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Effects of Medium Components on Isocitric Acid Production by *Yarrowia lipolytica* Yeast

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Abstract: The microbiological production of isocitric acid (ICA) is more preferable for its application in medicine and food, because the resulting product contains only the natural isomer—threo-D_S. The aim of the present work was to study ICA production by yeast using sunflower oil as carbon source. 30 taxonomically different yeast strains were assessed for their capability for ICA production, and *Y. lipolytica* VKM Y-2373 was selected as a promising producer. It was found that ICA production required: the limitation of *Y. lipolytica* growth by nitrogen, phosphorus, sulfur or magnesium, and an addition of iron, activating aconitate hydratase, a key enzyme of isocitrate synthesis. Another regulatory approach capable to shift acid formation to a predominant ICA synthesis is the use of inhibitors (itaconic and oxalic acids), which blocks the conversion of isocitrate at the level of isocitrate lyase. It is recommended to cultivate *Y. lipolytica* VKM Y-2373 under nitrogen deficiency conditions with addition of 1.5 mg/L iron and 30 mM itaconic acid. Such optimized nutrition medium provides 70.6 g/L ICA with a ratio between ICA and citric acid (CA) equal 4:1, a mass yield (Y_{ICA}) of 1.25 g/g and volume productivity (Q_{ICA}) of 1.19 g/L·h.

Keywords: microbial synthesis; (2*R*,3*S*)-isocitric acid (ICA); *Yarrowia lipolytica*; aconitate hydratase (AH); iron; isocitrate lyase (ICL); inhibitors; itaconic acid; oxalic acid

1. Introduction

Isocitric acid is a structural isomer of citric acid (CA), differing from the latter in the position of the hydroxyl group. Isocitric acid molecule has two asymmetric carbon atoms and hence exists in four stereoisomers, namely, threo-D_S-isocitric acid, threo-Ls-isocitric acid, erythro-D_S-isocitric acid, and erythro-L_S-isocitric acid [1], of which only threo-D_S-isocitric acid (or (2*R*,3*S*)-isocitric acid according to IUPAC Organic Nomenclature) (conventionally named as ICA) is an intermediate of the tricarboxylic acid (TCA) cycle, a common metabolite formed by microorganisms, plants, and animals [1].

ICA and its derivatives are widely used in industries producing pharmaceutical products, and cosmetics as surfactants, detergents, ion chelators, and biologically active additives to food products [2–10]. It was found that ICA surpasses the classical antioxidant ascorbic acid in the model of oxidative stress induced by the action of hydrogen peroxide and ecotoxicants (Cu, Pb, Zn, Cd) on infusorian cells [11]. In addition, ICA relieves the neurointoxication against the high concentration of lead and molybdenum salts, restores the memory and accelerates the learning [12]. It was also reported the positive influence of ICA on the spatial component of memory [13].

It is known that ICA accumulates in significant quantities in the juice of flowering showy stonecrop *Hylotelephium spectabile*, formerly called *Sedum spectabile*, which is sold at a price of 595 EUR per gram.

At present, microbiological production of ICA is more preferable for its applications in the medicine and food industries, because the resulting product contains only the natural isomer [2,4,5,9,10].

The effective processes for ICA production were developed on the cultivation of different wild, mutant and genetically engineered strains of *Yarrowia lipolytica* yeast on n-paraffin [14], ethanol [12,15], plant oils [3,4,6,16–19], and glycerol-containing waste of biodiesel industry [13,20–22]. It should be noted that CA accumulate simultaneously with ICA. The balance between CA and ICA can be shifted toward the overproduction of isocitrate by the activation of aconitate hydratase—enzyme, which isomerizes CA to ICA via *cis*-aconitate [14,16]. For the operation of aconitase, iron ions are required [14,15,17]. The ratio between citric acids toward the ICA production can be shifted by the inhibition of isocitrate lyase with itaconic and oxalic acid in *Y. lipolytica*, grown on rapeseed oil [23], while the genetically modified strain *Y. lipolytica* with the inactivated *ICL1* gene exhibited only a small increase in the synthesis of ICA in media with glucose and glycerol [24].

In recent years, sunflower oil has attracted increased interest as a renewable resource which can be obtained in any country. Sunflower oil is an excellent substrate for biotechnological processes because it provides for a high purity of the target product with the aim of its use in medicine and food industry. High ICA production from sunflower oil was revealed in wild and genetically modified yeast *Y. lipolytica* [3,4,6]. However, the knowledge on the physiology of isocitrate-producing strains *Y. lipolytica* and the fermentation conditions essential for ICA production from sunflower oil is still limited.

The aim of the present work was the selection of producer and the evaluation of critical factors affecting *Y. lipolytica* growth and ICA production for the optimal design of fermentation process.

