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Biochemistry of Citric Acid Production from Rapeseed Oil by *Yarrowia lipolytica* Yeast

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Abstract The growth of wild type strain *Yarrowia lipolytica* VKM Y-2373 and its mutant *Y. lipolytica* NG40/UV7 as well as the biosynthesis of citric and isocitric acid on rapeseed oil were studied. It was indicated that the initial step of assimilation of rapeseed oil in the yeast *Y. lipolytica* is its hydrolysis by extracellular lipases with the formation of glycerol and fatty acids, which appear in the medium in the phase of active growth. The concentrations of these metabolites were changed insignificantly upon further cultivation. Lipase and the key enzymes of glycerol metabolism (glycerol kinase) and the glyoxylate cycle responsible for the metabolism of fatty acids (isocitrate lyase and malate synthase) are induced just at the beginning of the growth phase and remain active in the course of further cultivation. These results, taken together, suggest that glycerol and fatty acids available in the medium do not suppress the metabolism of each other. Citric acid production and a ratio between citric and isocitric acids depended on the strain used. It was revealed that the wild strain produced almost equal amounts of citric and isocitric acids while the mutant produced only citric acid (175 g/L) with a yield of 1.5 g of CA per g of oil.

Keywords *Yarrowia lipolytica* · Assimilation of triacylglycerols · Glycerol · Fatty acids · Glyoxylate cycle · Citric acid production

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Introduction

Citric acid (CA) and its salts, sodium citrate, calcium citrate and potassium citrate have attracted increased interest due to their distinctive properties as acidulates, flavoring agents and antioxidants, and are used mainly in the 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 a growth of 5% per year [1].

CA is an intermediate of the tricarboxylic acid cycle (TCA) 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 are capable of CA production from various carbon sources, such as glucose (pure or industrial), glucose syrups, ethanol, methanol, *n*-hydrocarbons [1], mixtures of industrial saturated free fatty acids of animal origin [2], glycerol, and glycerol-containing waste [3]. Most wild strains of *Y. lipolytica* produce CA and *threo*-Ds-isocitric acid (ICA) simultaneously in the proportion that depends on the strain, carbon source, and composition of the growth medium [1]. The commercial demand for ICA has been expanding because of its use as a useful chiral building block for chemical synthesis and as a marker to detect the authenticity and quality of fruit products, most often citrus juices. In authentic orange juice, for example, the ratio of CA to ICA is usually less than 130, and ICA value higher than this may be indicative of fruit juice adulteration. Also, the 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 an alimentary additive have also been considered [1]. It is applied in medicine as an antistress, antihypoxic, and an immune active agent. Currently, ICA is isolated from plant tissue. The Sigma Company produces isocitrate in small amounts at a high price (250–280 €/g).

Recent years show an increasing interest in vegetable oils as substrates for the microbial production of practically important products, such as lipids [2, 4], lipases [5–7], and organic acids including CA and ICA [2, 8, 9].

The main component of vegetable oils and animal fats is triacylglycerol, which is entrained in microbial metabolism through the action of extracellular lipolytic enzymes, lipases. These enzymes hydrolyze triacylglycerols, forming glycerol and the respective fatty acids.

Microbial lipases are well studied and have been comprehensively reviewed [5–7, 10]. The lipolytic activity of *Y. lipolytica* has a taxonomic significance and is reflected in the systematic name of this species [11]. As early as in the 1970s, some lipases of *Y. lipolytica* (formerly, *C. lipolytica*) were found to be structurally bound to the cell wall of this yeast [5]. Later, *Y. lipolytica* was shown to produce various lipases (extracellular Lip2p, cell-bound lipases I and II, as well as intracellular lipolytic enzymes), depending on the medium composition and cultivation conditions [6]. The intracellular lipase Lip2 of *Y. lipolytica* was isolated, purified and characterized by Aloulou et al. [12]. Other researchers studied genes encoding the extracellular lipases of *Y. lipolytica* [7]. The study of the mRNAs derived from the yeast cells grown on oleic acid revealed a new group of the so-called SOA (specific for oleic acid) genes [13].

Lipases are practically important enzymes. They can be used for the production of glycerol and fatty acids from waste vegetable oils, as well as additives in the production of high-quality washing powders. There is an increasing interest in the use of lipases for the modification of oils and fats and for the production of novel stereospecific acylglycerides. Lipases can also be useful in the production of short-chain esters used as biofuel. Lipases have found a wide application in agrochemistry, medicine, and cosmetic formulations [10].

The mechanism of action of microbial lipases is well understood. However, little is known on the mechanism of consumption of the products of triacylglycerol hydrolysis, glycerol and fatty acids. Theoretically, glycerol and fatty acids can be consumed either simultaneously or successively (the latter mechanism of substrate consumption is known as diauxie).

The aim of this work was to study the metabolism of triacylglycerols and its regulation in the yeast *Y. lipolytica* grown on vegetable oil in order to improve the CA production.

Materials and Methods

Microorganism

Strains *Y. lipolytica* VKM Y-2373 and *Y. lipolytica* NG40/UV7 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 mutant strain *Y. lipolytica* NG40/UV7 was obtained in two steps. Initially, the strain *Y. lipolytica* VKM Y-2373 was exposed to different concentrations (20–100 μg/mL) of *N*-methyl-Ni-nitro-*N*-nitrosoguanidine. From 200 colonies treated with 40 μg/mL of *N*-methyl-Ni-nitro-*N*-nitrosoguanidine, the strain named *Y. lipolytica* NG40 was selected that displayed higher (up to 20%) biosynthetic ability as compared with the initial strain. Then, the selectant *Y. lipolytica* NG40 was exposed to a UV irradiation (distance, 10 cm; wavelength, 200–400 nm; exposure, 7 min). From 800 colonies, the strain named *Y. lipolytica* NG40/UV7 with an impaired ability to grow on acetate probably due to defects in the tricarboxylic acid cycle was selected; its biosynthetic activity exceeded that of the initial strain by 40%. 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). Rapeseed oil was purchased from the Kazan Seed Oil Processing Plant (Russia). The fatty acid profile of the rapeseed oil was (%, by mass): $C_{16:0}$, 4.0; $C_{18:0}$, 1.2; $C_{18:1}$, 58.8; $C_{18:2}$, 28.1; $C_{18:3}$, 5.9 with a total unsaturated fatty acid mass fraction of 93.6%.

