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Biosynthesis of isocitric acid in repeated-batch culture and testing of its stress-protective activity

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Abstract

Biosynthesis of Ds(+)-threo-isocitric acid from ethanol in the *Yarrowia lipolytica* batch and repeated-batch cultures was studied. Repeated-batch cultivation was found to provide for a good biosynthetic efficiency of the producer for as long as 748 h, probably due to maintenance of high activities of enzymes involved in the biosynthesis of isocitric acid. Under optimal repeated-batch cultivation conditions, the producer accumulated 109.6 g/L Ds(+)-threo-isocitric acid with a production rate of 1.346 g/L h. The monopotassium salt of isocitric acid isolated from the culture liquid and purified to 99.9% was found to remove neurointoxication, to restore memory, and to improve the learning of laboratory rats intoxicated with lead and molybdenum salts. Taking into account the fact that the neurotoxic effect of heavy metals is mainly determined by oxidative stress, the aforementioned favorable action of isocitric acid on the intoxicated rats can be explained by its antioxidant activity among other pharmacological effects.

Keywords Microbial synthesis · Ds(+)-threo-isocitric acid · Repeated-batch cultivation · Antioxidants · Stress-protective activity

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Introduction

There is an ever-increasing search for nontoxic original innovative compounds which can be produced by microbial synthesis at a relatively large scale and which can find wide application in medicine, food industry, and agriculture, that is, where there are high requirements to the purity of such compounds, to their stereoisomeric and isomeric composition, and to their ability to be metabolized in the cells to carbon dioxide and water without accumulation (Stottmeister et al. 2005; Sauer et al. 2008; Groenewald et al. 2014; Zinjarde 2014; Papanikolaou et al. 2017; Kawasaki and Ueda 2017; Morgunov et al. 2017; Koller 2018). Many countries even prohibit the relevant usage of chemically synthesized compounds.

According to data available in the literature (Demain and Martens 2017), up to 22,500 of biologically active compounds are produced by microbiological synthesis. Among them, there is Ds(+)-threo-isocitric acid or (1R,2S)-1-hydroxy-1,2,3-propanetricarboxylic acid, abbreviated as ICA, produced by the yeast *Yarrowia lipolytica*. ICA can be used (1) as a raw material for the production of ascorbic acid, (2) as a biochemical marker of some diseases, (3) as a therapeutic agent in the treatment of Fe-deficiency anemia, (4) for the resorption of blood clots, and (5) for treatment of drug and

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alcohol abuse (Finogenova et al. 2005; Aurich et al. 2012, 2017; Heretsch et al. 2008; Bullin et al. 2018). Recently, we also found that Ds(+)-threo-ICA is a good protector of damage caused by hydrogen peroxide and salts of some heavy metals (Cu, Pb, Zn, and Cd) in H_2O_2 in the infusorian *Paramecium caudatum*, being even more efficient than the classical antioxidant ascorbic acid (Morgunov et al. 2018). The antioxidant properties of ICA need further studies because of a wide distribution of heavy metals in the environment and their adverse biological effects, in particular, the disorder of mental functions, such as learning and memory (Karpukhina et al. 2014; Sripetchwandee et al. 2014; Inozemtsev et al. 2016; Karri et al. 2016; Kulikova et al. 2016; Inozemtsev et al. 2017). As found in the aforementioned works, the neurotoxicosis caused by heavy metals inhibits learning and memory probably because of the excessive formation of active oxygen species (in other words, heavy metals induce oxidative stress), which damage cellular membranes and cause many dysfunctions. It is the antioxidant activity of ICA that is probably responsible for its neuroprotective effect on the learning and memory inhibition in the laboratory rats intoxicated with heavy metals.

The application of ICA as an antioxidant is limited by the fact that chemically synthesized ICA represents an inseparable mixture of four isomers, of which only Ds(+)-threo-ICA is a naturally occurring central metabolite of all living cells, whereas the other three isomers are nonnatural inhibitory compounds (Finogenova et al. 2005). Thus, chemically synthesized ICA cannot be used in the food and pharmaceutical industries. It should be noted that the natural Ds(+)-threo-ICA produced by Sigma-Aldrich (USA) for the assay of isocitrate dehydrogenase is isolated from the juice of the specially cultivated *Sedum spectabile* plant and has a sale price of 538 EUR per 1 g of monopotassium salt. Such a high price makes this product unaffordable for medical applications.