2. Materials and Methods

All chemicals were of analytical grade (Sigma-Aldrich, St. Louis, MO, USA). Sunflower oil "Golden Seed Oil" was purchased from the "YugRossii" Processing Plant (Rostov, Russia). The fatty acid profile of the sunflower oil was (%, by mass): C14:0, 0.1; C16:0, 6.2; C18:0, 4.1; C18:1, 13.7; C18:2, 75.9.

For screening, 30 wild-type yeast strains were obtained from the Collection of the Laboratory of Aerobic Metabolism of Microorganisms, the Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences and from the All-Russia Culture Collection of Microorganisms, Russian Academy of Sciences (VKM). The strains were maintained at 4 °C on agar slants with 1% paraffin as the carbon source. The yeast strains were maintained on agar medium with paraffin at +4 °C. Yeasts were cultivated on an orbital shaker (180 ± 10 rpm) at 29 °C during 96 h in 750-mL shaking flasks with 50 mL of the Reader medium containing (g/L): sunflower oil, 10; $(NH_4)_2SO_4$, 0.3; KH_2PO_4, 1.0; K_2HPO_4, 0.1; $MgSO_4{\cdot}7H_2O$, 0.7; $Ca(NO_3)_2{\cdot}4H_2O$, 0.4; NaCl, 0.5; trace element solution containing (mg/L): KJ, 0.1; $Na_2B_4O_7{\cdot}7H_2O$, 0.08; $MnSO_4{\cdot}5H_2O$ 0.05; $ZnSO_4{\cdot}7H_2O$ 0.04; $CuSO_4$, 0.04; Na_2MoO_4, 0.03; $FeSO_4(NH_4)_2SO_4{\cdot}6H_2O$, 0.35; yeast extract "Difco", 0.5. During cultivation, the pH of the medium was determined each 12 h and, if necessary, corrected by adding a certain volume of 10% solution of either KOH or H_2SO_4.

In experiments on optimization of ICA production by the selected strain *Y. lipolytica* VKM Y-2373, five mineral media were used: (1) Reader medium; (2) nitrogen-deficient; (3) phosphorus-deficient; (4) sulfur-deficient; (5) magnesium-deficient. The first medium contained (g/L): $(NH_4)_2SO_4$, 12.0; $MgSO_4{\cdot}7H_2O$, 1.4; $Ca(NO_3)_2$, 0.8; NaCl, 0.5; KH_2PO_4, 2.0; K_2HPO_4, 0.2; double volume of Burkholder microelement solution; yeast autolysate, 8 mL/L; thiamine, 100 µg/L. Limitation of the yeast growth was performed by decreasing concentration of the salt containing limiting component. The second medium contained nitrogen, phosphorus, sulfur and magnesium in concentrations of 630, 492, 362 and 280 mg/L, respectively. The third medium decreased (almost 30-fold) the amount of phosphorus; concentrations of nitrogen, phosphorus, sulfur and magnesium were 1960, 16.4, 362 and 280 mg/L. In the fourth medium the sulfur amount was decreased by almost 30 times; concentrations of nitrogen, phosphorus, sulfur and magnesium were 1960, 492, 12 and 280 mg/L, respectively. The fifth medium decreased (almost 50-fold) the amount of magnesium; concentrations of nitrogen, phosphorus, sulfur and magnesium

were 1960, 492, 362 and 5.6 mg/L. The initial concentration of oil was 20 g/L; pulsed additions of oil (by 20 g/L) at 24, 48 and 72 h. The fermentation conditions were maintained automatically at the constant level: temperature (29 °C); dissolved oxygen concentration (pO_2) (55–60% from air saturation); pH 6.0 was adjusted with 20% KOH. These fermentation parameters were selected on the basis of data on optimization of the ICA synthesis from rapeseed oil by *Y. lipolytica* [17]. Cultivation was performed for 96 h.

The effect of inhibitors on the metabolization and production of ICA and CA was studied in experiments carried out in test tubes and in a fermenter. In the first case, *Y. lipolytica* VKM Y-2373 were cultivated on an orbital shaker (180 ± 10 rpm) at 29 °C during 48 h in large test tubes (20 cm long, 2 cm in diameter) with 5 mL Reader medium containing sunflower oil (5 g/L) as carbon substrate. In the second case, *Y. lipolytica* was cultivated in the 10-L fermenter with 5 L of medium containing (g/L): $(NH_4)_2SO_4$, 3; $MgSO_4 \cdot 7H_2O$, 1.4; $Ca(NO_3)_2$, 0.8; NaCl, 0.5; KH_2PO_4, 2.0; K_2HPO_4, 0.2; double volume of Burkholder microelement solution; yeast autolysate, 8 mL/L; thiamine, 100 µg/L and $FeSO_4 \cdot 7H_2O$, 7.5 mg/L. The fermentation conditions were maintained automatically at the constant level: temperature (29 °C); dissolved oxygen concentration (pO_2) (55–60% from air saturation); pH 6.0 was adjusted with 20% KOH. Cultivation was performed for 96 h.

