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Microbiological Production of Citric and Isocitric Acids from Sunflower Oil

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Summary

The growth of wild type strain *Yarrowia lipolytica* VKM Y-2373 and its mutant *Yarrowia lipolytica* N 15 as well the biosynthesis of citric and isocitric acids on sunflower oil were studied. It was indicated that cell growth was associated with the simultaneous utilization of glycerol and free fatty acids produced during oil hydrolysis. The activities of enzymes of glycerol metabolism (glycerol kinase), fatty acid assimilation enzymes of glyoxylate cycle (isocitrate lyase and malate synthase) and citric acid cycle were comparatively assayed in *Y. lipolytica* grown on sunflower oil, glycerol and oleic acid. Glycerol kinase ànd enzymes of glyoxylate cycle were active during the whole period of cell cultivation on sunflower oil. Citric acid production and a ratio between citric and isocitric acids depended on both the strain used and the medium composition. It was revealed that wild type strain *Y. lipolytica* VKM Y-2373 produced almost equal amounts of citric and isocitric acids at pH=4.5 and predominantly accumulated isocitric acid at pH=6.0. The mutant *Y. lipolytica* N 15 produced only citric acid (150 g/L with mass yield (Y_{CA}) of 1.32 g/g). Biochemical characteristics of mutant strain *Y. lipolytica* N 15 were discussed.

Key words: Yarrowia lipolytica, citric acid, isocitric acid, microbiological production, sunflower oil

Introduction

Citric acid (CA) and its salts, sodium citrate, calcium citrate and potassium citrate, are recommended as food ingredients and widely used in the production of jellies, jams, ice cream, cheese, ciders, and wines. Moreover, CA and its salts are applied extensively in medicine as ingredients of buffers and pharmaceutical syrups. In recent years, sodium citrate has attracted great interest for the application in the manufacture of detergents instead of phosphates. Moreover, CA and its salts (calcium, potassium, and sodium citrate) are natural components of plants and animals, which are easily degraded by water and soil microorganisms.

The total requirement for CA increases every year, and today the estimated annual world production reaches about 1 400 000 t (*1*) with annual increment of 3.5–4.0 % and the world market price of 0.80 €/kg.

The requirements for isocitric acid (ICA) and its salts are also increasing. Monopotassium salt of *threo*-D(*S*)-(+)--isocitric acid is used in several biochemical analyses (assays of aconitate hydratase, NAD-isocitrate dehydrogenase, NADP-isocitrate dehydrogenase, isocitrate lyase); the perspective of ICA as alimentary additive is also considered.

Traditionally, different strains of fungus, mostly belonging to *Aspergillus niger* have been used in the commercial production of CA from molasses, sucrose or glucose (*2*). There is a great interest in various yeasts belonging to the genus of *Candida,* which are capable of CA and ICA production from various carbon sources, such as *n*--alkanes (*3*), glucose (*4*), ethanol (*5*), and glycerol (*6,7*).

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At present, vegetable oils, animal fat, industrial or pure fatty acids are of great practical interest for various microbial transformations. These raw materials were intensively studied as carbon sources for the production of microbial lipids (*8–12*) and lipases (*13,14*). Little is known about the application of plant oils for CA and ICA production. When the yeasts were cultivated on plant oils, most strains of *Y. lipolytica* produced CA and ICA simultaneously in the proportion that depended on the strain, carbon source, and composition of the growth medium (*15–17*). Several scientific groups attempted to use mutant strains or to alter the growth medium in order to obtain high CA production from plant oils. In experiments with mutant strain *Y. lipolytica* H181, CA production up to 198 g/L with insignificant amount of ICA and the mass yield (Y_{CA}) of over 0.9 g/g was obtained (*18*). Recently, we have obtained CA production of 135 g/L and Y_{CA} of 1.55 g/g by using mutant *Y. lipolytica* 187/1 grown on rapeseed oil (*19*).

The present research is aimed at investigating the production of CA and ICA from sunflower oil using the wild type and mutant strains of *Y. lipolytica* yeast. The enzyme activities at different points of oil assimilation were also studied in order to elucidate the regime of oil feeding in the process of CA production. Biochemical characteristics of mutant strain *Y. lipolytica* N 15 were discussed.

Materials and Methods

Organisms

The wild strain *Y. lipolytica* VKM Y-2373 and its mutant *Y. lipolytica* N 15 were obtained 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 mutant *Y. lipolytica* N 15 was obtained by combined treatment of the wild strain with UV radiation and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and selected as a mutant incapable of citrate utilization.

The strains were maintained at 4 °C on the mineral agar medium supplemented with 1 % paraffin. The medium had the following composition (in g/L): agar 10, $(NH_4)_2SO_4$ 3.0, KH_2PO_4 1.0, K_2HPO_4 0.1, $MgSO_4 \cdot 7H_2O$ 0.7, $Ca(NO_3)_2$ 0.4, NaCl 0.5, yeast extract (Difco) 2.0, and trace elements according to Burkholder *et al.* (*20*).