Cultivation Conditions

In order to obtain yeast cells for the inoculation of a fermenter, a 750-mL Erlenmeyer flask containing 100 mL of cultivation medium (see below) was inoculated with a yeast colony grown on the agar medium. The flask was incubated on an orbital shaker (130 rpm) at 28 ± 1 °C for 36 h (to a cell mass density of 6.7 g/L). At regular intervals, the pH of the medium was adjusted to a value of 5.0 by adding an appropriate volume of 10% NaOH.

In growth experiments, *Y. lipolytica* VKM Y-2373 was cultivated in a 10-L ANKUM-2 M fermenter (SKB, Pushchino, Russia) half filled (to a volume of 5 L) in the medium containing (in g/L): $(NH_4)_2SO_4$, 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; trace elements as described by Burkholder et al. [14] with slight modifications (in 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 and thiamine-HCl 0.5 mg/L. Depending on the aim of the experiment, the medium was supplemented with one of the following growth substrate (g/L): rapeseed oil 10, glycerol 20, or oleic acid 10.

In growth experiments, *Y. lipolytica* VKM Y-2373 was cultivated in a 10-L ANKUM-2 M fermenter with an initial working volume of 5.0 L in media containing two carbon sources (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, yeast extract "Difco" 0.5; thiamine-HCl 0.5 mg/L and Burkholder's trace element solution [14]. The medium was supplemented with two carbon sources: glycerol and oleic acid, glucose and oleic acid; or hexadecane and glucose, as indicated in the text. The fermentation conditions were maintained automatically at a constant level: temperature (28 ± 0.5 °C); pH = 4.5 ± 0.1 was adjusted with 20% NaOH; dissolved oxygen concentration (pO_2) was 60% (from air saturation); agitation was 800 rpm. At regular intervals, the culture was sampled for analyses.

In CA production experiments, *Y. lipolytica* VKM Y-2373 and *Y. lipolytica* NG40/UV7 were cultivated in a 10-L ANKUM-2 M fermenter with an initial working volume of 5.0 L. The medium contained (in g/L): $(NH_4)_2SO_4$ 6.0, $MgSO_4$ $7H_2O$ 1.4, NaCl 0.5, $Ca(NO_3)_2$ 0.8, KH_2PO_4 2.0, K_2HPO_4 0.2, and Burkholder trace elements [14], Difco yeast extract 1.0, thiamine 0.02. Rapeseed oil was added, as indicated in the text. The fermentation conditions were maintained automatically at the constant level: temperature (28 ± 0.5 °C); pH = 4.5 ± 0.1 was adjusted with 25% NaOH; dissolved oxygen concentration (pO_2) was 60% (from air saturation); agitation was 800 rpm.

Analytical Methods

Biomass determination. From 10 to 50 mL of the culture liquid was filtered through Synpor filters No. 2 (Prague, Czech republic), and the yeast cells on the filters were washed with *n*-hexane to remove lipids probably adsorbed on the cells. Then membranes with the cells were dried in a vacuum desiccator at 110 °C to a constant weight. The mass of the dry cells was determined as the difference between the weights of the dried filters with cells and control dried filters without cells.

Lipid determination. The filtrate was extracted twice with *n*-hexane. The filtrate-hexane mixture was allowed to stay and separate into two phases. The upper phase contained extracted lipids in *n*-hexane, while the lower aqueous phase did not contain them. The hexane extract was collected in a glass flask and dried by passing it through a glass filter with anhydrous sodium sulfate; the solvent was evaporated to constant mass.

Glycerol was analysed enzymatically using a biochemical kit (Roche Diagnostics GmbH, Germany). 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 chromatography (HPLC) with an HPLC chromatograph (Pharmacia, LKB, Uppsala, Sweden) on an Inertsil ODS-3 reversed-phase column (250 × 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 °C.

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 the conversion of CA to oxaloacetate and its decarboxylation product pyruvate, and following the 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 the conversion of ICA to α-ketoglutarate, a reaction catalysed by isocitrate dehydrogenase.

Lipase Assay

The sample containing the culture medium and cells was filtered through a 0.20-μm filter in order to remove cells and analyze extra-cellular lipase. Lipase activity was measured by a titrimetric assay, described earlier with slight modification [8]. The substrate emulsion was prepared with rapeseed oil (m/V = 40%) and emulsifier (2% polyvinyl alcohol). The solution was emulsified in a Waring blender in order to obtain the dispersion of fat into the aqueous phase. The sample (0.2–0.5 mL) was added to the substrate emulsion (5 mL) and 50 mM phosphate buffer (pH = 8.0; 4.5 mL) and incubated for 1 h on a shaker (220 rpm) at 30 °C. Lipase activity was determined by titration of the released fatty acids with 50 mM sodium hydroxide (up to final pH = 10). The amount of enzyme that catalyzed the release of 1 μmol of fatty acids per mL per min at 30 °C was taken as the unit of lipase activity (U). Specific lipase activity was expressed as units per mg of cells (U/mg of cells).

Assay of Enzymes in Extracts of Yeast Cells

Yeast cells were collected by centrifugation at 3,000 *g* for 10 min (4 °C) and washed with an ice-cold 0.9% NaCl solution. The cell pellet was suspended in a proportion of 1:10 in 100 mM phosphate buffer (pH 7.4) supplemented with 1 mM EDTA. Cells in the suspension were disrupted with glass beads (d = 150–250 μm, BDH Chemicals LtD,

Poole, England) on a planetary mixer for 3 min at 1,000 rpm (0 °C). The cell homogenate was centrifuged at 5,000 *g* for 30 min (4 °C), and the supernatant was used for the assay 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- (EC 1.1.1.41) and NADP-(EC 1.1.1.42)-dependent isocitrate dehydrogenases, isocitrate lyase (EC 4.1.3.1), malate synthase (EC 4.1.3.2) and pyruvate dehydrogenase (EC 1.2.4.1). The activity of the enzymes was measured by standard methods with slight modifications.

