid production rate (q_{CA}).

The mass yield of CA production (Y_{CA}), expressed in g/g, was calculated by the formula:

$$Y_{CA} = \frac{P}{S},$$

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

$$Q_{CA} = \frac{P}{V \cdot t},$$

and the specific citric acid production rate (q_{CA}), expressed in g/g cells h, was calculated by the formula:

$$q_{CA} = \frac{P}{X \cdot t},$$

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

The volumetric citric acid productivity (Q_{CA}) and the specific citric acid production rate (q_{CA}) using *Y. lipolytica* N 15 reached 1.14 g/(L·h) and 0.076 g/g cell h, respectively. These values are close to the best values reported previously for the citrate-producing strains grown on various carbon sources. The mass yields (Y_{CA}) of *Y. lipolytica* N 15 was 0.7 g/g, which is close to the values reported in the literature for other citrate-producing strains (0.41 to 0.77 g/g). The highest values of the CA yield were observed for *n*-hexadecane (1.44 g/g), sucrose (0.82 g/g), ethanol (0.87 g/g) and rapeseed oil (1.55 g/g) [1].

It should be noted, however, that carbon substrates are characterized by different energy capacities and the direct comparison of mass yields for different substrates is inappropriate. It would be more correct to compare the energy yields of citric acid (η_{CA}) for different strains. The parameter η_{CA}, which characterizes the fraction of the energy content of the substrate (glycerol) that is incorporated into CA, was calculated in terms of the mass and energy balance theory [9]. The terms that characterize the mass and energy balance of cell metabolism are based on the generalized unit of reductivity, "redoxon". This term means the electron that can be transferred to oxygen; the former name of this term is "available electron". By definition, the energy yield of the product is the fraction of the total amount of the substrate redoxons (available electrons) that is incorporated into the product.

The parameter η_{CA} was calculated using the chemical composition of CA and glycerol:

$$\eta_{CA} = \frac{\gamma_{CA} \cdot \delta_{CA}}{\gamma_S \cdot \delta_S} \cdot Y_{CA},$$

where δ_S and δ_{CA} are the mass fractions of carbon in glycerol (S) and citric acid (CA), respectively; γ_S and γ_{CA} are the reductance degrees or the number of redoxons per one carbon atom of glycerol (S) and citric acid (CA), respectively.

For the substance (an individual compound or a mixture) having the formula $CH_pO_nN_q$, the reductance degree (γ) can be calculated as follows:

$$\gamma = 4 + p - 2n - 3q,$$

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 loss their energy when they were bound up with the oxygen and nitrogen atoms, respectively. The chemical formula of CA is $C_6H_8O_7$ or $CH_{8/6}O_{7/6}$ after calculation per one carbon atom, from which the reductance degree (γ_{CA}) is 4+1.333–2·1.167=2.999. The mass fraction of carbon in the CA molecule (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 CA from glycerol can be calculated as $(1.125/1.822)\cdot Y_{CA}$; the value of η_{CA} was found to be 0. 432 for *Y. lipolytica* N 15.

To compare, the maximum η_{CA} value calculated from the data available in the literature comprised 0.44 for the glucose-grown yeast cells and 0.41 for the rapeseed oil-grown yeast cells.