It is known that Ds(+)-threo-ICA can also be produced by microbial synthesis. Some researchers selected efficient ICA-producing *Y. lipolytica* strains from natural sources, while others used for this purpose classical mutagenesis and gene engineering (Förster et al. 2007; Heretsch et al. 2008; Holz et al. 2009; Aurich et al. 2012, 2017; Kamzolova et al. 2013, 2016, 2018; Liu et al. 2015; Morgunov et al. 2018; Timoumi et al. 2018; Hu et al. 2019; Rzechonek et al. 2019). In the above studies, it has been established that *Y. lipolytica* produced ICA and citric acid (CA) simultaneously, and the ratio of excreted ICA to CA greatly depends on the carbon source used for cultivation. For example, when the yeast is cultivated on glucose and glycerol, it mainly produces CA, whereas when grown on n-alkanes, ethanol, and fatty acids, it produces ICA and CA in approximately equal amounts.

At present, ethanol is considered a promising source of carbon, as it can be obtained from sugar cane, beet, maize, lignocellulose, and other renewable sources. Ethanol as a substrate for growth of producers of practically valuable compounds has several advantages over other substrates. It does not contain harmful impurities, it is well assimilated by yeast and dissolves in water in any proportions. Since ethanol is used in the human diet, the products derived from it do not require additional purification from the residual substrate. As reported by Weusthuis et al. (2011), the several companies in the USA and Switzerland created the food products based on microbial biomass produced from ethanol. In our experiments with the natural strain *Y. lipolytica* VKM Y-2373 with the employment of a metabolic inhibitor—itaconic acid, we reached the ICA concentration up to 90.5 g/L (Kamzolova et al. 2018; Morgunov et al. 2018).

It should be noted that the known microbiological processes of ICA production are based on batch cultivation, when microbial growth is limited by a deficiency of nutrients in the medium and acid formation is inhibited by metabolites gradually accumulated in the culture. As a result, after 5–6 days of the fermentation process, its continuation becomes unreasonable.

The aim of this work was to optimize the process of ICA production by *Y. lipolytica* in the repeated-batch mode and to study the protective effect of ICA on the learning and memory inhibition in the laboratory rats subjected to the neurotoxic action of heavy metals.

Materials and methods

Microorganisms and reagents

Experiments were carried out with the yeast strain *Y. lipolytica* VKM Y-2373, which was selected earlier and showed its ability to synthesize ICA from ethanol in sufficient amounts (Kamzolova et al. 2018; Morgunov et al. 2018). All chemicals were of analytical grade (Mosreactiv, Russia). Ethanol was purchased from the "Kupavnareaktiv" (Russia). Ammonium molybdate (para) tetrahydrate (Alfa Aesar, USA) and lead (II) acetate trihydrate (Acros Organics, USA) were used in the testing with ICA.

Batch cultivation

Batch cultivation was carried out in a 10-L ANKUM-2M fermentor manufactured in a town of Pushchino (Russia). The fermentor had 5 L of cultivation medium containing the following (g/L): $(NH_4)_2SO_4$, 3.0; $MgSO_4 \cdot 7H_2O$, 1.4; $Ca(NO_3)_2$, 0.8; NaCl, 0.5; KH_2PO_4, 2.0; K_2HPO_4, 0.2; double amount of microelements (Burkholder et al. 1944); and yeast autolysate "Difco," 0.5.

The cultivation temperature and the concentration of dissolved oxygen were 29.0 ± 0 .1 °C and 60% of saturation, respectively. The pH of the medium was monitored and

maintained at a level of 6.0 by adding the necessary amount of 20% KOH solution. The growth substrate ethanol was added as it was consumed from the medium.

Continuous repeated-batch cultivation

Repeated-batch cultivation was performed according to the following scheme: after 72 h of batch cultivation, the medium was refreshed by 20% at intervals of 24 h (5 cycles); then by 50% at 48-h intervals (4 cycles) and 72-h intervals (2 cycles); and then by 80% at 72-h intervals (2 cycles). Finally, the medium was refreshed by 50% and cultivation was continued for 72 h. Such cultivation regime provided for the maximum concentration of ICA in the medium.

Analytical methods

The assay of biomass, nitrogen, ICA, and CA and the calculation of the specific growth rate (μ), process productivity (Q_p), specific rate of ICA synthesis (q_p), and product yield (Y_p) were described in detail earlier (Kamzolova et al. 2013).

Methods for determining activities of citrate synthase, aconitate hydratase, NAD- and NADP-dependent isocitrate dehydrogenases, isocitrate lyase were described in detail (Morgunov et al. 2013).

Testing of ICA

Experiments on laboratory animals were carried out according to the rules of European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Council of Europe 1986) and of Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

Experiments were performed with outbred white male rats divided into six groups of ten animals each. The body weight of the rats was between 250 and 370 g. The rats were purchased from the Central Nursery of Laboratory Animals "Stolbovaya" (Russia). The rats were kept in a vivarium at 22–24 °C with free access to water and food.