The activity of glycerol kinase was determined at 340 nm as the result of NADH formation in the reaction mixture (2 mL) containing 2.5 mM glycerol, 2 mM $MgCl_2$, 2 mM ATP, 0.5 mM NAD, 0.5 U glycerol-3-phosphate-dehydrogenase, and 0.2 M glycine buffer (pH 9.8).

Citrate synthase was measured by forming the color of 5-thio-2-nitrobenzoic acid (TNB), which is generated from 5.5-dithiobis(2-nitrobenzoate) (DTNB) present in the reaction of citrate synthesis, and caused by the deacetylation of acetyl-CoA. The overall reaction product, TNB, absorbs at 412 nm. The reaction mixture (2 mL) contained 0.25 mM oxaloacetate, 0.25 mM acetyl CoA, 0.1 mM DTNB, and 100 mM Tris–HCl buffer (pH 8.5).

The activity of malate synthase was measured at 412 nm by determining the rate of glyoxylate-dependent release of free CoA from acetyl-CoA in the reaction mixture (2 mL) containing 10 mM $MgCl_2$, 0.08 mM acetyl CoA, 2.5 mM glyoxylate, 0.33 mM DTNB and 50 mM potassium phosphate buffer (pH 6.45).

Aconitate hydratase was assayed at 240 nm in the reaction mixture (2 mL) containing 5 mM monopotassium salt of *threo*-D(S)-(+)-isocitric acid and 50 mM potassium phosphate buffer (pH 7.5).

NAD-dependent isocitrate dehydrogenase was assayed at 340 nm as the result of reduction of NAD+ in the reaction mixture (2 mL) containing 0.25 mM *threo*-D(S)-(+)-isocitric acid, 4 mM NAD, 0.5 mM AMP, 10 mM $MgCl_2$, 2 mM antimycin, and 50 mM Tris–HCl buffer (pH 7.5).

NADP-dependent isocitrate dehydrogenase was assayed at 340 nm as the result of reduction of NADP+ in the reaction mixture (2 mL) containing 1 mM monopotassium salt of *threo*-D(S)-(+)-isocitric acid, 0.75 mM NADP, 10 mM $MgCl_2$, 2 mM antimycin, and 50 mM Tris–HCl buffer (pH 9.0).

Isocitrate lyase was assayed at 412 nm in the reaction mixture (2 mL) containing 4 mM *threo*-D(S)-(+)-isocitric acid, 8 mM phenylhydrazine-HCl, 4 mM cysteine-HCl, 10 mM $MgCl_2$, and 75 mM potassium phosphate buffer (pH 6.85).

Pyruvate dehydrogenase was assayed at 340 nm in the medium containing 6.4 mM NAD, 1 mM free CoA, 4.0 mM thiamin pyrophosphate, 10 mM $MgSO_4$, 10 mM cysteine, 100 mM potassium phosphate buffer (pH 7.3), 50 mM pyruvic acid potassium salt and 7.5 μg/mL antimycin A.

The amount of enzyme catalyzing the conversion of 1 μmol of substrate per min was taken as one unit of enzyme activity (U). Specific enzyme activities were expressed as units per mg protein (U/mg of protein). The amount of protein in cell-free extracts was determined by Bradford method [15].

Calculation of Fermentation Parameters

The biomass yield was calculated as follows: $Yx/s = X/S$, where X is the total amount of biomass in the culture liquid at the end of fermentation (g) and S is the amount of carbon source consumed (g).

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 oil, was calculated from:

$$Y_{CA} = \frac{P}{S} \quad (1)$$

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

$$Q_{CA} = \frac{P}{V \times t} \quad (2)$$

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

$$q_{CA} = \frac{P}{X \times t} \quad (3)$$

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

Statistical Analysis

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

Results and Discussion

The first step of utilization of oil by microorganisms is its hydrolysis by extracellular lipases with the formation of glycerol and fatty acids (Fig. 1). Glycerol is entrained into

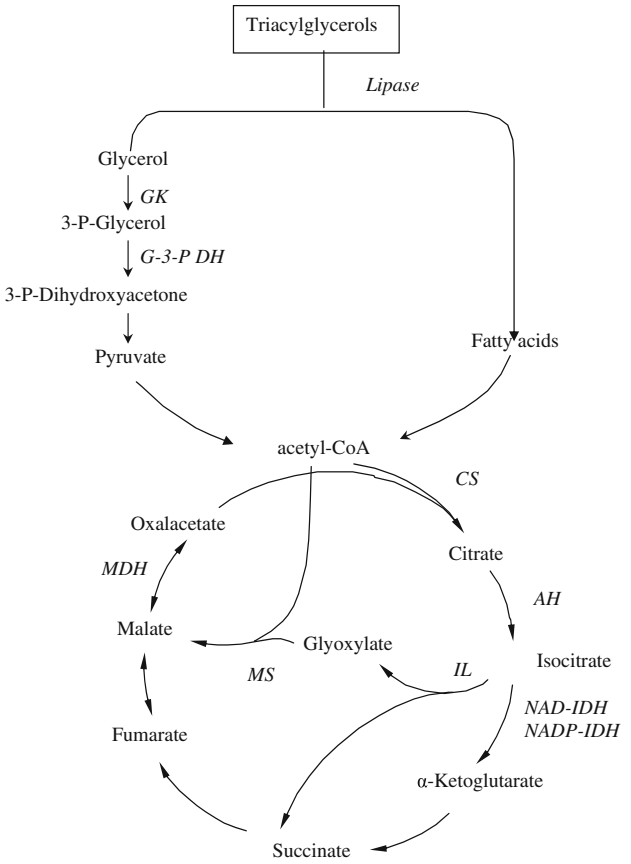

Fig. 1 Consecutive steps of triacylglycerols assimilation in *Y. lipolytica*. Enzymes: *GK* glycerol kinase, *G-3-P DH* glycerol-3-phosphate dehydrogenase, *CS* citrate synthase, *AH* aconitate hydratase, *NAD-ICDH* NAD-dependent isocitrate dehydrogenase, *NADP-ICDH* NADP-dependent isocitrate dehydrogenase, *IL* isocitrate lyase, *MS* malate synthase, *MDH* malate dehydrogenase