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

5. Citric acid production by the *Y. lipolytica* cells grown on the glycerol-containing waste of biodiesel industry

The both strains under study showed good growth on the glycerol-containing waste of biodiesel industry. This fact suggests that this waste contains easily available nutrients (Fig. 3). However, the production of CA from the glycerol-containing waste was lower than from pure glycerol. Indeed, after the cultivation of *Y. lipolytica* N 15 for 144 h, this strain accumulated 71.0 g/L CA and 5.6 g/L ICA, so that the CA:ICA ratio was equal to 12.7. The wild-type strain *Y. lipolytica* VKM Y-2373 produced a higher amount of ICA (46.6 g/L). This fact was likely due to contamination of the glycerol-containing waste by oil residues and some fatty acids. These data are in agreement with the data available in the literature [10] that the wild-type strains grown on triglycerides, ethanol and acetate secrete up to 70 g/L CA and ICA, the percentage of ICA being 35-50%. Recently, Heretsch et al. [11] reported on the production of 93 g/L ICA from sunflower oil by *Y. lipolytica* and demonstrated the possibility of using ICA as a chiral building block for chemical synthesis. The monopotassium salt of *threo*-Ds-(+)-isocitric acid is used in biochemical analyses (assays of aconitate hydratase, NAD-isocitrate dehydrogenase, NADP-dependent isocitrate dehydrogenase and isocitrate lyase); ICA is also considered as a promising food additive. It should be noted that it is difficult to isolate ICA from plant tissues. As a result, isocitrate is rather expensive and used not as widely as CA. For example, Sigma Company produces isocitrate in small amounts and sells it at a high price (250-280 EUR per gram).

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/g cell h for the *Y. lipolytica* N 15 cells grown on the glycerol-containing waste are lower than when grown on

pure glycerol, the situation being reverse with the mass yield (Y_{CA}) of 0.90 g/g and the energy yield coefficient 0.56. These results are likely due to contamination of industrially produced glycerol by oil residue and fatty acids.

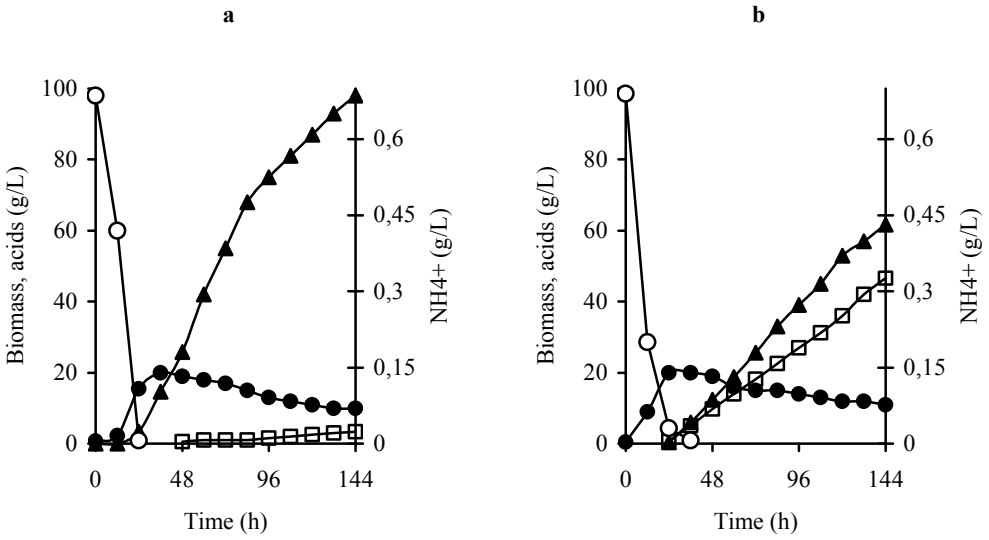

Fig. 3 (●) Growth, (O) ammonium consumption, excretion of (▲) CA and (□) ICA by the *Y. lipolytica* N 15 (a) and *Y. lipolytica* VKM Y-2373 (b) cells grown on the glycerol-containing waste of biodiesel industry.

Table 3. The composition of *Y. lipolytica* N 15 cells grown on the glycerol-containing waste of biodiesel industry

Item	Growth phase	CA production phase
*Content (% 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
Energy content of biomass (kJ/g)	25.5	21.0
*Fatty acid profile (% of total 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

*Protein was determined by the Lowry method; carbon, hydrogen, and nitrogen was measured on a C, H, N analyzer (Carlo Erba Strumentazione); the ash content was determined by burning samples in a muffle furnace. The oxygen content (O) was calculated from: O (%) = 100 %- (C + H + N + Ash), where C, H, N are the contents of carbon, hydrogen, and nitrogen (in %).