Chemicals

All chemicals and enzymes were of the highest purity commercially available and were purchased from Sigma (USA) or Boehringer Mannheim (Germany). Commercial sunflower oil was purchased from the Kazan Seed Oil Processing Company (Russia) and used as a carbon source. The fatty acid composition of sunflower oil was as follows (%, by mass): $C_{12:0}$ 2.0, $C_{14:0}$ 2.0, $C_{16:0}$ 4.0, $C_{18:0}$ 4.0, $\Delta^9C_{18:1}$ 36.0, $\Delta^{9,12}C_{18:2}$ 52.0.

Cultivation

In order to obtain the preculture, 750-mL flasks containing 100 mL of the medium were inoculated with a yeast colony grown on agar and incubated on an orbital shaker ((130±10) rpm) at (28±1) °C. Since growth was followed by decrease of 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. The medium had the following composition (in g/L): sunflower oil 10, or glycerol 20, or oleic acid 20, $(NH_4)_2SO_4$ 3.0, KH_2PO_4 1.0, K_2HPO_4 0.1, $MgSO_4 \cdot 7H_2O$ 0.7, $Ca(NO_3)_2$ 0.4, NaCl 0.5, yeast extract (Difco) 0.5, thiamine-HCL 0.5 mg/L, and trace elements as described by Burkholder *et al.* (*20*). The preculture was grown for 36 h up to the final cell concentration of 6.7 g/L.

The wild strain *Y. lipolytica* VKM Y-2373 and the mutant *Y. lipolytica* N 15 were cultivated in a 10-litre ANKUM-2M fermentor (Russia) with an operating volume of 5 L. Temperature of 28 °C and dissolved oxygen concentration (55–60 % of air saturation) were maintained automatically; pH was adjusted with 10–20 % NaOH to the values indicated in the text.

In order to investigate the growth characteristics, the wild strain *Y. lipolytica* VKM Y-2373 was cultivated in the medium containing (in g/L): $(NH_4)_2SO_4$ 5.0, KH_2PO_4 2.0, K_2HPO_4 0.2, $MgSO_4 \cdot 7H_2O$ 1.4, $Ca(NO_3)_2$ 0.8, NaCl 0.5, thiamine-HCL 0.5 mg/L, and trace elements as described by Burkholder *et al.* (*20*). Sunflower oil (10 g/L) or glycerol (20 g/L) or oleic acid (20 g/L) were used as the sole carbon and energy source. Cultivation was performed as indicated in the text.

In order to investigate CA production, the wild strain *Y. lipolytica* VKM Y-2373 and its mutant *Y. lipolytica* N 15 were cultivated in the medium containing (in g/L): $(NH_4)_2SO_4$ 6.0, KH_2PO_4 2.0, K_2HPO_4 0.2, $MgSO_4 \cdot 7H_2O$ 1.4, $Ca(NO_3)_2$ 0.8, NaCl 0.5, thiamine-HCL 0.5 mg/L, and trace elements as described by Burkholder *et al.* (*20*). Sunflower oil (20 g/L) was used as the sole carbon and energy source; pulsed additions of oil were made when oil concentration decreased below 5.0 g/L. Cultivation was performed as indicated in the text.

Measurement techniques

For extraction of extracellular fat from the culture liquid, the modified method of Kates was performed (*21*). For biomass determination, 10–50 mL of the culture liquid were filtered through membrane filters and yeast cells were washed with *n*-hexane. The complete removal of extracellular fat from the yeast was confirmed by the absence of lipids in hexane phase collected after the last wash of cells. Also, in order to verify that no losses of cell components occurred during washing with *n*-hexane, yeast cells produced on glucose were washed twice with distilled water or as previously described (with *n*--hexane). In both cases, yeast cells were dried at 110 °C to constant mass.

The filtrate was used for the oil content analysis; it was washed twice with *n*-hexane, the mixture was divided into two layers, the upper phase of which was composed of *n*-hexane and lipids, while the lower phase contained a water layer. Hexane extract was collected into a glass flask with precision. Lipids from the water layer were extracted by chloroform and combined with the *n*--hexane extract. The combined lipid extract was dried by passing it through a glass filter with anhydrous sodium sulphate; solvents were evaporated to constant mass.

In order to determine individual lipid fractions, thin layer chromatography was carried out on 60G silica gel plates (Merck) by using a solvent system *n*-hexane/diethyl ether/acetic acid at volume ratio of 85:15:1. Lipid fractions were visualized by iodine vapour.

Methyl esters of fatty acids were obtained by the method of Sultanovich *et al.* (*22*) and analysed by gas-liquid chromatography on a Chrom-5 chromatograph (The Czech Republic) with a flame-ionisation detector. The column (2 m×3 mm) was packed with 15 % Reoplex-400 applied to Chromaton N-AW (0.16–0.2 mm). The temperature of the column was 200 °C. The lipid content in the biomass was determined from the total fatty acid content with *n*-docosane ($C_{22}H_{46}$) or heptadecanoic acid as internal standards.

Filtered aliquots of the culture liquid were used for the analysis of nitrogen, CA, ICA and glycerol.