cellular metabolism by means of its phosphorylation by glycerol kinase [16, 17]. In microbial cells grown on glycerol, the activity of pyruvate carboxylase is high, whereas the glyoxylate cycle is almost inactive (as in the microbial cells grown on glucose) [16, 18–20]. In contrast, fatty acids enter yeast metabolism just via the glyoxylate cycle [4, 21, 22], which actively operates due to great amounts of acetyl-CoA, the main product of lipid oxidation. The high concentrations of acetyl-CoA suppress the oxidation of pyruvic acid and thus the functioning of the Krebs cycle. However, the key enzymes of the glyoxylate cycle (isocitrate lyase and malate synthase) actively catalyze the breakdown of isocitrate to succinic and glyoxylic acids with the formation of the final product, malate.

In this study, the pathway of oil utilization in the yeast *Y. lipolytica* was studied by a comparison of growth dynamics, the production of lipase, the production and consumption of glycerol and fatty acids, as well as by measuring the activity of the enzymes of glycerol metabolism and the glyoxylate cycle.

Growth of *Y. lipolytica* on Rapeseed Oil and the Dynamics of Lipase Activity

The wild-type strain *Y. lipolytica* VKM Y-2373 was grown in the medium with rapeseed oil as the single carbon source. For comparison, this strain was also grown on glycerol and oleic acid. The results are presented in Fig. 2, which shows the accumulation of biomass and lipase, and the consumption of the carbon sources. As evident from Fig. 2, immediately after the inoculation of the cultivation media, the yeast culture began to grow and the concentrations of the carbon sources began to decrease. *Y. lipolytica* showed significant growth on all substrates: rapeseed oil (10 g/L of biomass, $Y_{X/S}$ of 1.1), glycerol (9.3 g/L of biomass, $Y_{X/S}$ of 0.47), and oleic acid (8.7 g/L of biomass, $Y_{X/S}$ of 0.87). The above findings confirm the potential of *Y. lipolytica* VKM Y-2373 for the transformation of fats and the production of single-cell protein from fatty materials. In the literature, it is suggested that yield values ($Y_{X/S}$) of around 0.9 ± 0.2 g/g are very satisfactory for the single-cell protein fermentation from fatty substrates [23–26].

When *Y. lipolytica* was grown on rapeseed oil, the activity of extracellular lipase began to increase just from the start of yeast growth and remained at a high level (approximately 10 U/mg cells) during the whole cultivation period (Fig. 2). With yeast cultivation on glycerol, the activity of the lipase began to rise only when the concentration of glycerol fell to 4.0 g/L. In this case, the activity of lipase did not exceed 3.0–3.2 U/mg cells. With yeast cultivation on oleic acid, the activity of the lipase slowly increased to about the same level as during the yeast cultivation on glycerol. The inhibitory action of glycerol on lipase activity has also been reported for *Candida rugosa* [27]. As for the effect of fatty acids, experimental data available in the literature are contradictory. Some authors observed that fatty acids (namely, capric, lauric, and oleic acids) inhibited lipase activity in *C. lipolytica* [5], whereas other authors reported that oleic acid stimulated lipase activity in *C. lipolytica* [6, 7].

The early induction of lipase and the maintenance of its activity at a high level during the yeast cultivation on rapeseed oil suggest that the primary products of oil hydrolysis did not accumulate in the medium in noticeable amounts. Indeed, the concentration of glycerol in the cultivation medium varied at a very low level between 0.028 and 0.056 g/L (Fig. 2) as rapeseed oil was consumed and no accumulation of glycerol was observed. It is in agreement with our results obtained with *Y. lipolytica* yeast grown on sunflower oil: the residual glycerol remained at the trace level as sunflower oil was consumed and no accumulation of glycerol was observed [28].

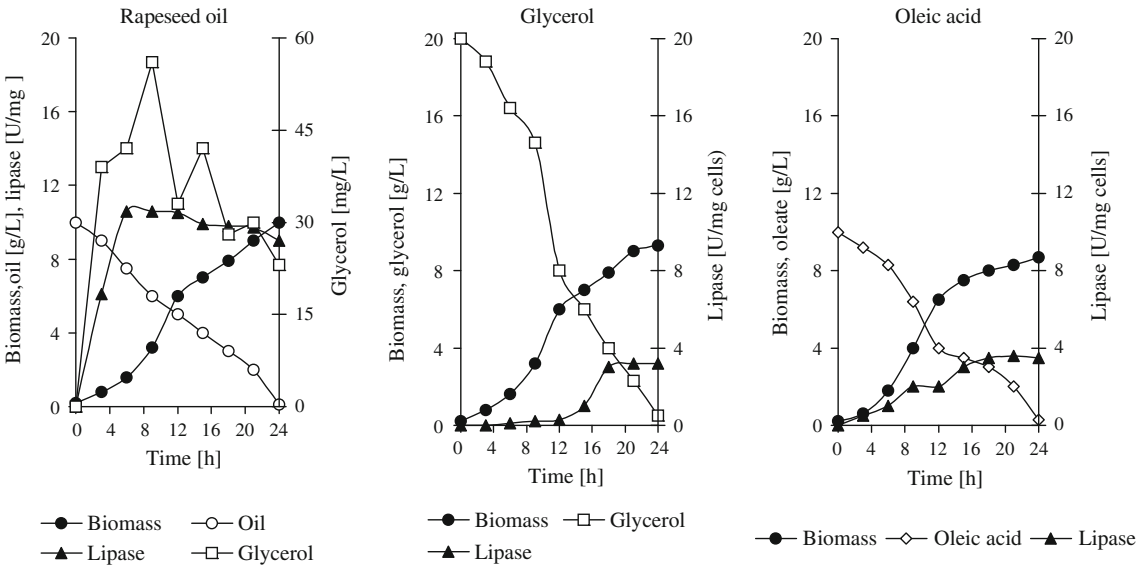

Fig. 2 Growth of *Y. lipolytica* on various carbon sources

On the basis of these results, one can suggest that glycerol and fatty acids formed from oil are consumed by yeast cells concurrently rather than by the type of diauxie, which means that the cells first effectively assimilate one of the two available substrates (commonly, a carbohydrate), whereas the assimilation of the other substrate starts only after the first substrate is fully consumed from the medium, resulting in two separate growth phases.