The amount of biomass, oil was determined as described earlier [17].

Concentration of organic acids (pyruvic acid, citric acid, α-ketoglutaric acid, fumaric acid, malic acid, oxaloacetic acid, itaconic acid, oxalic acid) 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, Moscow, 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. Acids were also identified using the standard solutions (Roche Diagnostics GmbH. Germany). Glucose 6-phosphate was determined by enzymatic assay with glucose-6-phosphate dehydrogenase. Fructose 1,6-bisphosphate was determined by fructose based on the Seliwanoff color reaction.

For the enzyme assay, yeast cells were collected by centrifugation at 3000× *g* for 10 min (4 °C) and washed with an ice-cold 0.9% NaCl solution. The cell pellet was suspended in a proportion of 1:10 in 100 mM phosphate buffer (pH 7.4) supplemented with 1 mM EDTA. Cells in the suspension were disrupted with Ballotini glass beads (d = 150–250 µ) on a planetary mixer for 3 min at 1000 rpm (0 °C). The cell homogenate was centrifuged at 5000 g for 30 min (4 °C), and the supernatant was used for the assay of citrate synthase (CS), aconitate hydratase (AH), NAD-isocitrate dehydrogenase (NAD-ICDH) and isocitrate lyase (ICL). CS was assayed in the reaction mixture containing 0.25 mM oxaloacetate, 0.25 mM acetyl CoA, 0.1 mM 5.5-dithiobis(2-nitrobenzoate), and 100 mM Tris-HCl buffer (pH 8.5). AH was assayed in the reaction mixture containing 5 mM ICA and 50 mM potassium phosphate buffer (pH 7.5). NAD-ICDH was assayed in the reaction mixture containing 0.25 mM ICA, 4 mM NAD, 0.5 mM AMP, 10 mM $MgCl_2$, 2 mM antimycin, and 50 mM Tris-HCl buffer (pH 7.5). ICL was assayed in the reaction mixture containing 4 mM ICL, 8 mM phenylhydrazine-HCl, 4 mM cysteine-HCl, 10 mM $MgCl_2$, and 75 mM potassium phosphate buffer (pH 6.85). The amount of enzyme catalyzing conversion of 1 µmol of substrate per min per mg of protein was taken as one unit of enzyme activity (U/mg of protein).

The mass yield coefficient of ICA production (Y_{ICA}), expressed in g of ICA per g of sunflower oil consumed, was calculated from:

$$Y_{ICA} = P/S$$

The volume productivity (Q_{ICA}), expressed in g/L·h, was calculated from:

$$Q_{ICA} = P/(V \cdot t)$$

where P is the total amount of ICA in the culture liquid at the end of cultivation (g), S is the total amount of sunflower oil consumed (g), V is the initial volume of culture liquid (L), t is the fermentation duration (h).

All the data presented are the means values of three experiments and two measurements for each experiment; standard deviations were calculated (S.D. < 10%). The ICA/CA ratio, the mass yield coefficient of ICA production (Y_{ICA}) and the volume productivity (Q_{ICA}) are calculated using the mean value of ICA.

3. Results and Discussion

3.1. Selection ICA Producers

Screening of yeasts actively producing ICA from sunflower oil was carried out 30 natural strains belonging to 18 species. Yeasts were cultivated under limitation of cell growth by nitrogen (67 mg/L) and excess of sunflower oil (10 g/L). It should be noted that in this study, for the first time, a wide range of yeast species was tested for their ability to produce ICA from sunflower oil, whereas until now, the studies on ICA production were carried out mainly with Yarrowia lipolytica (syn. Candida lipolytica) [3,4,6]. On other substrates (n-alkanes, glucose, rapeseed oil), the acid-producing activity was found in yeast species *Meyerozyma guilliermondii* (syn. *Candida guilliermondii*), *Candida ravautii*, *Diutina catenulata* (syn. *Candida brumptii*, *C. catenulata*) and *Y. lipolytica* [17,25–27].

As seen from Table 1, all strains grew well in a medium with sunflower oil as only carbon source. However, the strains were heterogeneous in ICA production. Thirteen strains belonging to species *Aciculoconidium aculeatum*, *Babjeviella inositovora*, *Candida intermedia*, *C. parapsilosis*, *C. tropicalis*, *C. utilis*, *Diutina catenulata, D. rugosa*, *Pichia besseyi*, *P. media*, *P. membranifaciens*, *Sugiyamaella paludigena* and *Wickerhamomyces anomalus* not produced ICA. Strains belonging to species *Blastobotrys adeninivorans*, *Candida zeylanoides, Meyerozyma guilliermondii* and *Y. lipolytica* produced ICA in the amounts from 0.14 to 6.3 g/L. The strain *Y. lipolytica* VKM Y-2373 synthesized the maximum amount of ICA (6.3 g/L). It should be noted that, acid-producing yeasts synthesized also a by-product, CA; a ratio of ICA to CA varied from 1.3: 1 to 1: 2.9, and was the best (1.3:1) in *Y. lipolytica* VKM Y-2373.