Tested substances (ICA, heavy metals, and water) were injected intraperitoneally. Rats of control group 1 were given sterile distilled water (2 mL) 1 h before the start of the training session. Members of group 2 were given ICA in a dose of 20 mg/kg. Groups 3 and 4 were given 2 mL of aqueous solutions of lead diacetate (10^{-7} M) and ammonium molybdate (10^{-5} M), respectively, 5 h before the start of the training session. Groups 5 and 6 were given lead diacetate and ammonium molybdate as described above for groups 3 and 4 and then (after 4 h) ICA in the same dose as for group 2.

The conditioned reaction of active avoidance response (CAAR), which served as the experimental model of learning and memory (Bures et al. 1983), was developed in rats by application of stimuli for 5 days (25 trials per day). Experiments were carried out using a 60 × 30 × 30-cm shuttle box divided into two equal compartments by a wall with a 10 × 10-cm hole. The conditioned stimulus was an acoustic signal lasting 10 s. Whenever the rat crossed from one compartment to the other, that is, made avoidance within this 10-s interval, the sound was switched off and the electrical shock was not applied. Otherwise, the electrical shock was applied in the compartment occupied by the animal. The electrical shock caused motor reaction and the rat crossed to the opposite compartment, thus exhibiting shock escape response. After that, both stimuli were terminated. The interstimulus interval was 30 s. CAAR was evaluated as the percentage of successful avoidances relative to the number of applied stimuli.

On the fifth day of training, when avoidance behavior became well established, CAAR was deliberately smashed. For this purpose, the stimuli were not switched off even if the rat crossed to the other compartment in response to the stimulus (this was repeated successively five times) so that the rat was exposed to current shocks regardless of the correct reaction.

After the fifth crossing, the electrical current was switched off immediately and the sound 2 s later. Then, CAAR was estimated as usually in 20 trials. As the effect of the smash was most pronounced immediately after it, we compared the results of the five last trials before the smash and the five first trials after it.

The results were expressed as the arithmetic means of avoidance responses with the standard deviation of the mean. The dynamics of training in the groups was estimated using the Kruskal-Wallis test, and the difference between the groups was estimated using the Wilcoxon test. The differences were considered to be statistically significant at $p < 0.05$.

Results

Fed-batch cultivation

Figure 1 shows the dynamics of nitrogen consumption and the accumulation of biomass, ICA, and CA in the *Y. lipolytica* VKM Y-2373 culture grown under the condition of growth limitation by nitrogen (panel a), as well as the logarithmic growth curve, specific growth rate (μ), and the specific rate of ICA biosynthesis (q_{ICA}) (panel b). The logarithmic growth curve exhibited a clear-cut exponential phase from 0 to 12 h of cultivation, growth retardation phase from 12 to 72 h, and stationary phase (from 72 to 144 h). After 6 h of cultivation, the specific growth rate (μ_{max}) reached the maximum value 0.231 h^{-1}. ICA appeared in the medium only in the growth retardation phase (after 12 h of cultivation) caused by the exhaustion of a nitrogen source. The accumulation of ICA continued in the stationary phase until the medium was