Enzyme Activities in *Y. lipolytica* VKM Y-2373 Grown on Rapeseed Oil, Glycerol, and Oleic Acid

The suggestion made at the end of the previous section was confirmed by the results of enzyme assay in the *Y. lipolytica* cells grown on rapeseed oil, glycerol, and oleic acid (Table 1). As early as after 6 h of yeast cultivation on the oil, the activity of glycerol kinase in the yeast cells was notably higher than that in the yeast cells grown on oleic acid, although lower by 30% than in the yeast cells grown on pure glycerol. The key enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase, which are involved in the metabolism of fatty acids, were also induced early during the yeast cultivation on the oil. The activities of these two enzymes in the 6-h-old yeast cells grown on oleic acid were higher (by 14.7 and 10 times, respectively) than those in the glycerol-grown yeast cells. In older yeast cells (12- and 24-h-old) grown on the oil, the activity of glycerol kinase was also high, probably due to the fact that glycerol produced from the oil was actively utilized during the whole cultivation period. Similarly, the high activities of isocitrate lyase and malate synthase in the 12- and 24-h-old yeast cells grown on the oil suggest that the fatty acids produced from the oil are actively metabolized during the whole cultivation period.

The active functioning of the glyoxylate cycle upon the assimilation of rapeseed oil and oleic acid is confirmed by the high activities of citrate synthase and aconitate hydratase, which operate both in the glyoxylate cycle and in the tricarboxylic acid cycle of yeast. For comparison, NAD- and NADP-dependent isocitrate dehydrogenases, which are not involved in the glyoxylate cycle, exhibit higher activities in the yeast cells grown on glycerol than on oleic acid.

These results, taken together, suggest that mixtures of glycerol and fatty acids in the medium do not suppress the metabolism of each other.

It should be noted that the concurrent utilization of two different substrates is not typical of microorganisms, which first assimilate one of the two available substrates (commonly, a carbohydrate), whereas the assimilation of the other substrate starts only after the first substrate is fully consumed from the medium.

The utilization of two different substrates was thoroughly studied in the bacterium *Escherichia coli* and, though not so well, in the yeast *Saccharomyces cerevisiae* [29, 30]. Glucose and related sugars repress the transcription of genes encoding enzymes required for the utilization of alternative carbon sources. The different sugars produce signals, which modify the conformation of certain proteins that, in turn, directly or through a regulatory cascade affect the expression of the genes subject to repression. These genes are not all controlled by a single set of regulatory proteins, but there are different circuits of repression for different groups of genes [31]. Repression allows the organism to use effectively the readily-metabolized

Table 1 Activities of enzymes of the tricarboxylic acid cycle and the glyoxylate cycle during the growth of *Y. lipolytica* VKM Y-2373 on various carbon sources

Carbon source	Time (h)	GK	TCA cycle				Glyoxylate cycle	
			CS	AH	NAD-ICDH	NADP-ICDH	IL	MS
Rapeseed oil	6	0.254	2.04	0.92	0.063	0.058	0.089	0.054
	12	0.250	2.44	1.02	0.062	0.068	0.109	0.064
	24	0.244	1.96	0.87	0.058	0.068	0.093	0.035
Glycerol	6	0.363	0.82	0.33	0.105	0.120	0.009	0.012
	12	0.350	0.90	0.43	0.115	0.132	0.009	0.014
	24	0.312	0.87	0.40	0.117	0.152	0.011	0.010
Oleic acid	6	0.112	1.70	0.53	0.110	0.072	0.133	0.105
	12	0.112	1.90	0.59	0.112	0.068	0.134	0.131
	24	0.112	1.80	0.48	0.105	0.071	0.144	0.121

Activities are expressed in U/mg protein

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

GK glycerol kinase, *CS* citrate synthase, *AH* aconitate hydratase, *NAD-ICDH* NAD-dependent isocitrate dehydrogenase, *NADP-ICDH* NADP-dependent isocitrate dehydrogenase, *IL* isocitrate lyase, *MS* malate synthase

carbohydrates in the growth medium before metabolizing other carbon sources.

The degree of repression varies very significantly in microorganisms. For example, glucose suppresses the expression of invertase in *S. cerevisiae* by 800 times, whereas the expression of aconitate hydratase, cytochrome *c* oxidase, and isocitrate dehydrogenase are suppressed no more than 10-fold [29]. The phenomenon of utilization of two different substrates in *Y. lipolytica* is poorly studied.

Growth of *Y. lipolytica* VKM Y-2373 in Media Containing Two Carbon Sources

The yeast *Y. lipolytica* was cultivated in media containing two different carbon sources: glycerol + oleic acid; glucose + oleic acid; glucose + hexadecane. The concentration of glycerol and glucose was equal to 10 g/L, the concentration of oleic acid was 5.2 g/L, and the concentration of hexadecane was 4 g/L.