Ammonium concentration was assayed with an ionometer (Orion, USA). Concentration of organic acids was determined using high-performance liquid chromatography (HPLC) with an HPLC chromatograph (LKB, Sweden) on an Inertsil ODS-3 reversed-phase column (250×4 mm, Elsiko, 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 ICA were identified using the standard solutions (Boehringer Mannheim, Germany). Moreover, diagnostic kits (Roche Diagnostics GmbH, Germany) were used for the assay of CA and ICA. The determination of CA was based on the measurement of the NADH produced during conversion of CA to oxaloacetate and its decarboxylation product pyruvate, and following conversion to L-malate and L-lactate. Reactions were catalysed by citrate lyase, malate dehydrogenase and L-lactate dehydrogenase. The determination of ICA was based on the measurement of the NADPH produced during conversion of ICA to α-ketoglutarate, a reaction catalysed by isocitrate dehydrogenase.

Glycerol was analysed enzymatically using biochemical kit (Boehringer Mannheim/R-Biopharm, Germany). The determination of glycerol was based on the measurement of NADH produced during conversion of glycerol to L-lactate in coupled reactions. The reactions were catalyzed by glycerol kinase, pyruvate kinase and L-lactate dehydrogenase.

Enzyme assays

Glycerol-grown cells were centrifuged (3000×*g*, 10 min, 4 °C); those grown on sunflower oil or oleic acid were paper-filtered and washed with ice-cold 0.9 % NaCl solution to remove residual fat. All types of cells were washed with 100 mM phosphate buffer (pH=7.4), centrifuged at 3000×*g* (10 min, 4 °C) and used to prepare 10 % suspension in the same buffer (pH=7.4) containing 1 mM EDTA. Cells were disintegrated with Ballotini™ glass beads (*d*=150–250 μ) on a planetary mill for 3 min at 1000 rpm (0 °C). The homogenate obtained was centrifuged (5000×*g*, 30 min, 4 °C), and the supernatant was used for determining activities of cytoplasmic, mitochondrial and peroxisomal enzymes: glycerol kinase (EC 2.7.1.30), citrate synthase (EC 4.1.3.7), aconitate hydratase (EC 4.2.1.3), NAD-dependent (EC 1.1.1.41) and NADP-dependent isocitrate dehydrogenase (EC 1.1.1.42), isocitrate lyase (EC 4.1.3.1) and malate synthase (EC 4.1.3.2).

The activity of glycerol kinase was analysed according to the method described by Wieland and Suyter (*23*) in the reaction mixture containing glycerol 2.5 mM, $MgCl_2$ 2 mM, ATP 2 mM, NAD 0.5 mM, glycerol-3-phosphate-dehydrogenase 0.5 U in 2 mL of reaction mixture, and 0.2 M glycine buffer (pH=9.8).

The activity of citrate synthase was determined according to the method of Srere (*24*). The reaction mixture contained oxaloacetate 0.25 mM, acetyl CoA 0.25 mM, 5,5-dithiobis(2-nitrobenzoate) 0.1 mM, and Tris-HCl buffer 100 mM (pH=8.5).

Malate synthase (EC 4.1.3.2) was assayed in the reaction mixture containing $MgCl_2$ 10 mM, acetyl CoA 0.08 mM, glyoxylate 2.5 mM and potassium phosphate buffer 50 mM (pH=6.45) by the method described by Ornston and Ornston (*25*).

The aconitate hydratase activity was assayed according to the method described by Anfinsen (*26*). The reaction mixture contained monopotassium salt of *threo*-D(*S*)-isocitric acid 5 mM and potassium phosphate buffer 50 mM (pH=7.5).

The activities of NAD- and NADP-dependent isocitrate dehydrogenases were determined according to the method described by Kornberg and Pricer (*27*). The reaction mixture for the analysis of NAD-dependent isocitrate dehydrogenase contained monopotassium salt of *threo*-D(*S*)-isocitric acid 0.25 mM, NAD 4 mM, AMP 0.5 mM, $MgCl_2$ 10 mM, antimycin 2 mM, and Tris-HCl buffer 50 mM (pH=7.5). The reaction mixture for the analysis of NADP-dependent isocitrate dehydrogenase contained monopotassium salt of *threo*-D(*S*)-isocitric acid 1.0 mM, NADP 0.75 mM, $MgCl_2$ 10 mM, antimycin 2 mM, and Tris-HCl buffer 50 mM (pH=9.0).

The activity of isocitrate lyase was measured using the method described by Dixon and Kornberg (*28*). The reaction mixture contained monopotassium salt of *threo*-D(*S*)-isocitric acid 4 mM, phenylhydrazine-HCl 8 mM, cysteine-HCl 4 mM, $MgCl_2$ 10 mM, potassium phosphate buffer 75 mM (pH=6.85).

The amount of enzyme catalyzing the conversion of 1 μmol of substrate per min was taken as the unit of enzyme activity (U). The enzyme activities were expressed as units per mg of protein (U/mg). Protein amount in cell-free extract was determined by the Bradford method (*29*). All the data presented are the means of three experiments and two measurements for each experiment; standard deviations were calculated (SD<10 %).