Out of 30 strains studied, we selected strain *Y. lipolytica* VKM Y-2373 for further optimization of ICA production.

3.2. Effect Limiting Factors on ICA Production

Numerous studies have noted that the ability of the yeast *Y. lipolytica* to produce citric acids was found under nitrogen deficiency [2,4,5,9,10,28–30].

The present study deals with ICA synthesis in the selected strain by using various growth-limiting components such as nitrogen, phosphorus, sulfur and magnesium. As seen from Table 2, when *Y. lipolytica* VKM Y-2373 was grown in a Reader medium without limitation, no acid formation occurred; under these conditions the cells grew well, and the biomass reached 27.7 g/L. When growth of *Y. lipolytica* VKM Y-2373 was limited by nitrogen, phosphorus, sulfur and magnesium, then concentration of these elements was selected so that the biomass did not exceed 10–12 g/L, since at a higher biomass, the main factor limiting growth is the oxygen deficiency. To fulfill this condition, the nitrogen concentration was decreased 4-fold, phosphorus and sulfur—30-fold, magnesium—50-fold. Under nitrogen limitation, *Y. lipolytica* VKM Y-2373 synthesized 46.97 g/L of ICA and 35.67 g/L of CA; a ratio of ICA to CA was 1.3:1. Similar data were found with a deficiency of phosphorus and sulfur; a ratio of ICA to CA varied from 1.1:1 to 1.2:1. The limitation of *Y. lipolytica* VKM Y-2373 growth by magnesium led to the predominant accumulation of ICA (a ratio of a ratio of ICA to CA was 3:1); however, the amount of ICA was decreased in 2.3 times compared with one under nitrogen deficiency.

Table 1. Screening of yeasts producing of isocitric acid (ICA) from sunflower oil.

Strains	Biomass (g/L)	ICA (g/L)	CA (g/L)	ICA/CA Ratio
Aciculoconidium aculeatum VKM Y-1301	4.43 ± 0.35	0	0.53	n.d.
Babjeviella inositovora VKM Y-2494	4.60 ± 0.53	0	0	n.d.
Blastobotrys adeninivorans VKM Y-2676	4.90 ± 0.56	0.60 ± 0.10	1.64 ± 0.07	1:2.7
C. intermedia	4.20 ± 0.60	0	0	n.d.
C. parapsilosis	4.10 ± 0.66	0	0	n.d.
C. tropicalis 303	3.00 ± 0.56	0	0	n.d.
C. utilis VKM Y-33	1.47 ± 0.50	0	0	n.d.
C. zeylanoides VKM Y-6	2.21 ± 0.50	0.61 ± 0.12	1.22 ± 0.12	1:2
C. zeylanoides VKM Y-14	2.20 ± 0.20	1.11 ± 0.20	2.22 ± 0.13	1:2
C. zeylanoides VKM Y-2324	2.57 ± 0.21	1.12 ± 0.12	1.90 ± 0.20	1:1.7
Diutina catenulata VKM Y-5	4.70 ± 0.20	1.30 ± 0.20	2.60 ± 0.20	1:2
D. catenulata VKM Y-36	2.30 ± 0.20	1.10 ± 0.20	1.1 ± 0.20	1:1
D. rugosa VKM Y-67	2.03 ± 0.35	0	0	n.d.
Meyerozyma guilliermondii	5.20 ± 0.56	0.80 ± 0.10	2.35 ± 0.10	1:2.9
Pichia besseyi VKM Y-2084	2.15 ± 0.15	0	0	n.d.
P. media VKM Y-1381	2.55 ± 0.15	0	0	n.d.
P. membranifaciens VKM Y-292	3.01 ± 0.40	0	0	n.d.
Sugiyamaella paludigena VKM Y-2443	2.65 ± 0.35	0	0	n.d.
Wickerhamomyces bisporus VKM Y-1065	2.90 ± 0.25	0	0	n.d.
Y. lipolytica VKM Y-2412	2.00 ± 0.21	2.10 ± 0.35	1.10 ± 0.16	1:1
Y. lipolytica VKM Y-2373	1.70 ± 0.17	6.30 ± 0.61	4.84 ± 0.39	1.3:1
Y. lipolytica 68	2.37 ± 0.23	0.14 ± 0.01	0.16 ± 0.02	1:1.1
Y. lipolytica 69	2.60 ± 0.22	2.90 ± 0.26	2.21 ± 0.21	1.3:1
Y. lipolytica 374/4	2.40 ± 0.24	2.46 ± 0.40	2.66 ± 0.29	1:1.1
Y. lipolytica 571	3.50 ± 0.29	1.1 ± 0.18	1.07 ± 0.10	1:1
Y. lipolytica 581	2.40 ± 0.21	1.26 ± 0.24	1.73 ± 0.27	1:1.4
Y. lipolytica 585	1.95 ± 0.20	1.20 ± 0.19	1.52 ± 0.25	1:1.3
Y. lipolytica 607	2.20 ± 0.20	1.80 ± 0.60	1.7 ± 0.44	1:1.1
Y. lipolytica 684	2.78 ± 0.40	2.10 ± 0.60	2.0 ± 0.24	1.1:1
Zygoascus hellenicus VKM Y-2007	2.70 ± 0.20	0	0	n.d.