As seen from Fig. 3, when *Y. lipolytica* was cultivated on the mixture of glycerol and oleic acid, the concentration of these substrates started to decrease even from the first hours of cultivation. Moreover, the utilization of these two substrates occurred concurrently, although glycerol was utilized at a higher rate than oleic acid. Glycerol kinase (the key enzyme of glycerol metabolism) and two key enzymes of the glyoxylate cycle responsible for the metabolism of fatty acids (isocitrate lyase and malate synthase) were induced from the first hours of cultivation and remained active (approximately 0.105 U/mg of protein for glycerol kinase, 0.133 U/mg of protein for isocitrate lyase and 0.1 U/mg of protein for malate synthase) until the exhaustion of glycerol and oleic acid in the medium. The

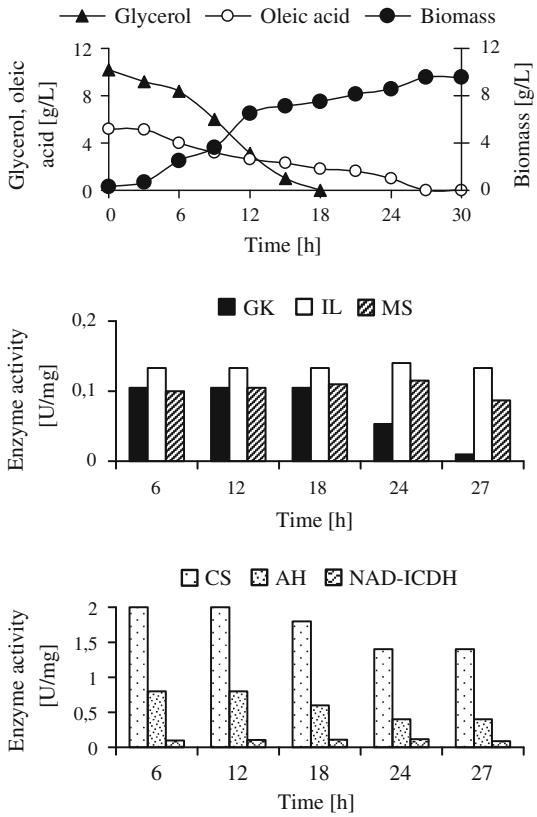

Fig. 3 The growth parameters of *Y. lipolytica* and activities of enzymes *GK* glycerol kinase, *IL* isocitrate lyase, *MS* malate synthase, *CS* citrate synthase, *AH* aconitate hydratase, *NAD-ICDH* NAD-dependent isocitrate dehydrogenase in the medium containing glycerol and oleic acid as carbon sources

active functioning of the glyoxylate cycle induced from the first hours of cultivation is confirmed by the high activities of citrate synthase (1.4–2.0 U/mg of protein) and aconitate

hydratase (0.4–0.8 U/mg of protein), which operate both in the glyoxylate cycle and in the tricarboxylic acid cycle of yeast.

These results suggest that glycerol is a more easily utilizable substrate than oleic acid and probably other fatty acids; however, glycerol does not suppress the metabolism of fatty acids. It is in agreement with the results obtained with other strains *Y. lipolytica* yeast grown on the mixture of free saturated fatty acid and glycerol [32].

In contrast, upon the cultivation of *Y. lipolytica* on the mixture of glucose and oleic acid, the latter substrate began to be utilized only when the concentration of glucose decreased from 10 to less than 2.5 g/L (at the 12th h of cultivation) (Fig. 4). The glycolytic enzyme pyruvate dehydrogenase was induced from the first hours of cultivation and remained at high levels (0.044–0.151 U/mg of protein) until the exhaustion of glucose in the medium. At the same time, the activities of isocitrate lyase and malate synthase were very low (0.01 and 0.014 U/mg of protein, respectively) during the metabolism of glucose, but were rapidly induced (approximately by 10 times) after the exhaustion of glucose in the medium. These data can be interpreted in such a manner that glucose at rather high concentrations suppresses enzymes involved in the metabolism of fatty acids.

When *Y. lipolytica* was grown on the mixture of glucose and hexadecane, the dynamics of growth and substrate consumption was typical of the diauxie phenomenon (Fig. 5). Indeed, the utilization of hexadecane began only several hours after the complete exhaustion of glucose. In this case, the exhaustion of glucose delayed growth and the culture resumed growth only after a lag period. The assay of enzymes showed that the glycolytic enzyme pyruvate dehydrogenase was active during the phase of growth on glucose, whereas the enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase, were active during the phase of growth on hexadecane. Similar results were reported by Iske et al. [33], who observed the incorporation of the radioactive label of *n*-tetradecane at later hours of cultivation; glucose as substrate was used only for the organism growth, whereas the *n*-paraffin fraction was used exclusively for the acid production. Kinetic studies of the substrate mixtures assimilation by means of a *n*-alkanes and glucose and the shift from carbohydrate substrates to hydrocarbons and reversely was the basis of the technology for CA production by *Y. lipolytica* developed in the former German Democratic Republic (GDR) in the 1980s [33–36], however, the development of this technology was completed after 1990.

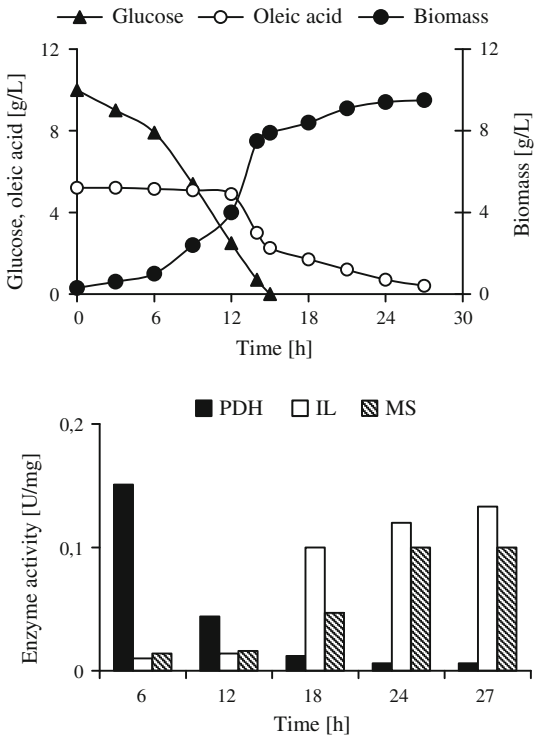

Fig. 4 The growth parameters of *Y. lipolytica* and activities of key enzymes: *PDH* pyruvate dehydrogenase, *IL* isocitrate lyase, *MS* malate synthase in the medium containing glucose and oleic acid as carbon sources