Calculations of parameters of cell growth and acid production

To take into account the medium dilution due to the addition of NaOH solution for maintaining the constant pH value, the total amounts of CA, ICA and biomass in the culture liquid were used for calculations.

The specific growth rate (μ) was calculated using the following equation:

$$\mu = 2.3(\log X_2 - \log X_1)/(t_2 - t_1) \qquad /1/$$

where X_2 and X_1 are the biomass at the moment of time t_2 and t_1, respectively.

The biomass yield was calculated as follows:

$$Y_{X/S}=X/S \qquad /2/$$

where X is the total amount of biomass in the culture liquid at the end of fermentation (g/L) and S is the amount of the consumed oil (g/L).

The specific rates of CA and ICA production (q_{CA} or q_{ICA}, respectively) were calculated using the following equation:

$$q=(P/X)\cdot t \qquad /3/$$

where P is the total amount of CA or ICA in the culture liquid at the end of fermentation (g), X is biomass content in a fermentor at the end of fermentation (g), and t is fermentation time (h).

The mass yield coefficients of CA or ICA production were calculated as follows:

$$Y=P/S \qquad /4/$$

where P is the total amount of CA or ICA in the culture liquid at the end of fermentation (g), and S is the amount of consumed oil (g).

The volumetric productivity of the process was calculated using the following equation:

$$C=(P/V)\cdot t \qquad /5/$$

where P is the total amount of CA or ICA in the culture liquid at the end of fermentation (g), V is the volume of fermentor (L), and t is fermentation time (h).

Results and Discussion

Properties of wild type strain Y. lipolytica VKM Y-2373 and the development of regime of oil feeding

The wild type strain *Y. lipolytica* was cultivated in the complete medium containing 10 g/L of sunflower oil and 5.0 g/L of $(NH_4)_2SO_4$ in a 10-litre fermentor with initial working volume of 5 L; pH=5.0 was adjusted automatically with 10–15 % NaOH. Fig. 1 illustrates a typical time course of growth of *Y. lipolytica.* Cell growth was accompanied by a decrease in the content of nitrogen and oil in the medium; 10 g/L of biomass were accumulated at the end of fermentation (27 h) (Fig. 1a).

The maximum specific growth rate (μ_{max}) calculated from the linear segment of the growth curve (Fig. 1) amounted to 0.230 h^{-1}; a value comparable with literature data obtained for *Y. lipolytica* grown on fatty acids (*8,9*). The biomass yield from the consumed oil ($Y_{X/S}$) was 1.1 g/L. The above findings support the potential of *Y. lipolytica* for the production of single-cell protein from fatty material. High $Y_{X/S}$ of (1.1±0.3) g/L has been reported for different strains of *Y. lipolytica* cultivated on various types of crude fat (*8,9,30,31*). Recently, in experiments with *Y. lipolytica* grown on completely saturated fatty acid mixture (*9*), it has been demonstrated that an increase in dissolved oxygen concentration from 5–15 to 60–70 % of air saturation resulted in the increase of $Y_{X/S}$ from 1.1 to 1.6, whereas the lipid production decreased at the same time. Moreover, the growth parameters appeared to be critically influenced by fatty acid composition of the carbon source. It was indicated that yeast strains (*Saccharomycopsis lipolytica* and *Apiotrichum curvatum*) did not grow sufficiently well on saturated fat due to inadequate dispersion of these substrates into the liquid medium; solid fat required considerable agitation (1200 rpm) for dispersal in the growth medium (*32,33*). In other reports, it was observed that *Y. lipolytica* demonstrated significant growth without differentiations, regardless of the fatty acid composition of the culture medium (saturated free fatty acids, rapeseed oil and mixture of these two substrates) (*8*).

The utilization of sunflower oil involved its hydrolysis by lipase that resulted in the formation of di- and monoglycerides, glycerol, and free fatty acids. The glycerol concentration rapidly increased during the first 9 h of cultivation (up to 0.125 g/L) (Fig. 1b) and then remained at this level. It should be noted that residual glycerol remained at the trace level as sunflower oil was consumed and no accumulation of glycerol was observed. Also, that a ratio among di-, monoglycerides, and free fatty acids in the culture broth remained at the constant level in the course of cultivation (data not shown). Analysis of free fatty acid composition revealed that in the course of cultivation, the fractions of oleic acid ($\Delta^9C_{18:1}$) and linoleic acid ($\Delta^{9,12}C_{18:2}$) decreased from 36 to 27 % and from 52.1 to 35.0 % (by mass), respectively, while the amount of stearic acid ($C_{18:0}$) increased from 4.0 to 26.0 % (by mass). It may be considered that the strong fatty acid specificity of *Y. lipolytica* was probably

