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The effect of oxalic and itaconic acids on *threo*-Ds-isocitric acid production from rapeseed oil by *Yarrowia lipolytica*

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HIGHLIGHTS

- *Threo*-Ds-isocitric acid for food industry and sport medicine.
- The microbiological synthesis of isocitric acid from rapeseed oil by yeast.
- Oxalic and itaconic acids are the strong inhibitors of isocitrate lyase of *Y. lipolytica*.
- Inhibition of isocitrate lyase shifts metabolism toward the isocitrate production.

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ABSTRACT

The effect of oxalic and itaconic acids, the inhibitors of the isocitrate lyase, on the production of isocitric acid by the wild strain *Yarrowia lipolytica* VKM Y-2373 grown in the medium containing rapeseed oil was studied. In the presence of oxalic and itaconic acids, strain *Y. lipolytica* accumulated in the medium isocitric acid (70.0 and 82.7 g/L, respectively) and citric acid (23.0 and 18.4 g/L, respectively). In control experiment, when the inhibitors were not added to the medium, the strain accumulated isocitric and citric acids at concentrations of 62.0 and 28.0 g/L, respectively. Thus, the use of the oxalic and itaconic acids as additives to the medium is a simple and convenient method of isocitric acid production with a minimum content of citric acid.

1. Introduction

Isocitric acid has four stereoisomers, from which only *threo*-Ds-isocitric acid (from now on, ICA) is a natural biologically active compound involved in the tricarboxylic acid (TCA) cycle.

ICA is used for the diagnosis of some diseases, and, like citric acid (CA), for the conservation and acidification of food products. ICA can be applied in subsequent chemical transformations to provide new chiral entities for further usage in total synthesis and pharmaceutical research (Heretsch et al., 2008; Aurich et al., 2012). A promising application of ICA is its use in sport medicine. This application is based on the fact that the oxidation of ICA in the TCA cycle provides reducing equivalents (NADH and $FADH_2$) for the substrate- and oxidative phosphorylation. The intracellular concentrations of ICA and other intermediates of the TCA cycle regulate the energetic state of the cell and the organism as a whole. In preliminary experiments, we showed that ICA has a marked energetic and antihypoxic effect and can be used as a physiological stimulant of sportsmen undergoing intensive long-term physical training in such sports as athletics, swimming, skiing, skating, rowing, cycling, etc. The stimulation of cell respiration and oxidative phosphorylation by ICA activates physiological processes in various tissues and organs, enhances the physical endurance and efficiency of organism, stimulates its adaptive and compensatory capacity, and mitigates the chronic fatigue and stress of sportsmen after intensive physical loads.

The wide application of ICA in medicine and sport is restricted by high cost of its production. At present, biologically active ICA is produced from blackberry juice and from the specially cultivated flowering plant *Sedum spectabile*. However, the most promising method of its production is considered to be microbiological synthesis (Finogenova et al., 2005; Moeller et al., 2007; Heretsch et al., 2008; Holz et al., 2009; Aurich et al., 2012; Kamzolova

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et al., 2015a,b; Kamzolova and Morgunov, 2015; Morgunov and Kamzolova, 2015).

It is known that the yeast *Yarrowia lipolytica* actively accumulates ICA and CA in the cultivation medium when its growth is limited by some nutrients. 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 (Stottmeister et al., 1982; Barth and Gaillardin, 1996; Mauersberger et al., 2003; Förster et al., 2007a,b; Levinson et al., 2007; Heretsch et al., 2008; Papanikolaou et al., 2008; Kamzolova et al., 2008, 2013; Kamzolova and Morgunov, 2015; Rymowicz et al., 2010; Aurich et al., 2012; Morgunov and Kamzolova, 2015).

Recent years show an increasing interest to vegetable oils as substrates for biotechnological processes (Heretsch et al., 2008; Kamzolova et al., 2008, 2013; Kim et al., 2009; Aurich et al., 2012; Mathew et al., 2014). Heretsch et al. (2008) 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.

Our experiments, as well as the data available in the literature, show an important role of the glyoxylate cycle in the biosynthesis of organic acids. The involvement of this cycle in the biosynthesis of ICA and CA is shown for the yeast *Candida lipolytica* grown on n-alkanes, plant oils and the glycerol-containing waste products of biodiesel manufacture (Kamzolova et al., 2011; Morgunov et al., 2013). The high activity of isocitrate lyase (ICL), a key enzyme of the glyoxylate cycle, was first found in the oxalate-accumulating plant *Oxalis pes-caprae*; in the presence of ^{14}C-glyoxylic and ^{14}C-glycollic acids, the radioactive label was incorporated into oxalate from ^{14}C-glyoxylic acid rather than from ^{14}C-glycollic acid (Millerd et al., 1963). The biosynthesis of succinate and malate by the mycelial fungus *Aspergillus niger* was increased by the overexpression of the *ICL1* gene encoding isocitrate lyase (Meijer et al., 2009). At the same time, a recombinant strain of *Y. lipolytica* with a deletion in the ICL1 gene, when grown on glycerol or glucose, showed only a small enhancement (by 2–5%) of ICA synthesis over CA synthesis (Förster et al., 2007a). Another recombinant strain of *Y. lipolytica* with overexpression of the *ICL1* gene accumulated mainly CA whereas the relative content of ICA in the medium was as low as 5–7% of the total acids (Förster et al., 2007b). On the basis of these data, it can be anticipated that the addition of inhibitors of isocitrate lyase to the culture of *Y. lipolytica* grown under the condition of oversynthesis of citric acids should change equilibrium between ICA and CA toward ICA.