n.d.—not determined.

Table 2. Effect of limiting component on the growth of *Y. lipolytica* VKM Y-2373 and ICA production.

Parameter	Limiting Component (mg/L)				
	Full Medium	N	P	S	Mg
	2545 (N) 492 (P) 362 (S) 280 (Mg)	630 (N) 492 (P) 362 (S) 280 (Mg)	1960 (N) 16.4 (P) 362 (S) 280 (Mg)	1960 (N) 492 (P) 12 (S) 280 (Mg)	1960 (N) 492 (P) 362 (S) 5.6 (Mg)
Time (h)	48	96	96	96	96
Biomass (g/L)	27.7 ± 3.3	9.83 ± 0.15	8.23 ± 0.15	8.40 ± 0.20	9.80 ± 0.20
ICA (g/L)	1.37 ± 0.12	46.97 ± 2.73	38.0 ± 1.1	40.36 ± 1.86	20.83 ± 0.57
CA (g/L)	1.37 ± 0.10	35.67 ± 1.05	32.36 ± 2.25	38.46 ± 0.31	6.40 ± 0.36
ICA/CA ratio	1:1	1.3:1	1.2:1	1.1:1	3.3:1
Y_{ICA} (g/g)	n.d.	0.83	0.67	0.71	0.37
Q_{ICA} (g/L·h)	n.d.	0.79	0.64	0.68	0.35

n.d.—not determined.

The obtained data demonstrate that limitation of *Y. lipolytica* growth by biogenic macroelements (N, P, S and Mg) is the effective method for regulation of the acid production. At the same time, it is noted that the requirement of limiting this components in the nutrition medium can be a problem for citric acids production using carbon sources enriched by these elements [30]. All further experiments were carried out under nitrogen deficiency because it showed the maximum values of the ICA yield (Y_{ICA} = 0.83 g/g) and volume productivity (Q_{ICA} = 0.79 g/L·h).

3.3. Effect of Iron

Aconitate hydratase (AH) is a key enzyme which involved in the synthesis of isocitrate in TCA cycle. It was reported the genetically modified strain *Y. lipolytica* with the superexpressed *ACO1* gene cultivated on sunflower oil showed a higher content of ICA (66–71%) in comparison with the wild-type strain *Y. lipolytica* H222 (35–49% ICA) [16]. This transformant produced 68.4 g/L ICA (75.6% of total acids) with the yield Y_{ICA} = 0.64 g/g [6].

It is known that iron is an essential element for active functioning AH [2,9]. As can be seen from Table 3, the cell growth was observed in iron-free medium that apparently due to a low amount of iron available from the sunflower oil. An iron concentration in the range 0–0.05 mg/L was growth-limiting. This is confirmed by a direct correlation between iron concentration and biomass density. An increase in iron concentration from 0.05 to 6 mg/L showed no effect on biomass level. An increase iron concentration up to 10 mg/L led to a forced growth and the biomass production at level of 15.73 g/L. Earlier it was reported that an increased iron content (10–50 mg/L) promotes the accumulation of biomass in the erythritol-producing *Y. lipolytica* grown on glycerol [31].

Table 3. Effect of iron on the growth of *Y. lipolytica* VKM Y-2373 and ICA production.

Parameter	Concentration of Iron (mg/L)					
	Without Fe	**0.05**	**1.5**	**3.0**	**6.0**	**10.0**
Biomass (g/L)	2.83 ± 0.15	9.83 ± 0.15	10.67 ± 0.15	11.10 ± 0.36	10.73 ± 0.78	15.73 ± 0.15
ICA (g/L)	10.97 ± 0.73	46.97 ± 2.73	51.50 ± 1.5	48.90 ± 3.57	48.50 ± 0.50	35.67 ± 2.04
CA (g/L)	10.67 ± 1.25	35.67 ± 1.05	30.23 ± 0.60	28.60 ± 0.62	28.73 ± 1.15	20.58 ± 2.31
ICA/CA ratio	1:1	1.3:1	1.7:1	1.7:1	1.7:1	1.7:1
Y_{ICA} (g/g)	0.19	0.83	0.91	0.86	0.86	0.63
Q_{ICA} (g/L·h)	0.18	0.79	0.87	0.80	0.81	0.62
Enzyme activity (U/mg protein)						
CS	2.20 ± 0.30	2.25 ± 0.35	2.66 ± 0.20	2.62 ± 0.40	2.70 ± 0.30	2.75 ± 0.25
AH	0.20 ± 0.02	0.25 ± 0.03	0.95 ± 0.15	0.93 ± 0.25	0.85 ± 0.16	0.88 ± 0.07
NAD-ICDH	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
ICL	0.11 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.12 ± 0.01