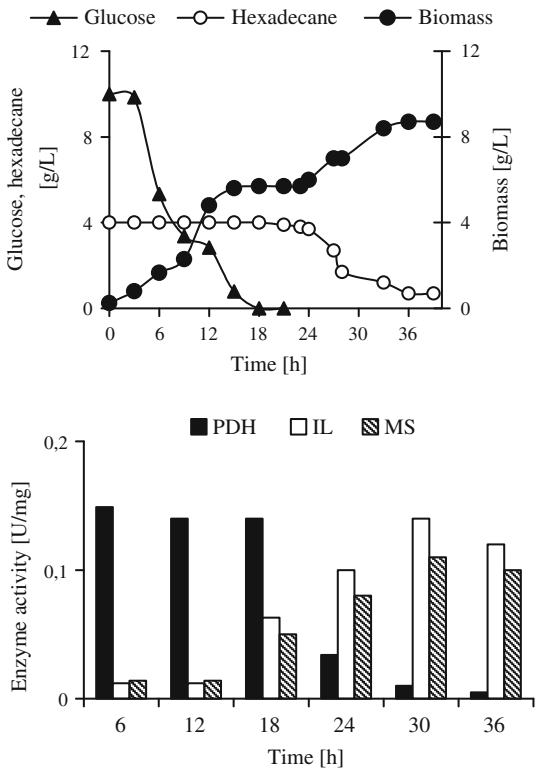

Fig. 5 The growth parameters of *Y. lipolytica* and activities of key enzymes *PDH* pyruvate dehydrogenase, *IL* isocitrate lyase, *MS* malate synthase in the medium containing glucose and hexadecane as carbon sources

The Production of Citric Acid by *Y. lipolytica*

The fact that glycerol and fatty acids can be consumed simultaneously is of special importance for the development of an efficient regime of oil feeding in CA production process by *Y. lipolytica*. According to the auxiliary concept of Babel et al. [37, 38] the mixed utilization of physiologically analogous substrates (in our case, glycerol and fatty acids) could increase the yield of the process, which is of a very important economic consideration.

The necessary condition for the synthesis of CA and its excretion into the medium is the retardation of growth of *Y. lipolytica* under the conditions of carbon excess and nitrogen deficiency in the cultivation medium. The wild strain *Y. lipolytica* VKM Y-2373 and its mutant *Y. lipolytica* NG40/UV7 were studied for CA production under optimal conditions of oil feeding under nitrogen limitation. The curves of growth of both strains, nitrogen and oil consumption, lipase activity, synthesis of CA and by-product threo-Ds-isocitric acid (ICA) are presented in Fig. 6.

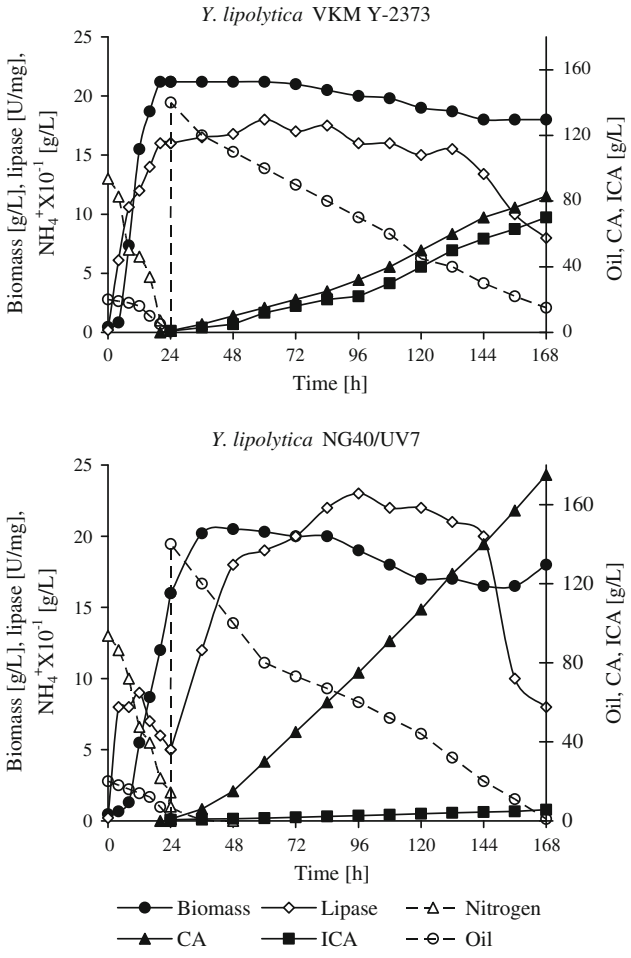

Fig. 6 Time courses of growth, lipase activity, oil and ammonium consumption, CA and ICA production by *Y. lipolytica*, grown on rapeseed oil

The initial concentration of rapeseed oil was 20 g/L and then the addition of oil (140 g/L) was performed as the pO_2 value increased by 5% indicating a decrease in respiratory activity of cells due to the total consumption of oil. Both strains showed good growth on rapeseed oil; biomass reached 21.2 g/L for *Y. lipolytica* VKM Y-2373 at 24 h and 20.0 g/L for *Y. lipolytica* NG40/UV7 at 36 h; then the yeast cultures transited to the stationary phase because of the nitrogen exhaustion in the medium. The developed regime of oil feeding provided the high lipase activity and, hence, the high rate of the oil transformation into CA during the whole process.

CA and ICA accumulated in the medium only during the stationary growth phase. At the end of cultivation (168 h) the wild strain *Y. lipolytica* VKM Y-2373 produced almost equal amounts of CA and ICA. It should be noted that no microorganisms are known to produce ICA abundantly and extracellularly except wild strains *Y. lipolytica*. Recently, Heretsch et al. [39] 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.