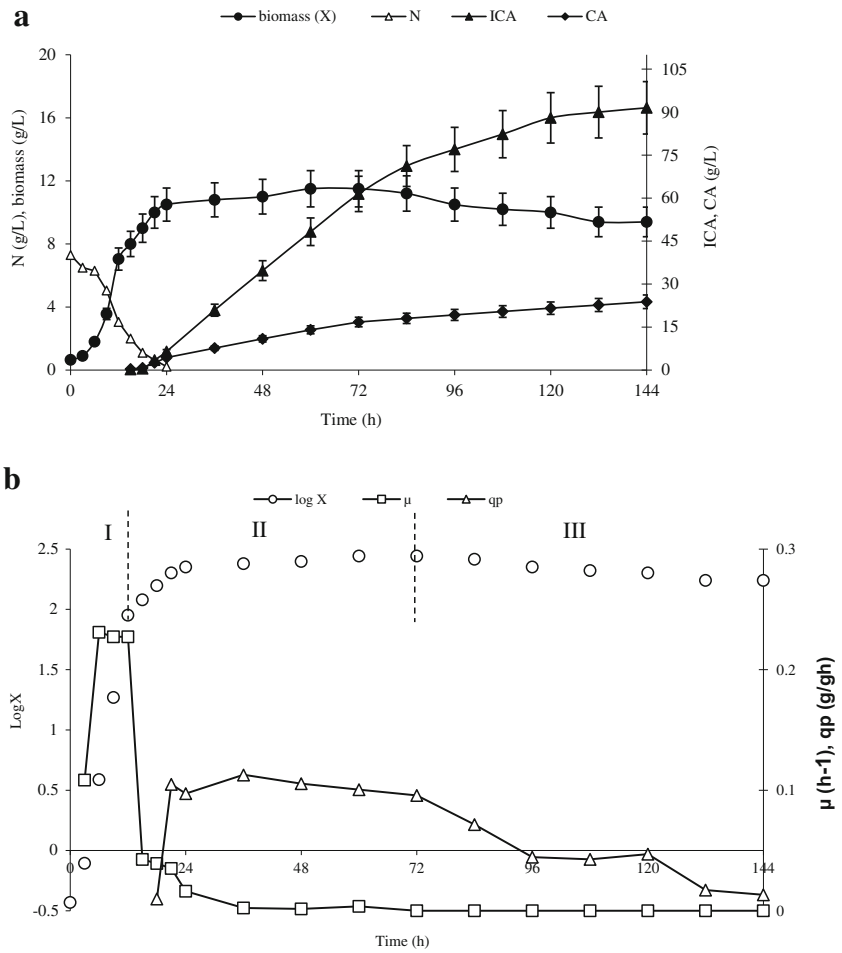

Fig. 1 Fig. 1 Time courses of growth, nitrogen consumption, and ICA and CA production by Y. lipolytica VKM Y-2373 (**a**) and the calculated parameters of the process (**b**): I, exponential phase; II, growth retardation phase; and III, stationary phase

supplied with the growth substrate ethanol. After 144 h of cultivation, the concentration of ICA in the medium was 91.5 g/L. The biosynthesis of ICA was accompanied by formation of the only byproduct CA at a concentration of 23.8 g/L, the ICA to CA ratio being 3.8:1. The average productivity of the fermentation process calculated with allowance for the medium dilution with the KOH solution used for the control of pH turned out to be 0.896 g/L h. The yield of the target product (Y_{ICA}) was 0.75 g/g ethanol.

As seen from Fig. 1b, the specific rate of ICA biosynthesis (q_{ICA}) was maximum from 21 to 72 h of cultivation and comprised 0.095–0.104 g/g h. From 72 to 144 h, q_{ICA} gradually decreased by approximately 4.5 times.

To find the reason of lower culture productivity during batch cultivation, the major enzymes of ethanol and ICA metabolism were assayed in the cell-free homogenate in the course of cultivation. Cells for analysis were withdrawn in the phase of active growth (12 h of cultivation), in the phase of growth retardation and active ICA formation (24, 48, and 72 h), and in the phase of stationary growth and slow ICA formation (120 and 144 h). The results of the assay of enzyme activities in the *Y. lipolytica* VKM Y-2373 cells grown in the batch mode are shown in Table 1. As seen from Table 1, *Y. lipolytica* cells in the phase of active growth (12 h of cultivation) showed high activities of enzymes of the initial oxidation of ethanol, that is, alcohol dehydrogenase and aldehyde dehydrogenase (0.070 and 0.060 U/mg protein, respectively). The activity of citrate synthase, which is involved in the formation of CA, was considerably higher than that of the subsequent enzymes of the tricarboxylic acid (TCA) cycle, aconitate hydratase and NAD-dependent isocitrate dehydrogenase (by 3.4 and 20 times, respectively). The high activity of isocitrate lyase (0.200 U/mg protein) indicated the operation of the glyoxylate cycle upon the assimilation of ethanol. In the phase of retarded growth (caused by a deficiency of nitrogen in the medium) and active biosynthesis of ICA (24, 48, and 72 h of cultivation), alcohol dehydrogenase, aldehyde dehydrogenase, and citrate synthase remained active. The activity of aconitate hydratase (this enzyme is responsible for ICA formation in the TCA cycle) increased by 1.3 times, whereas the activity of isocitrate lyase (enzyme responsible for ICA oxidation in the glyoxylate cycle) decreased by approximately 2.2 times. In this case, the activity of NAD-dependent isocitrate dehydrogenase, which oxidizes ICA in the TCA cycle, remained at a relatively high level of 0.118–0.121 U/mg protein. In the stationary phase (120 and 144 h of

Table 1 Dynamics of enzyme activities (U/mg protein) in *Y. lipolytica* cells cultivated in the batch mode

Enzymes	Time (h)					
	12	24	48	72	120	144
Alcohol dehydrogenase	0.070 ± 0.015	0.080 ± 0.02	0.075 ± 0.020	0.052 ± 0.010	0.014 ± 0.010	0.010 ± 0.010
Aldehyde dehydrogenase	0.060 ± 0.010	0.060 ± 0.010	0.060 ± 0.010	0.060 ± 0.010	0.070 ± 0.010	0.070 ± 0.010
Citrate synthase	2.210 ± 0.170	2.000 ± 0.160	1.800 ± 0.230	1.800 ± 0.230	0.900 ± 0.080	0.840 ± 0.080
Aconitate hydratase	0.650 ± 0.130	0.73 ± 0.150	0.880 ± 0.160	0.880 ± 0.160	0.530 ± 0.119	0.450 ± 0.120
NAD-dependent isocitrate dehydrogenase	0.110 ± 0.010	0.121 ± 0.011	0.118 ± 0.020	0.120 ± 0.031	0.060 ± 0.020	0.020 ± 0.010
Isocitrate lyase	0.200 ± 0.060	0.100 ± 0.020	0.087 ± 0.010	0.080 ± 0.015	0.067 ± 0.015	0.040 ± 0.010