As seen from Table 3, high ICA concentration (46.97–51.5 g/L) and the mass yield (Y_{ICA}) (0.83–0.91 g/g) were found at iron concentration between 0.05 and 0.6 mg/g. An increase in iron from 6 to 10 mg/L imposed a decrease in ICA production for 25%.

As seen from Table 3, under iron limitation in the range 0–0.05 mg/L, AH was insignificant (0.2–0.25 U/mg protein). An increase in iron concentration from 0.05 to 10 mg/L resulted in AH activity in 3.4–3.8 times. In this case, CS activity increased only for 22%. NAD-ICDH remained practically constant at low level (0.02–0.03 U/mg protein). ICL which is involved in the metabolism of fatty acids, was high (0.11–0.12 U/mg protein) in all variants of experiment.

Further experiments were carried out with the optimal concentration of iron in the cultivation medium equal to 1.5 mg/L.

3.4. Inhibition of Isocitrate Lyase

It is known that the ratio between citric acids toward the ICA production can be shifted by the inhibition of isocitrate lyase (ICL) [2,13–15,23,24]. Yeast ICL is a constitutive enzyme subject to catabolite repression [32,33]. ICL is induced when the yeast grows on acetate, n-alkanes, ethanol and fatty acids [21,32–38]. At the same time, glucose in the cultivation medium suppresses ICL [36,37,39]. If the medium contains a mixture of hexadecane and glucose, ICL is suppressed until glucose is exhausted and then is induced providing the consumption of hexadecane [37]. In contrast, the glyoxylate cycle in *Candida tropicalis* functions even in the presence of glucose in the medium; this yeast first transforms carbohydrates to fatty acids, which then are utilized through β-oxidation and the glyoxylate

cycle [40]. The study of the genetic mechanisms of ICL in *Escherichia coli* showed that the *aceA* gene of the acetate operon by the special repressor protein IclR [41]. The ICL gene responsible for the synthesis of ICL was isolated and cloned from the yeast *Saccharomyces cerevisiae* [42], the fungus *Aspergillus nidulans* [43], the yeast *Y. lipolytica* [44] and other microorganisms. The genetic studies of *S. cerevisiae* showed that glucose suppresses ICL with the aid of ten amino acids inside the polypeptide chain with the involvement of the cAMP-dependent protein kinase [45,46]. The promoter region necessary for the activation of ICL synthesis is common to the genes encoding the enzymes involved in gluconeogenesis [47]. The deletion of the *ICL1* gene from the genome of *Y. lipolytica* not only inhibits the assimilation of acetate, ethanol and fatty acids, but also reduces the rate of growth of this yeast on glucose [44].

The task of this section was to find an inhibitor of ICL, which could not be metabolized by cells. The published data on ICL inhibitors are discussed in detail below. ICL of *C. guillermondii* was found to be inhibited by low concentrations of glucose-6-P (Ki = 0.8 mM), the degree of inhibition being decreased by high concentrations of ICA. Glucose-6-P also inhibits the ICL of *C. tropicalis* but Ki is considerably higher (Ki = 4–5 mM) [48]. The effect of glucose-6-P on ICL is explained by the inhibition of the first enzyme of gluconeogenesis from C2-compounds by the end product, and hence can be considered as retroinhibition [49]. The strong inhibitor of yeast ICL is 6-phosphogluconate, whose inhibitory action is complete and competitive. This fact is explained by the structural and steric similarity of 6-phosphogluconate and ICA [49]. Fructose-1,6-bisphosphate is an inhibitor of ICL; Ki for *C. tropicalis* is equal to 5 mM [48]. Phosphoenolpyruvate is a strong allosteric inhibitor of ICL. When yeasts are cultivated in the medium with glucose or intermediates of gluconeogenesis, phosphoenolpyruvate can be an intermediate of glycolysis provided that the yeast cells utilize noncarbohydrate carbon sources [32]. The inhibitor constant (Ki) for phosphoenolpyruvate is low: 0.22 mM for *E. coli* [50], and 0.6 mM for *Phycomyces blakesleeanus* [51]. On the other hand, ICL of *Ashbya gossypii* is weakly (by 55%) inhibited by 10 mM phosphoenolpyruvate [52]. The data available in the literature for yeasts show that phosphoenolpyruvate is a noncompetitive inhibitor of ICL, the inhibitor constant Ki being different for different yeasts. For example, Ki for *S. cerevisiae* is equal to 1–4 mM [49], for *C. guilliermondii*, 1.6 mM [53], for *C. tropicalis*, 5 mM [48]. Pyruvate is also a strong inhibitor of ICL, Ki for ICL from *C. guilliermondii* being equal to 0.8 mM [53], and that from *C. tropicalis*, 5.0 mM [48]. ICL is also inhibited by TCA cycle intermediates: citrate (≥5 mM for *C. tropicalis* [48] and 10 mM for *C. guilliermondii* [53]); α-ketoglutarate (≥5 mM for *C. tropicalis* [48]); fumarate (9 mM for *C. guilliermondii* [53]) and malate 9 mM for *C. guilliermondii* [53]).The ICL of *C. guilliermondii* is strongly inhibited by 0.1 mM oxaloacetate (Ki = 0.05 mM) [53]. But in *C. tropicalis*, ICL is also inhibited by oxaloacetate, but to a lower degree (Ki = 5.0 mM) [48]. ICL is inhibited by the excess of its substrate and products (ICA, glyoxylate and succinate) [33,48,53–56].