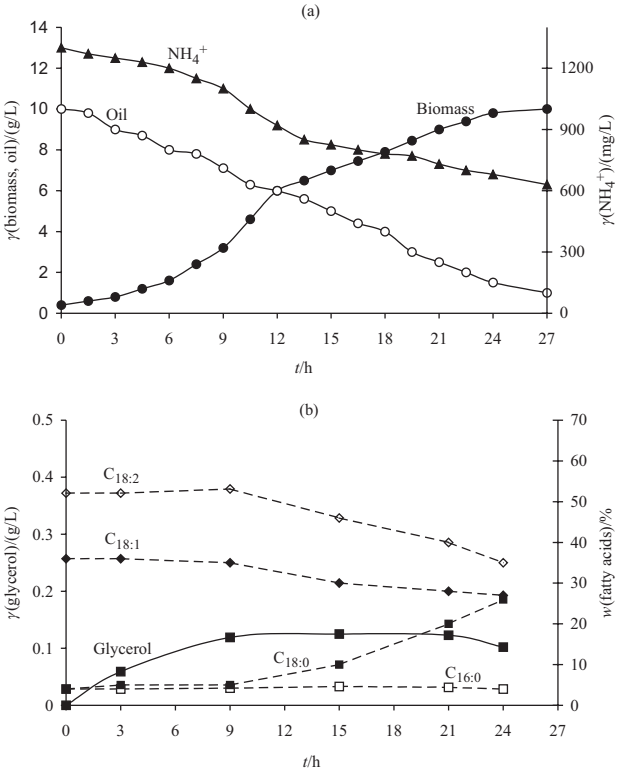

Fig. 1. The growth of *Y. lipolytica* VKM Y-2373. Variation of sunflower oil, biomass, nitrogen concentrations (a), glycerol and free fatty acids (b) with time. Cultivation conditions: 10 g/L of sunflower and 5.0 g/L of $(NH_4)_2SO_4$; pH=5.0

due to the different fatty acid carriers. Papanikolaou *et al.* (*8*), using *Y. lipolytica* LGAM S(7)1 grown on a mixture of saturated, unsaturated and polyunsaturated fatty acids ($C_{12:0}$, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, $\Delta^9C_{18:1}$, $\Delta^{9,12}C_{18:2}$), observed that the uptake rates of $C_{12:0}$, $C_{14:0}$, $\Delta^9C_{18:1}$ and $\Delta^{9,12}C_{18:2}$ were significantly higher than that of $C_{18:0}$ (and in a lesser extent that of $C_{16:0}$). Similar discrimination against stearic acid has been observed during growth of another strain of *Y. lipolytica* when industrial saturated fat was used as substrate (*9*). In that case, in spite of the rapid uptake of unsaturated fatty acids, the microorganism accumulated a totally saturated lipid with the composition similar to cocoa butter $C_{18:0}$ (*8,9,11*).

Since no accumulation of any products of oil hydrolysis was observed in our experiments, it can be assumed that the consumption of glycerol and fatty acids by the yeast *Y. lipolytica* occurred simultaneously. It is in agreement with the results obtained with *Y. lipolytica* yeast grown on the mixture of saturated free fatty acid and glycerol (or glucose) (*9,11*). It should be noted that simultaneous consumption of two substrates from the environment is not typical for microorganisms. It is generally accepted that yeasts utilize sugars preferentially than any other carbon source (*34,35*). However, recently the numeric models describing simultaneous uptake of sugars and fat in *Y. lipolytica* have been developed (*36*).

Activities of enzymes were determined in *Y. lipolytica*, grown in the complete medium (5.0 g/L of $(NH_4)_2SO_4$), containing different carbon sources like sunflower oil (10 g/L), glycerol (20 g/L) or oleic acid (10 g/L) in a 10-litre fermentor with initial working volume of 5 L; pH=5.0 was adjusted automatically with 10–15 % NaOH. The cultivation was carried out up to complete consumption of the carbon source. *Y. lipolytica* showed significant growth in all cases (sunflower oil: 10 g/L of biomass, μ_{max} of 0.230 h^{-1}, $Y_{X/S}$ of 1.1 g/L; glycerol: 9.3 g/L of biomass, μ_{max} of 0.276 h^{-1}, $Y_{X/S}$ of 0.47 g/L; oleic acid: 8.7 g/L of biomass, μ_{max} of 0.316 h^{-1}, $Y_{X/S}$ of 0.87 g/L).

Activities of glycerol kinase (involved in glycerol metabolism), four enzymes of the Krebs cycle (citrate synthase, aconitate hydratase, NAD- and NADP-dependent isocitrate dehydrogenases) and two key enzymes of glyoxylate cycle (isocitrate lyase and malate synthase) involved in the utilization of free fatty acids were assayed at 6, 12 and 24 h of cultivation (Table 1).

In the first hours of cultivation, when active assimilation of sunflower oil occurred, the induction of glycerol kinase (a key enzyme of glycerol metabolism) was achieved; the activity of glycerol kinase was 70 % of that observed in glycerol-grown cells but much higher than that in oleate-grown cells. In the subsequent hours of cultivation (12 and 24 h), the activity of glycerol kinase remained very high. These results suggest that in the course of oil assimilation, glycerol, which was produced continuously, was consumed actively.