growth), all key enzymes of the ethanol and ICA metabolism became less active (alcohol dehydrogenase by 7.2 times, citrate synthase by 2.2 times, aconitate hydratase by 1.8 times, NAD-dependent isocitrate dehydrogenase by 6 times, and isocitrate lyase by 2.2 times) as compared with their activities in the phase of active acid formation (24, 48, and 72 h of growth).

Repeated-batch cultivation

With the repeated-batch cultivation technique, a part of the culture liquid is withdrawn from the fermentor and the same volume of fresh cultivation medium which contains all necessary for growth nutrients, including nitrogen, is added to the fermentor. This procedure is repeated at regular intervals.

In experiments with the ICA-producing *Y. lipolytica* VKM Y-2373 strain, we varied the volume of replaced liquid and the cycle duration. Figure 2 shows the dynamics of biomass and ICA during the continuous repeated-batch cultivation of the ICA producer. As seen from Fig. 2, even after 748 h of cultivation, the concentration of ICA in the broth was as high as 103 g/L.

To analyze the activity of the major enzymes involved in the metabolism of the growth substrate ethanol and of the target product ICA, microbial cells were sampled during the batch period (48 h of cultivation) and in the course of different stationary phases of the repeated-batch cultivation period (192, 384, 528, 672, and 748 h of cultivation). The results of the enzyme assay in the cell-free homogenate of *Y. lipolytica* VKM Y-2373 are summarized in Table 2. As seen from Table 2, the activities of alcohol dehydrogenase, aldehyde dehydrogenase, citrate synthase, aconitate hydratase, NAD-dependent isocitrate dehydrogenase, and isocitrate lyase remained at a high level during the whole cultivation period. By the end of the repeated-batch cultivation (Table 2, 748 h), the activities of almost all assayed enzymes, except aldehyde dehydrogenase, were higher than those by the end of the batch cultivation (Table 1, 144 h). Namely, alcohol dehydrogenase was more active by 7 times, citrate synthase by 2.1 times, aconitate hydratase by 1.7 times, NAD-dependent isocitrate dehydrogenase by 5 times, and isocitrate lyase by 1.5 times.

Table 3 shows data on the mean concentration of biomass and ICA in the culture broth, specific rate of ICA biosynthesis (q_p), process productivity (Q_p), and ICA yield relative to the ethanol consumption (*Y*p) in different regimes of repeated-

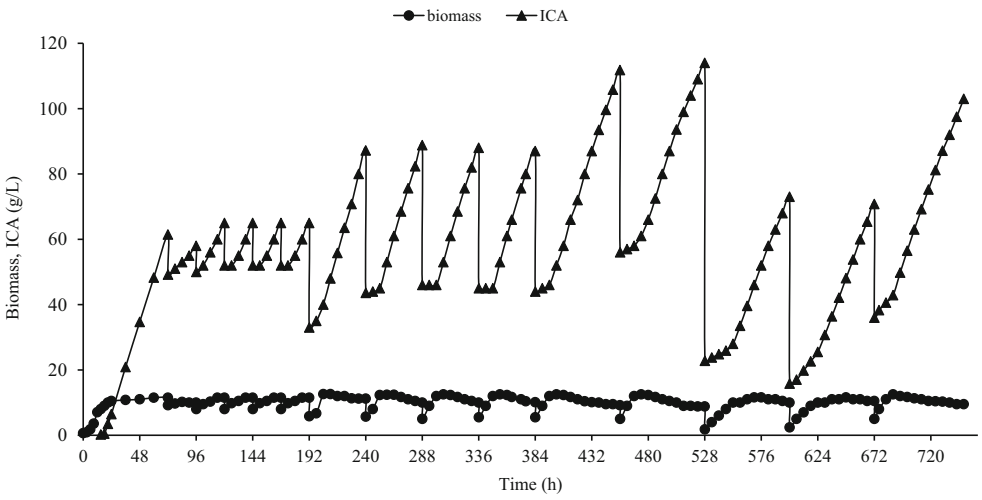

Fig. 2 Biosynthesis of ICA in the repeated-batch culture of *Y. lipolytica*

Table 2 Dynamics of enzyme activities (U/mg protein) in *Y. lipolytica* cells cultivated in the repeated-batch mode