The aim of this work was to study the effect of inhibitors of isocitrate lyase (oxalic and itaconic acids) on its activity in the cell-free homogenate of *Y. lipolytica* VKM Y-2373, as well as on the synthesis of ICA and CA from rapeseed oil by this strain.

2. Methods

2.1. Microorganism

Experiments were carried out with the yeast strain *Y. lipolytica* VKM Y-2373, able to grow on plant oils (rapeseed and sunflower) and to produce ICA and CA in marked amounts (Kamzolova et al., 2008, 2013).

2.2. Chemicals

The reagents used in the work were of super pure- and chemically pure (Sigma–Aldrich, St. Louis, MO, USA, and Mosreactiv, Moscow, Russia). Rapeseed oil was purchased from the "Russian seeds" Processing Plant (Vinev, 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%.

2.3. Effect of inhibitor concentrations in the reaction mixture on the activity of the isocitrate lyase

To study the effect of inhibitors on the activity of isocitrate lyase in the cell-free homogenate of *Y. lipolytica*, the strain was cultivated in a 10-L fermentor ANKUM-2M (SKB, Pushchino, Russia) containing 5 L of complete nutrient medium. Cultivation conditions were as follows: temperature, 29.0 ± 0.1 °C; concentration of dissolved oxygen, 20–25%; pH of the medium, 5.0. The medium contained (g/L): rapeseed oil, 20; $(NH_4)_2SO_4$, 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; the double amount of trace elements (Burkholder et al., 1944); and yeast autolysate "Difco", 0.5. The cells from the exponential growth phase (14–16 h of cultivation) were harvested by centrifugation, washed with 0.9% NaCl and then with a phosphate buffer (pH 7.0), and then disrupted with glass beads "ballotini" (d = 150–250 μ, BDH Chemicals Ltd., Poole, England).

Isocitrate lyase was assayed in the reaction mixture (2 ml) containing 4 mM Ds-*threo*-isocitric acid, 8 mM phenylhydrazine-HCl, 4 mM cysteine-HCl, 10 mM $MgCl_2$, and 75 mM potassium phosphate buffer (pH 6.85) at 412 nm. Inhibitors were added to the reaction mixture before the registration reaction. Oxalic acid was used in the form of its dihydrate; itaconic acid was applied in free form. The inhibitors were used at concentrations from 0.1 to 2 mM. In control experiments, the reaction mixture did not contain inhibitors.

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 protein). The amount of protein in the cell-free extracts was determined by Bradford method.

2.4. Effect of inhibitors on the production of ICA and CA by Y. lipolytica in flasks and in a fermentor

The effect of inhibitors on the production of ICA and CA was studied in experiments carried out in flasks and in a fermentor.

In the case of flask experiments, the strain was grown at 28–30 °C for 144 h in Erlenmeyer flasks under the condition of growth limitation by nitrogen source. The cultivation medium contained (g/L): rapeseed oil, 10; $(NH_4)_2SO_4$, 0.3; $MgSO_4{\cdot}7H_2O$, 0.7; $Ca(NO_3)_2$, 0.4; NaCl, 0.5; KH_2PO_4, 1.0; K_2HPO_4, 0.1; the double amount of trace elements (Burkholder et al., 1944); and yeast autolysate ("Difco", USA), 0.5. The 750-ml Erlenmeyer flasks containing 50 mL of the cultivation medium were shaken at 180 rpm. Under these conditions, the aeration of the medium was 1 g O_2/L h. pH of the medium was adjusted to 6 with 10% KOH twice a day. Cells for inoculation were grown for 2 days in the same manner and inoculated in a dose equivalent to 100 mg cells (dry weight)/L.

In the second case, *Y. lipolytica* was cultivated in the 10-L fermentor with 5 L of medium containing (g/L): $(NH_4)_2SO_4$, 3.0; $MgSO_4{\cdot}7H_2O$, 1.4; $Ca(NO_3)_2$, 0.8; NaCl, 0.5 г; KH_2PO_4, 2.0; K_2HPO_4, 0.2; the double amount of trace elements (Burkholder et al., 1944); yeast autolysate, 8 mL/L; thiamine, 1.2 μg/L; Fe^{2+}, 1.2 mg/L. Rapeseed oil was added initially at a concentration of 20 g/L and then at the same concentration when pO_2 in the medium exceeded its basal level by 5–10%. The temperature was maintained at a level of 29.0 ± 0.1 °C, the concentration of dissolved oxygen was maintained at 20–25% in the growth phase and at 50–55% in the phase of acid formation. The pH was maintained