Moreover, in the first hours of oil assimilation, the induction of enzymes of glyoxylate cycle, isocitrate lyase and malate synthase was revealed. It should be noted that isocitrate lyase and malate synthase were induced simultaneously with the formation of free fatty acids; this phenomenon was also observed in cells grown on oleic acid; however, in the case of glycerol assimilation, activities of isocitrate lyase and malate synthase were extremely low. In later hours of oil assimilation, the activities of malate synthase and isocitrate lyase were maintained at high level, which is possibly associated with active consumption of free fatty acids produced during the whole process.

Physiologically, the glyoxylate cycle in yeast operates to maintain continuous resynthesis of oxaloacetate needed for the Krebs cycle. In yeast grown on glucose and other substrates for glycolysis, resynthesis of oxaloacetate may occur during carboxylation of pyruvate and phosphoenolpyruvate (*37–39*) and the functioning of glyoxylate cycle is not necessary. It has been observed that during *Y. lipolytica* growth on glucose (*37,38,40*) or glycerol (*41*) the activity of isocitrate lyase is low, while during growth on hydrocarbons its activity is much higher (*37,38,42*). In contrast, the glyoxylate cycle enzymes are shown to be involved in fatty acid utilization by *Y. lipolytica* (*43*). It is known that yeast grown on lipids hydrolyses them to fatty acids, which are then β-oxidised

Table 1. Enzyme activities in *Y. lipolytica* grown on sunflower oil and products of its hydrolysis – glycerol and oleic acid

Carbon source	Time/h	Enzyme activity/(U/mg protein)						
		GK	CS	AH	NAD-IDH	NADP-IDH	IL	MS
Sunflower oil	6	0.254	2.040	0.420	0.060	0.058	0.109	0.074
	12	0.244	2.440	0.460	0.060	0.068	0.129	0.084
	24	0.250	1.960	0.473	0.058	0.068	0.093	0.075
Glycerol	6	0.363	0.820	0.135	0.105	0.120	0.009	0.012
	12	0.360	0.900	0.130	0.115	0.132	0.009	0.014
	24	0.300	0.835	0.120	0.117	0.152	0.010	0.010
Oleic acid	6	0.095	1.700	0.530	0.100	0.070	0.133	0.100
	12	0.100	1.900	0.530	0.100	0.060	0.134	0.130
	24	0.110	1.900	0.520	0.100	0.070	0.144	0.120

GK – glycerol kinase; CS – citrate synthase; AH – aconitate hydratase; NAD-IDH – NAD-dependent isocitrate dehydrogenase; NADP-IDH – NADP-dependent isocitrate dehydrogenase; IL – isocitrate lyase; MS – malate synthase
The values are the mean of three experiments and two measurements for each which varied by no more than 10 %

to acetyl CoA, a substrate for both the Krebs and glyoxylate cycles.

Hildebrandt and Weide (*44*) consider yeast isocitrate lyase as a constitutively activated enzyme which undergoes catabolite repression. Products of glycerol oxidation such as pyruvate and phosphoenolpyruvate inhibit isocitrate lyase and impair the glyoxylate cycle-mediated resynthesis of oxaloacetate. Our experiments indicated that in the course of sunflower oil assimilation, the glycerol level was very low (no more than 0.125 g/L), and, hence, concentrations of pyruvate and phosphoenolpyruvate were also very low and could not affect the functioning of glyoxylate cycle.

During the active functioning of glyoxylate cycle under the utilization of sunflower oil and oleic acid, the high activities of citrate synthase (CS) and aconitate hydratase (AH), enzymes involved in the Krebs and glyoxylate cycles, were indicated, while the activities of NADP-isocitrate dehydrogenase and NAD-isocitrate dehydrogenase, enzymes not participating in glyoxylate cycle, were considerably higher in the cells grown on glycerol.

Thus, the high activities of glycerol kinase, isocitrate lyase, and malate synthase in *Y. lipolytica* grown on sunflower oil allowed us to suggest the simultaneous consumption of products of oil hydrolysis (glycerol and fatty acids) in the course of cultivation. This fact is of special importance for the development of the efficient regime of oil feeding in the process of CA production.

The production of citric and isocitric acids by wild type strain Y. lipolytica VKM Y-2373

To study the production of citric acids by *Y. lipolytica* VKM Y-2373 under nitrogen limitation, yeasts were grown in a 10-litre fermentor with working volume of 5 L at pH=4.5 or 6.0. The initial concentration of sunflower oil in the medium was 20.0 g/L; pulsed additions of oil were made when oil concentration decreased below 5.0 g/L. The parameters of the cell growth and acid synthesis at pH=4.5 and 6.0 by the end of cultivation (144 h) are shown in Table 2. At pH=4.5, the total concentration of acids comprised 123.0 g/L; the yield of the both acids from the oil consumed (Y_{CA+ICA}) was 1.15 g/g; concentrations of ICA and CA reached 55.0 and 68.0 g/L, respectively; the ICA/CA ratio was 1.00:1.24. It was shown that the yield of CA (Y_{CA}) was 0.64 g/g; the specific rate of CA synthesis (q_{CA}) amounted to 0.058 g/(g·h), the volume productivity of the CA production was 1.05 g/(L·h).