Enzymes	Time (h)					
	48	192	384	528	672	748
Alcohol dehydrogenase	0.075 ± 0.020	0.052 ± 0.010	0.060 ± 0.015	0.080 ± 0.020	0.070 ± 0.020	0.070 ± 0.020
Aldehyde dehydrogenase	0.060 ± 0.010	0.060 ± 0.010	0.060 ± 0.010	0.075 ± 0.010	0.060 ± 0.010	0.060 ± 0.010
Citrate synthase	1.800 ± 0.230	1.630 ± 0.170	1.850 ± 0.100	1.700 ± 0.200	1.630 ± 0.200	1.780 ± 0.200
Aconitate hydratase	0.880 ± 0.160	0.800 ± 0.220	0.790 ± 0.200	0.810 ± 0.200	0.800 ± 0.200	0.750 ± 0.200
NAD-dependent isocitrate dehydrogenase	0.118 ± 0.020	0.120 ± 0.031	0.110 ± 0.020	0.110 ± 0.020	0.110 ± 0.020	0.110 ± 0.020
Isocitrate lyase	0.087 ± 0.010	0.080 ± 0.015	0.077 ± 0.015	0.057 ± 0.010	0.057 ± 0.010	0.060 ± 0.010

batch cultivation. When the volume of replaced liquid was 50% with the cycle duration of 72 h, the concentration of ICA, the process productivity (*Q*p), and the ICA yield (Y_p) reached the maximum values of 109.6 g/L, 1.346 g/L h, and 0.8 g/g ethanol, respectively. The increase in the volume of replaced liquid from 50 to 80% diminished the ICA concentration by 1.5 times, process productivity (Q_p) by 1.2 times, and product yield by 20%, which probably was related to excessive cell renewal. When the replacement cycle duration was decreased from 72 to 48 h with the optimal volume of replaced liquid equal to 50%, the ICA concentration slightly decreased by 1.3 times, whereas the process productivity (Q_p) and the product yield (Y_p) practically did not change. The decrease in the volume of replaced liquid from 50 to 20% diminished the ICA concentration by 1.7 times, process productivity (Q_p) by 1.2 times, and product yield (Y_p) by 15%, which probably was related to an insufficient level of cell renewal. It should be noted that the specific rate of ICA synthesis (q_{ICA}) remained relatively high (within 0.065–0.102 g/g h) during the whole period of repeated-batch cultivation. As for the major byproduct CA, its content did not exceed 25% of the sum of ICA and CA irrespective of the cultivation mode used.

For further experiments on laboratory animals, ICA was isolated from the culture liquid in the form of monopotassium salt (Morgunov et al. 2018). The isolated and purified product represented white crystalline preparation containing 99.9% ICA.

Testing of ICA in model experiments on learning and memory in rats

The effect of ICA and heavy metals was studied in experiments on the development of reaction of active avoidance response (CAAR) in laboratory rats. The results are shown in Fig. 3.

As seen from Fig. 3, ICA favorably influenced the training of rats within the first 2 days, whereas then its influence became negligible. The peculiarity of this period is that the animals are exposed to more electric shocks than in other days. For example, the control rats showed less than 10% of avoidance reactions during the first training day (that is, they received at least nine electrical shocks of the ten possible). On the third day of training and later, the number of CAAR increased and number of electrical shocks respectively diminished.

As seen from Fig. 3, the heavy metals under study inhibited the learning of the rats, lead being more inhibitory than molybdenum. In the first experimental day, half of the animals injected with lead diacetate did not made a single correct response; in the next 2 days, there was a slight acceleration of learning, and then it began to slow down. As a result, on the last fifth day of training, the rats received more than 20 electrical shocks of the 25 possible. These results testify in favor of the strong inhibition of learning and memory by lead. In contrast to lead, molybdenum exerted an insignificant inhibitory action on the learning response. Indeed, ammonium

Table 3 Biosynthesis of ICA by *Y. lipolytica* VKM Y-2373 cultivated in the repeated-batch mode

Volume of replaced liquid (%)	Cycle duration (h)	Number of cycles	Biomass (g/L)	ICA (g/L)	Q_p (g/L h)	q_{ICA} (g/g h)	Y_p (g/g)
20	24	5	11.20 ± 0.67	63.60 ± 3.13	1.09	0.065	0.68
50	48	4	10.33 ± 0.59	87.20 ± 0.82	1.300	0.102	0.74
50	72	3	9.17 ± 0.35	109.60 ± 5.82	1.346	0.094	0.80
80	72	2	10.25 ± 0.35	71.40 ± 0.85	1.119	0.086	0.64