The inhibitory action of itaconate (a structural analogue of succinate) and oxalate (a structural analogue of glyoxylate) is well known. Experiments with the permeabilized cells of *Pseudomonas indigofera* showed that itaconic acid specifically inhibits ICL and is not metabolized by the cells [55]. Ki for ICL from different cultures was found to be very different. For example, Ki = 100 mM for the fungus *Mucor racemosus* grown on acetate [57]. Oxalate and itaconate strongly inhibited the ICL of *Ashbya gossypii* (Ki = 4 μM and 127 μM, respectively) [52] and that of the basidiomycete *Fomitopsis palustris* (Ki = 37 μM and 68 μM, respectively) [58]. The inhibitory action of itaconate on ICL and the formation of ICA were found in the wild strain *C. lipolytica* grown on n-alkanes [2,59]. We reported that itaconate shifts of acid formation toward the preferential synthesis of ICA from rapeseed oil and increased the ratio ICA/CA by 6 times [23]. However, this inhibitor elevated the ICA/CA ratio only by 1.5 times for *Y. lipolytica*, grown on biodiesel waste [13].

Up to yet the metabolization of all the aforementioned inhibitors by *Y. lipolytica* were not studied in details.

We carried out experiments to study the metabolization of putative inhibitors noted by *Y. lipolytica* VKM Y-2373.

As can be seen from Table 4, when inhibitors were added to the medium in an amount of 0.2–2.1 g/L simultaneously with the main source—sunflower oil (5 g/L), putative ICL inhibitors were quickly assimilated and included in the metabolism. However, *Y. lipolytica* VKM Y-2373 does not metabolize only two compounds—itaconic and oxalic acids. These acids introduced into the medium at the beginning of cultivation are found in the medium after the complete consumption of sunflower oil.

Table 4. The metabolization of putative inhibitors by *Y. lipolytica* VKM Y-2373, grown on sunflower oil.

Inhibitor	Concentration in Cultural Medium (g/L)	
	Initial	Residual
Glucose 6-phosphate	1.1	0
Fructose 1,6-bisphosphate	2.1	0
Pyruvic acid	0.2	0
Citric acid	0.6	0
α-Ketoglutaric acid	0.4	0
Fumaric acid	0.3	0
Malic acid	0.5	0
Oxaloacetic acid	0.4	0
Itaconic acid	0.4	0.4
Oxalate	0.4	0.4

In subsequent experiments we examined the effect of itaconate, oxalate, as well as their combined effect on the growth and ICA production of *Y. lipolytica* VKM Y-2373.

As seen from Table 5, the concentration of itaconate in the medium should not exceed 40 mM, since at higher concentrations (50 mM), the biomass accumulation decreased by 1.3 times, as compared to control experiment, when the inhibitor was not added to the medium. With the addition of 30 mM itaconic acid, ICA production increased from 51.5 to 70.60 g/L. A further increase in the concentration of itaconate to 50 mM and more led to inhibition of ICA synthesis in 2.3 times. At 30 mM itaconate *Y. lipolytica* VKM Y-2373 shifted towards ICA (ICA/CA = 4:1 in comparison with a ratio of 1.7:1 without inhibitor. The maximum values of the ICA yield (Y_{ICA} = 1.25 g/g) and volume productivity (Q_{ICA} = 1.19 g/L·h) were obtained at 30 mM itaconic acid. The study of enzyme activity revealed that an addition of 20–50 mM itaconic acid resulted in a 12-fold decrease in activity of ICL, while CS, AH and NAD-ICDH slowly down.

Table 5. Effect of itaconic acid on the growth of *Y. lipolytica* VKM Y-2373 and ICA production.