A change in the pH value from 4.5 to 6.0 showed no marked effect on the biomass accumulation and the total acid production (Table 2). The total concentration of acids and Y_{CA+ICA} amounted to 120.5 g/L and 1.29 g/g, respectively. However, a ratio between citric acids depended considerably on the pH value; at pH=6.0, the concentrations of ICA and CA were 70.0 and 50.5 g/L respectively, with the ICA/CA ratio of 1.39:1.00, whereas at pH=4.5, the ratio of ICA to CA was 1.00:1.24. Similar effect of pH on CA and ICA synthesis was observed earlier with this strain grown on rapeseed oil; at pH=4.5, yeasts actively produced both CA and ICA (the ICA/CA ratio was 1.00:1.18), whereas at pH=6.0, ICA synthesis prevailed (the ICA:CA ratio was 2.55:1.00) (*45*). It is known that CA transport across the membrane is stimulated by low pH values (*46*), whereas ICA transport is independent of pH; this may explain why at pH=4.5 the ratio of ICA to CA changed in favour of CA.

The parameters of ICA production were calculated for *Y. lipolytica*; specific rate of ICA synthesis (q_{ICA}) amounted to 0.06 g/(g·h). The volume productivity of the ICA production was as high as 1.14 g/(L·h). The yield of ICA from the consumed oil (Y_{ICA}) was 0.75 g/g. It can be assumed that physiological or genetic manipulations undertaken to reduce the synthesis of CA would increase the ICA production by *Y. lipolytica* VKM Y-2373. It should be noted that no production of isocitrate by microbial synthesis has been reported before and several companies produce ICA by complete chemical synthesis; Sigma Company produces isocitrate in small amounts by its isolation from plant tissues; the price of product is 320 €/g. In the literature, there are only two communications on predominant ICA production from ethanol (*47*) and *n*-alcanes (*48*), the concentrations of ICA achieved were 66 and 80 g/L, respectively.

Biosynthesis of citric acid by mutant Y. lipolytica N 15

To study the production of CA by mutant *Y. lipolytica* N 15, yeasts were grown in a 10-litre fermentor with working volume of 5 L, at pH=4.5. The initial concentration of sunflower oil in the medium was 20.0 g/L; pulsed additions of oil were made when oil concentration decreased below 5.0 g/L. Fig. 2 illustrates a typical time course of CA production by the mutant. The CA production started after the depletion of nitrogen from the medium (to 70 mg/L) and reached 150.0 g/L at 144 h of cultivation, while the amount of ICA was insignificant (the ratio between CA and ICA was 30.0:1.0). This is the first time that *Y. lipolytica* grown on sunflower oil has been shown to produce CA at high concentration.

Table 2. The production of citric and isocitric acids from sunflower oil by wild type strain *Y. lipolytica* VKM Y-2373

pH	γ(biomass) g/L	γ(total acids) g/L	Y_{CA+ICA} g/g	γ(ICA) g/L	γ(CA) g/L	ICA/CA	CA production			ICA production		
							q_{CA} g/(g·h)	Productivity g/(L·h)	Y_{CA} g/g	q_{ICA} g/(g·h)	Productivity g/(L·h)	Y_{ICA} g/g
4.5	18.4	123.0	1.15	55.0	68.0	1.00:1.24	0.058	1.05	0.64	n.d.	n.d.	n.d.
6.0	18.0	120.5	1.29	70.0	50.5	1.39:1.00	n.d.	n.d.	n.d.	0.06	1.14	0.75

Parameters were determined at 144 h of cultivation
The values are the mean of three experiments and two measurements for each which varied by no more than 10 %
n.d. – not determined