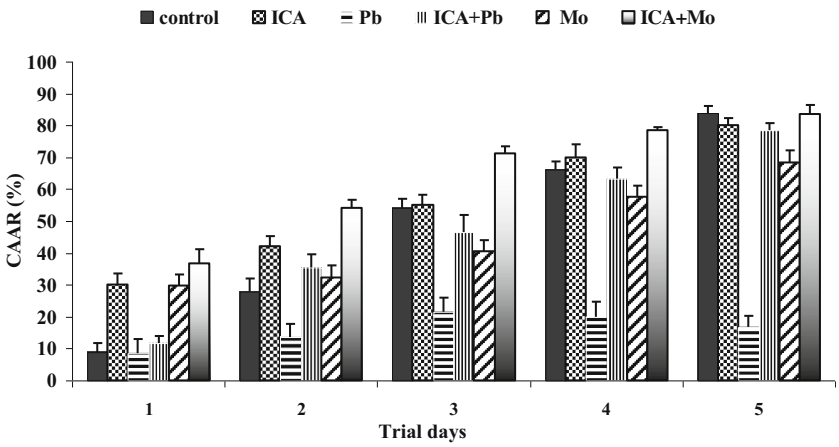

Fig. 3 Effect of ICA and heavy metal salts on the development of the reaction of active avoidance response (CAAR)

molybdate decreased the number of CAAR relative to the control rats only during the three last days of training, its effect being even positive in the first training day.

As seen from Fig. 3, ICA prevented the neurotoxic action of the heavy metals on learning and memory. Indeed, the number of CAAR in the rats given a combined injection of ICA with lead and molybdenum salts was higher than in the case of injection of the heavy metal salts without ICA. Especially significant was the effect of ICA against the negative influence of lead. Thus, on the last day, rats coexposed to ICA and lead performed an average of 4 times more CAAR than rats without ICA. In the first training day, lead acetate reduced the favorable effect of ICA on the learning and memory of rats. At the same time, the number of CAAR in the rats injected with both ICA and ammonium molybdate was higher than that in the control rats injected with water, in the rats injected with ICA alone, and in the rats injected with molybdenum alone. These data are a strong indication that molybdenum, in contrast to lead, enhances the neuroprotective effect of ICA.

The effect of ICA was further investigated by analyzing the reversible functional failure of CAAR caused by the unexpected change of causal relationships between the stimuli, response, and its effect, named as "smash of avoidance responses" (Inozemtsev 2009). As seen from Table 4, the smash of avoidance responses (SAR) caused a considerable failure of the developed skill in the control rats so that the number of CAAR after the smash was by 31.3% lower than that before the smash. SAR also drastically increased the number of aimless motor reactions in animals so that the rats run to the opposite chamber compartment not only in response to the stimuli (for example, electrical shock) but also spontaneously. This type of motor activity is named as intersignal responses (ISR).

Besides the described intersignal responses in the form of running from one chamber compartment to the other, the rats exhibited a variety of other reactions typical to stressed animals, such as multiple jumps to the chamber ceiling, chaotic running, vocalization, and urination. The decrease in the number of avoidances after SAR and the increase in the number of ISR in the presence of ICA were small (10.9% and 50%, respectively) and statistically insignificant.

Discussion

This study shows that the batch experiments indicated that ICA is actively produced within 72 h, and then the formation of isocitrate decreases (Fig. 1). These data are in good agreement with our earlier data (Kamzolova et al. 2013, 2016, 2018; Morgunov et al. 2018), as well as with the results of other authors (Förster et al. 2007; Holz et al. 2009; Aurich et al. 2012, 2017; Rzechonek et al. 2019), showing the existence of two clear-cut phases of ICA biosynthesis in the yeast *Y. lipolytica* with different acid formation rates.

The data, presented in Table 1, clearly show that the decrease in the culture productivity (q_p) during batch cultivation well coincides with the decrease in the activity of the enzymes involved in the ethanol oxidation and ICA biosynthesis. Makri et al. (2010) also observed a decrease in enzyme activities, in particular, NAD-dependent isocitrate dehydrogenase activity to minimal levels during the citric acid production phase.

Table 4 Effect of ICA on the number of CAAR and ISR before and after smash

Substance	Before smash	After smash
	CAAR	
Control (H_2O)	98.0 ± 6.3	66.0 ± 28.4*
ICA	92.0 ± 14.0	82.0 ± 14.8
	ISR	
Control (H_2O)	6.0 ± 13.5	54.0 ± 15.2*
ICA	24.0 ± 24.6	36.0 ± 37.5

Data are the mean and standard deviation

*Stands for $p < 0.05$ relative to the number before smash

The low activities of enzymes, involved in metabolism of ICA, can be related to the aging of microbial cells because of inhibition of their growth by nitrogen limitation. Some modification of the cultivation technique presumably can provide for microbial cell renewal. Recent research into this problem showed promise of repeated-batch cultivation in this respect. The advantage of this type of cultivation has been demonstrated on the CA-producing *Y. lipolytica* strains (Anastassiadis and Rehm 2006; Arzumanov et al. 2000; Makri et al. 2010; Rywińska and Rymowicz 2010; Rymowicz et al. 2010; Moeller et al. 2011; Kamzolova et al. 2015).