Parameter	Concentration of Itaconic Acid (mM)				
	0	20	30	40	50
Biomass (g/L)	10.67 ± 0.15	10.1 ± 1.15	10.85 ±1.15	9.7 ± 1.10	7.7 ± 1.20
ICA (g/L)	51.50 ± 1.5	62.70 ± 4.45	70.60 ± 2.15	51.00 ± 3.45	31.00 ± 6.15
CA (g/L)	30.23 ± 0.60	23.50 ± 2.15	17.50 ± 1.15	12.75 ± 2.25	7.75 ± 2.15
ICA/CA ratio	1.7:1	2.8:1	4:1	4:1	4:1
Y_{ICA} (g/g)	0.91	1.11	1.25	0.90	0.55
Q_{ICA} (g/L·h)	0.87	1.06	1.19	0.86	0.52
Enzyme activity (U/mg protein)					
CS	2.66 ± 0.20	2.30 ± 0.35	2.20 ± 0.35	2.20 ± 0.35	2.10 ± 0.20
AH	0.95 ± 0.15	0.85 ± 0.07	0.85 ± 0.07	0.80 ± 0.17	0.75 ± 0.10
NAD-ICDH	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
ICL	0.12 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01

As seen from Table 6, oxalic acid had a similar, but even weaker, effect for ICA production. An addition of 30 mM oxalic acid resulted in inhibition of isocitrate lyase (8-fold), whereas a shift in ratio between formed ICA and CA consisted of 2.85:1 and ICA production was only 23% higher

than in the absence of the inhibitor. It can be assumed that the weaker effect of oxalate in comparison with itaconate on *Y. lipolytica* was associated with the different permeability of the cell wall for these two anions.

Table 6. ICA production by *Y. lipolytica* cultivated on sunflower oil with and without inhibitors of ICL.

Parameter	With Inhibitor			
	Without Inhibitor	**Itaconic Acid 30 mM**	**Oxalic Acid 30 mM**	**Itaconic Acid + Oxalic Acid**
Biomass (g/L)	10.67 ± 0.15	10.85 ± 1.15	9.80 ± 0.75	6.10 ± 0.65
ICA (g/L)	51.50 ± 1.5	70.60 ± 2.15	62.80 ± 4.75	68.00 ± 2.20
CA (g/L)	30.23 ± 0.60	17.50 ± 1.15	22.20 ± 2.15	17.00 ± 2.15
ICA/CA ratio	1.7:1	4:1	2.9:1	4:1
Y_{ICA} (g/g)	0.91	1.25	1.11	1.2
Q_{ICA} (g/L·h)	0.87	1.19	1.05	1.15
Enzyme activity (U/mg protein)				
CS	2.66 ± 0.20	2.20 ± 0.35	2.70 ± 0.20	2.20 ± 0.25
AH	0.95 ± 0.15	0.85 ± 0.07	0.78 ± 0.10	0.80 ± 0.10
NAD-ICDH	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.02	0.03 ± 0.01
ICL	0.12 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01

The combined effect of itaconic and oxalic acids on the production of ICA by *Y. lipolytica* VKM Y-2373) was first studied in this work. As evident from the comparison of biotechnological parameters shown in Table 6, the combined effect of inhibitors practically do not affect ICA production.

The presented results demonstrate that ICA synthesis is associated with high activities of citrate synthase and aconitate hydratase and low activities of NAD-isocitrate dehydrogenase and isocitrate lyase. Recently it was reported the genetically modified strain with the superexpressed *CIT1* and *CIT2* genes produced citric and isocitric acid from vegetable oil in a ratio close to 1:1while CA/ICA ratio for wild-type strain was 4.12:1 [19].

Y. lipolytica VKM Y-2373 is recommended to cultivate under nitrogen deficiency conditions with addition of iron (1.5 mg/L) and 30 mM itaconic acid. Such nutrition medium provides 70.6 g/L ICA with a ratio of 4:1, a mass yield (Y_{ICA}) of 1.25 g/g and volume productivity (Q_{ICA}) of 1.19 g/L·h.

4. Conclusions

Thus, *Y. lipolytica* VKM Y-2373 is as a perspective producer of ICA from sunflower oil. The intensive ICA production occurred only under the limitation of cell growth by nitrogen, phosphorus, sulfur or magnesium and the excess sunflower oil. The concentration of iron (activator of AH) and itaconic acid (inhibitor of ICL) are effective factors controlling the ICA synthesis in *Y. lipolytica*. Under chosen concentration of iron (1.5 mg/L) and itaconic acid (30 mM), the selected strain produced up to 70.6 g/L ICA with a ratio between ICA and CA equal 4:1, a mass yield (Y_{ICA}) of 1.25 g/g and volume productivity (Q_{ICA}) of 1.19 g/L·h.

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