**The methyl esters of fatty acids were prepared by the method of Sultanovich *et al.* [12] and analyzed by gas-liquid chromatography on a Chrom-5 chromatograph with a flame-ionization detector. The column (2 m × 3 mm) was packed with 15% Reoplex-400 on Chromaton N-AW (0.16–0.20 mm). The temperature of the column was 200°C. The lipid content of the biomass was determined as the total fatty acid content using docosane ($C_{22}H_{46}$) as the internal standard.

The chemical composition of the yeast cells grown on the glycerol-containing waste is shown in Table 3. In the growth phase, the waste was mainly converted into protein and lipids (30.4 and 29.9% of the dry biomass, respectively). The transition of the culture to the stationary phase caused by nitrogen deficiency was accompanied by a decrease in the protein content (to 21.9%) and by a 2.9-fold reduction in the lipid content. The carbon content of the dry biomass

decreased from 55.7 to 45.6%, the oxygen content increased from 22.6 to 30.1 %, and the hydrogen content changed insignificantly during the cultivation period (Table 3). The decrease in the lipid content of the biomass in the citric acid-production phase is not surprising since lipid synthesis and intense CA production compete for acetyl-CoA.

It should be noted that the cellular content of nitrogen in the dry biomass decreased from 6.2% in the growth phase to 3.6% in the acid-production phase. This fact demonstrates the importance of both nitrogen deficiency and balance between the concentrations of nitrogen and other nutrients for the citrate excretion by yeast cells. Similar data on the decrease in the nitrogen content of *Saccharomycopsis lipolytica* D1805 cells upon their transition to the stationary growth phase (from 8.5% in the trophophase to 4% in the late exponential phase) have been reported by Briffaud and Engasser (*23*). Moresi (*24*) revealed a reduction from 7–8 to 2.3–4.4% in the cellular nitrogen content of *Y. lipolytica* ATCC 20346 cells. There is evidence on the importance of nitrogen deficiency for the production of CA by *Candida oleophila* ATCC 20177. The optimum concentration of NH_4^+ for the production of CA was found to be 1.2 mg/g (*21*). Under growth limitation by nitrogen, the energy content of the biomass decreased from 25.5 to 21 kJ/g. This fact can be explained by a positive correlation between the energy content and the lipid content of biomass because lipids are a major group for energy storage in cells.

The lipid profile of *Y. lipolytica* N 15 was mainly represented by oleic ($^{\Delta 9}$C18:1), linoleic ($^{\Delta 9,12}$C18:2), palmitic (C16:0) and palmitoleic ($^{\Delta 9}$C16:1) fatty acids (Table 3). The transition of the yeast to the citric acid-production phase resulted in a notable alteration of the fatty acid profile of cells: 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 contents of palmitoleic acid ($^{\Delta 9}$C16:1) increased from 1.8 to 9.03 %. Linoleic acid ($^{\Delta 9,12}$C18:2) did not markedly change. The fatty acid desaturase activities during the yeast cultivation were estimated by calculating the ratios of the desaturase product to its precursor (namely, C16:1/C16:0; C18:1/C18:0; C18:2/C18:1). The high values of the C18:1/C18:0 ratio revealed in all experimental variants are indicative of the high activity of Δ9-desaturase in the yeast cells, especially in the citric acid-production phase.

Conclusions

To conclude, the active production of CA by the glycerol-grown yeast *Y. lipolytica* demonstrates that yeast strains can efficiently be used instead of commonly used moulds for the CA production on an industrial scale. Indeed, the common CA producer *A. niger* can produce CA from molasses with a volumetric productivity of 0.8 g/(L·h) and Y_{CA} equal to 0.9 g/g, whereas the yeast mutant *Y. lipolytica* N 15 produces CA from crude glycerol with a productivity of 0.89 g/(L·h) and Y_{CA} of 0.9 g/g. Another advantage of the selected yeast species for the production of CA is due to the fact that the cultivation of *A. niger* is associated with the accumulation of solid and liquid wastes in great amounts. Moreover, the selected yeast strains exhibit a greater resistance to high substrate concentrations and a greater tolerance to detrimental metal ions than the mould *A. niger*. This circumstance allows the use of less refined substrates in the case of the yeast producers.

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