Our data, presented in Fig. 2, show that the production of ICA by the repeated-batch fermentation of ethanol is more effective than that by batch fermentation. During the continuous fermentation process, its productivity varied from 1.09 to 1.346 g/L h and the ICA accumulation values from 63.6 to 109.60 g/L (Table 3). Moreover, the activities of enzymes involved in the synthesis of ICA remained at a high level during the whole cultivation period (Table 2).

It should be noted that the repeated-batch microbiological process of ICA production developed by us does not have analogs in the literature and that its parameters can be compared only with those of the batch processes of ICA production. There is evidence that the natural strain *Y. lipolytica* EH59 cultivated in the batch mode can accumulate in the medium up to 93 g/L ICA; however, this advantage is deteriorated by the high content of the byproduct CA (82.3 g/L) (Heretsch et al. 2008). Aurich et al. (2017) described the efficient process of ICA production from rapeseed oil by the recombinant strain *Y. lipolytica* with the superexpressed aconitate hydratase gene *ACO1*. That process is characterized by the following parameters: the ICA concentration equal to 68.4 g/L, the process productivity (Q_p) equal to 0.47 g/L h, and the ICA:CA ratio equal to 3.1:1. Citric acids were also manufactured from biodiesel waste using the recombinant *Y. lipolytica* AWG7 strain with the superexpressed *Gut1* and *Gut2* genes. The achieved ICA concentration was 42.5 g/L and the product cost was relatively low (Rzechonek et al. 2019). In our experiments with the natural, mutant, and genetically modified microbial strains with the employment of such metabolic inhibitors as itaconic and oxalic acids, we reached the ICA concentration from 70.6 to 90.5 g/L depending on the carbon source used for fermentation (either rapeseed oil or ethanol) (Kamzolova et al. 2013, 2016, 2018; Morgunov et al. 2018). The process productivity in the aforementioned works was between 0.97 and 1.15 g/L h, while in the present process (by 50% at 72-h intervals), the process productivity (*Q*p) consisted of 1.346 g/L h.

In the present study, we continued to study the pharmaceutical properties of biosynthetic ICA. Earlier, we found that ICA is a promising natural compound for prevention of oxidative stress induced by hydrogen peroxide or heavy metals in the infusorian *Paramecium caudatum* (Morgunov et al. 2018). Testing of ICA in the model of learning and memory showed that it increased the number of CAAR relative to the control value but only at the beginning of the experiment (Fig. 3), when the control animals received the maximum number of electric shocks. As shown earlier (Berezhnoy et al. 2016), CAAR development induced both pain stress and oxidative one; meanwhile, the antioxidant carnosine diminished the oxidative stress and enhanced learning and memory. Therefore, the positive effect of ICA in our experiment can be explained by its stress-protective properties. For this reason, when on the third day of training and later, the number of CAAR increased and the number of electrical shocks respectively diminished, the favorable effect of ICA disappeared (Fig. 3).

As seen from Table 4, SAR diminished the number of CAAR; meanwhile, it increased the value of ISR by nine times. As shown elsewhere (Inozemtsev 2009), this behavior indicates emotional stress development. ICA prevents the sharp decrease in the number of CAAR and the increase in the number of ISR. Consequently, our data indicate that ICA counteracts emotional stress. Since anxiolytics prevent the development of such responses (Inozemtsev et al. 1996), it should be concluded that ICA prevents the development of emotional stress and the failure of learning and memory caused by SAR.

As seen from Fig. 3, ammonium molybdate and lead acetate inhibited the learning of the rats. This result is in agreement with our earlier data (Inozemtsev et al. 2017). Many researchers consider that the main cause of neurotoxic activity of heavy metals is the oxidative stress which they induce (Jan et al. 2015). According to earlier research, cadmium induces oxidative stress in rats and in the cell culture, whose development is prevented by the antioxidant carnosine (Kulikova et al. 2016). ICA also promotes the survival of infusoria impaired by heavy metals, such as Cu, Pb, Zn, and Cd (Morgunov et al. 2018). Therefore, the prevention of the neurotoxic effect of the heavy metals by ICA shown in this paper can reasonably be explained by its antioxidant activity.

Thus, the testing of ICA produced by microbial synthesis in the model of learning and memory showed its efficiency in the prevention of pain stress induced by electrical shock on the first stage of CAAR development, of emotional stress induced by smash of avoidance responses, and of oxidative stress caused by lead acetate and ammonium molybdate.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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