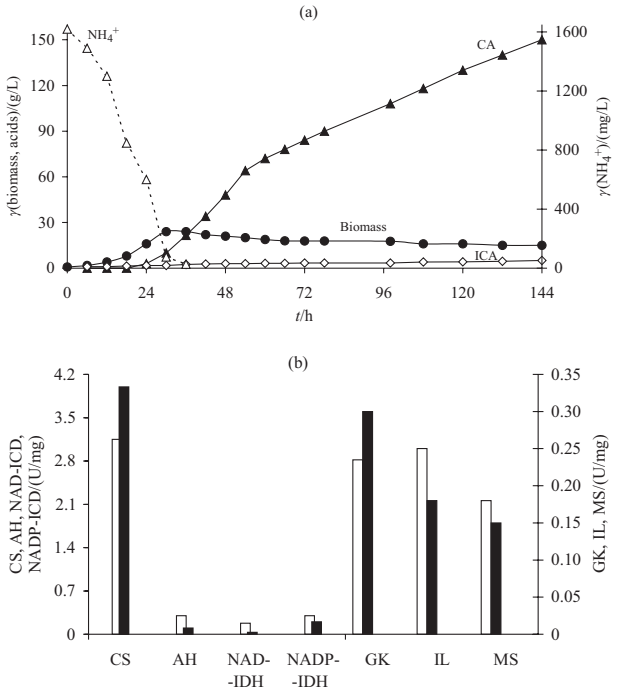

Fig. 2. Biosynthesis of CA by mutant *Y. lipolytica* N 15 at pH=4.5 (a) and activity of enzymes in growth phase (black bars) and acid-formation phase (white bars) (b)
CS – citrate synthase; AH – aconitate hydratase; NAD-IDH – NAD-dependent isocitrate dehydrogenase; NADP-IDH – NADP-dependent isocitrate dehydrogenase; GK – glycerol kinase; IL – isocitrate lyase; MS – malate synthase

Earlier, the maximum CA concentration of 18.7 g/L was obtained by using the wild type strain *Y. lipolytica* grown on sunflower oil (*49*). Using other carbon sources (rapeseed oil, soybean oil, palm oil, glucose, or glycerol), relatively high CA concentration was achieved with selected mutant strains of *Y. lipolytica*. In experiments with mutant strain *S. lipolytica* NTG9 grown on rapeseed oil, the CA concentration reached 152.3 g/L, while ICA production was significant and a ratio of CA/ICA was 5.34: 1.00 (*16*). Aurich *et al.* (*18*) obtained the CA concentration of 198 g/L, which was achieved after a 300-hour fed-batch cultivation of mutant strain *Y. lipolytica* H181. The use of rapeseed oil resulted in the CA production of 135 g/L by *Y. lipolytica* 187/1 (*19*). High CA production was reported when mutant strain *Y. lipolytica* was grown on glucose (150–200 g/L) in batch, fed-batch and continuous culture (*4,46,50–53*) and on *n*-paraffins (*54*). Using the raw glycerol (which can become an important feedstock of biodiesel production from rapeseed oil), Rymowicz *et al.* (*7*) achieved CA production of 124.5 g/L by acetate-negative mutant *Y. lipolytica*.

In the present study, the volumetric productivity of CA synthesis was rather high (1.56 g/(L·h)) and Y_{CA} was 1.32 g/g (data not shown), which is in agreement with the data reported previously for various *Y. lipolytica* strains cultivated on fatty material (*15–19*).

To elucidate the mechanism of CA production from sunflower oil by *Y. lipolytica* N 15, the activities of enzymes were determined. Fig. 2b shows typical data on enzyme activities examined in homogenates of cells taken from the exponential growth phase (12 h) (black bars) and at 48 h, when active acid formation occurred (white bars). Activities of glycerol kinase, isocitrate lyase and malate synthase remained unchanged in the course of the whole process of cultivation, indicating that the transition of cells to the acid-producing phase had no effect on the activities of enzymes involved in the oxidation of products of oil hydrolysis (glycerol and fatty acids). In the exponential phase the cells exhibited high activities of citrate synthase (3.15 U/mg protein), comparable with wild type strain shown in Table 1, but activities of aconitate hydratase (0.3 U/mg protein), NAD-isocitrate dehydrogenase (0.18 U/mg protein) and NADP-isocitrate dehydrogenase (0.3 U/mg protein) were lower than that of the wild type strain (Table 1). The transition of the mutant from the growth phase to the phase of active biosynthesis of CA caused by the exhaustion of nitrogen in the medium was accompanied by an increase in the citrate synthase activity (by 30 %) and a decrease in the activities of aconitate hydratase and NAD-isocitrate dehydrogenase (by 3 and 6 times, respectively). A high activity of citrate synthase, in distinction from other enzymes of citric acid cycle, including aconitate hydratase and NAD-isocitrate dehydrogenase, is necessary for the intensive CA production in view of the fact that CA formed in citric acid cycle can be presumably excreted from the yeast cell rather than metabolized *via* the cycle. NAD-isocitrate dehydrogenase probably plays a key role in the CA production by the yeast *Y. lipolytica.* The limitation of yeast growth by nitrogen restricted the biosynthesis of nitrogen-containing compounds (proteins and nucleotides) and diminished their content in cells. This is accompanied by a decrease in the intracellular level of AMP with a concurrent increase in the ATP/AMP ratio (*55*). The exhaustion of nitrogen sources from the medium also leads to an increased NADH/NAD^+ ratio. Low concentration of the allosteric regulator AMP suppresses NAD-isocitrate dehydrogenase; the suppression is enhanced by a high NADH/NAD^+ ratio. As a result, yeast cells produce ICA, whereas the disturbed equilibrium of the aconitase reaction leads to the overproduction of CA.

Conclusions

To conclude, the results of the efficient CA production by oil-grown yeast *Y. lipolytica* obtained in the present work and results by other authors (*17–19,45,46, 51–53*) confirm that the application of yeasts has considerable promise for the industrial CA production. The use of yeasts instead of moulds for the CA production also represents a novel approach, since the traditional production of CA by using *Aspergillus 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 a greater tolerance to metal ions that allows the use of less-refined substrates.

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