After 168 h of cultivation, the mutant strain *Y. lipolytica* NG40/UV7 had produced 175 g/L of CA, and 5.6 g/L of ICA, so that the CA:ICA ratio was 32:1. This is the first time that *Y. lipolytica* grown on rapeseed oil has been shown to produce CA at high concentrations with insignificant amount of the by-product ICA. Earlier, we indicated that the use of rapeseed oil resulted in the CA production of 135 g/L by *Y. lipolytica* 187/1 [8]. In experiments with the mutant strain *Saccharomycopsis 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 equal to 5.34:1.00 [40]. Aurich et al. [41] obtained a CA concentration of 198 g/L, which was achieved after a 300-h fed-batch cultivation of the mutant strain *Y. lipolytica* H181. High CA production has been reported for a mutant strain *Y. lipolytica* growing on glucose hydrols [42] and *n*-paraffins [43]. Using the raw glycerol (a byproduct of biodiesel production from rapeseed oil), Rywińska and Rymowicz [44] achieved CA production of 124.5 g/L by acetate-negative mutant *Y. lipolytica* under long-term repeated-batch cultivation.

The volumetric CA productivity (Q_{CA}) and the specific CA production rate (q_{CA}) using mutant *Y. lipolytica* NG40/UV7 reached 1.34 g/(L·h) and 0.063 g/(g/h), respectively, which corresponded to the best values reported previously for citrate-producing strains grown on fatty acid materials [8, 28, 40, 41]. The mass yield (Y_{CA}) of *Y. lipolytica* NG40/UV7 was 1.5 g of CA per g of rapeseed oil, which is comparable to those reported in the literature for citrate-producing strains [28, 40, 41].

To elucidate the mechanism of CA production from rapeseed oil by *Y. lipolytica*, the activities of enzymes were determined. Table 2 shows the enzyme activities examined in homogenates of *Y. lipolytica* VKM Y-2373 and *Y. lipolytica* NG40/UV7 taken from the exponential growth phase (12 h, no CA production) and at 72 h when active acid formation occurred. For both strains, the 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 mutant *Y. lipolytica* NG40/UV7 exhibited higher activity of citrate synthase (2.75 U/mg of protein) comparable with wild type strain *Y. lipolytica* VKM Y-2373. Activities of aconitate hydratase (0.2 U/mg of protein) and NAD-isocitrate dehydrogenase (0.018 U/mg of protein) in mutant *Y. lipolytica* NG40/UV7 were lower than that of the wild type strain *Y. lipolytica* VKM Y-2373.

The transition of both strains 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 citrate synthase (by 1.4 times for the mutant and by 1.2 times for the wild type strain) and a decrease in the activities of aconitate hydratase (by twofold for the mutant and by 1.3 times for the wild type strain) and NAD-isocitrate dehydrogenase (by 1.8 times for the mutant and by 3 times for the wild type strain).

A high activity of citrate synthase, in distinction from the other enzymes of TCA, including aconitate hydratase and NAD-isocitrate dehydrogenase, is necessary for the intensive CA production in view of the fact that CA formed in TCA can be presumably excreted from the yeast cell rather than metabolized *via* the cycle.

It seems likely that the ratio between CA and ICA is defined by the activities of citrate synthase, aconitate hydratase and NAD-isocitrate dehydrogenase. In the mutant, high activity of citrate synthase (3.881 U/mg of protein) and extremely low activity of aconitate hydratase (0.2 U/mg of protein) led to a presumable CA synthesis. At the same time, in the wild strain *Y. lipolytica* VKM Y-2373, rather high activity of aconitate hydratase (0.805 U/mg of protein) and simultaneously a low activity of NAD-isocitrate dehydrogenase (0.02 U/mg of protein) led to the excretion of both CA, and ICA.

It seems likely that NAD-isocitrate dehydrogenase plays a key role in the CA production by both strains. 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 [45]. 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; this process is enhanced by a high NADH/NAD^+ ratio. As a result, the wild type strain accumulates and excretes ICA, whereas in the mutant strain the reduced aconitate hydratase activity prevents ICA production that promotes the accumulation and excretion of CA.

Table 2 Activities of enzymes during CA production by different strains of *Y. lipolytica*

Enzymes	*Y. lipolytica* NG40/UV7		*Y. lipolytica* VKM Y-2373	
	Growth	CA production	Growth	CA production
GK	0.310	0.330	0.250	0.235
CS	2.750	3.881	2.440	2.910
AH	0.200	0.100	1.020	0.805
NAD-ICDH	0.018	0.010	0.060	0.020
NADP-ICDH	0.300	0.200	0.080	0.051
MDH	4.200	4.100	3.700	3.800
IL	0.150	0.180	0.111	0.121
MS	0.070	0.081	0.070	0.102

Activities are expressed in U/mg protein

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

GK glycerol kinase, *CS* citrate synthase, *AH* aconitate hydratase, *NAD-IDCH* NAD-dependent isocitrate dehydrogenase, *NADP-ICDH* NADP-dependent isocitrate dehydrogenase, *MDH* malate dehydrogenase, *IL* isocitrate lyase, *MS* malate synthase

Conclusions

The data on the efficient CA production from rapeseed oil by yeast *Y. lipolytica* obtained in the present work and the results reported by other authors indicate that the application of yeast organisms has considerable promise for industrial CA production. The traditional CA producer *A. niger* grown on molasses can provide for the volumetric productivity of the process of 0.8 g CA/(L·h) and Y_{CA} of 0.9 g CA per g of molasses [46], while the mutant *Y. lipolytica* NG40/UV7 synthesized CA from rapeseed oil with productivity of 1.34 g/(L·h) and yield (Y_{CA}) of 1.5 g of CA per g of rapeseed oil.

The use of yeasts instead of molds 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.

Y. lipolytica is considered as nonpathogenic organism and the CA production based on this culture may be classified as "generally recognized as safe" (GRAS) by the Food and Drug Administration (FDA, USA).

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