Comparative Study of the Effect of Iron on Citrate-producing Yeast Growing on Different Substrates

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Little is known about the regulatory mechanisms for citric acid production by different yeast. It is suggested the important role of iron ions in this process; iron is the integral component of many metalloenzymes; such as aconitate-hydratase, catalase, peroxidases and components of mitochondrial electron transfer chain. The response of citric acid metabolism to different iron concentrations was studied with ethanol- and glucose-grown yeast; the emphasis was focused on the physiological parameters of yeast growth and citric acid production. The effect of iron on citric acid production by yeasts has been shown to appear in a variable profile when grown on different carbon sources – glucose and ethanol. It was shown, that increased iron promoted growth on the medium containing glucose; but citric acid production decreased with an increase in iron concentration. As contrast, yeast grown on ethanol required the sufficient iron supplementation for citric acid production. The enzyme activities of yeast at different iron concentrations were studied in order to elucidate the role of iron ions in citrate metabolism.

Keywords citric acid production; yeast; trace elements; iron ions

1. Introduction

It is known that iron is the integral component of many metalloenzymes, such as aconitate-hydratase, catalase, peroxidases and components of mitochondrial electron transfer chain [1].

The effect of iron on citric acid production has been studied in several yeast. It has been found [2 - 4] that citric acid production by *Yarrowia (*syn. *Candida) lipolytica,* grown on *n*-alkanes or ethanol increased significantly with an increase in the concentration of iron ions in the physiological range. It was investigated the effect of iron concentration on the enzymatic activities and reported the certain intracellular iron concentration, which is necessary for citric acid accumulation [3]. Moreover, intracellular concentration of iron ions was shown to determine yeast requirements for oxygen [4]. In experiments with *C. lipolytica,* grown on *n*-paraffin [5] has been found that the optimum concentration for citric acid production consisted of 10 mg/L ferric nitrate whereas 10 -18 mg/L of iron were required for biomass accumulation. It should be noted that the information on iron effect on citric acid production from glucose in yeast is not presented in literature. The role of iron as a factor influencing citric acid production from glucose has been investigated only with filamentous fungi *Aspergilllus niger* [6, 7]; and it has been observed that the strong limitation of cell growth by iron is the basic condition of citric acid production.

The purpose of this study was to elucidate the iron effect on physiological and biochemical characteristics of yeast grown on glucose or ethanol at regime of continuous cultivation that makes it possible to analyze the effect of a single parameter, keeping all the other factors constant.

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2. Materials and methods

2.1. Yeast and cultivation conditions

The study with *Candida oleophila* ATCC 20177 has been carried out at the Institute of Biotechnology 2 of Research Centre Jülich (Germany). The study with *Yarrowia lipolytica* N 1 has been carried out G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms (Pushchino, Moscow region, Russia).The influence of iron on CA production by glucose-grown mutant strain *C. oleophila* was investigated in a 1 litter magnetically stirred glass fermenter (Research Centre Jülich, RCJ, Germany) at a dilution rate of D = 0.0286 h^{-1}, 460 ml working volume, 30.0 ± 0.1ºC, pH 5.0 ± 0.1, 1100 rpm and a constant aeration rate of 0.145 vvm oxygen. The adapted medium of following composition was used in chemostat cultivation of *C. oleophila* (limit of nitrogen) (g/L): NH_4Cl – 3,0; glucose – 120,0; KH_2PO_4 – 0,7, $MgSO_4$ $7H_2O$ – 0,35; $CaCl_2$ – 0,1; NaCl – 0,1; trace elements (mg l^{-1}): $MnSO_4$ $4H_2O$ – 110; $CuSO_4$ $5H_2O$ – 1,0; $ZnSO_4$ $7H_2O$ – 21,0; $CoSO_4$ $7H_2O$ – 4,0; H_3BO_3 – 40,0; potassium iodide (KJ) – 0.1; Na_2MoO_4 $2H_2O$ – 0.2; vitamins (mg/L): thiamine-HCl – 2; biotin – 0.25, pyridoxine-HCl – 0.625; Ca-D-pantothenate – 0.625; nicotinic acid – 0.5. The concentrations of iron varied from 0 to 278.1 μM. The influence of iron on citric acid production by ethanol-grown mutant strain *Y.lipolytica* was studied in the chemostat experiments on the following medium (g/L): $(NH_4)_2SO_4$ – 1,32; KH_2PO_4 – 1,38; $MgSO_4$ $5H_2O$ – 1,4; $CaCl_2$ $6H_2O$ – 0,007; rectified ethanol, 5.6 vol%; trace elements (mg/L): $ZnSO_4$ $5H_2O$ 8.45; $CuSO_4$ $5H_20$ - 0.59; vitamins (g/L) - yeast extract "Difco" 0.5. The concentrations of iron varied from 0.89 to 178.6 μM. The value of pO_2 was 60 % (from air saturation). Dilution rate was maintained at 0.01 h^{-1}). When a steady state was achieved, the cultivation was continued for three changes of medium volume in the fermenter.

2.2. Biochemical assays

Yeast biomass, ethanol, glucose, ammonium concentration were analysed as described earlier [3, 10-12]. Concentrations of citric acid and isocitric acid were measured by using HPLC (column stable-C_{18}) (Elsiko, Russia) analysis and enzymatic assay. The injection volume of the sample was 50 μl; 20 mM phosphoric acid was used as mobile phase with the flow rate of 1.0 ml min^{-1}. The wavelength of UV detector was 210 nm and the column temperature was controlled at 35^0C. CA (Roche Diagnostics GmbH, Germany) and isocitric acid (ICA) were used as the standard. Diagnostic kits (Roche Diagnostics GmbH, Germany) were used for the enzymatic assay of citric acid and isocitric acid. The enzymatic determination of citric acid was based on the measurement of NADH concentration produced during conversion of citric acid to oxaloacetate and its decarboxylation product, pyruvate, and following conversion to L-malate and L-lactate; reactions are catalysed by citrate lyase, malate dehydrogenase and L-lactate dehydrogenase. The determination of isocitric acid was based on the measurement of the NADPH produced during conversion of isocitric acid to α-ketoglutarate, a reaction catalysed by isocitrate dehydrogenase. Cytochrome content in intact cells and respiration intensity were determined as described earlier [4]. Analysis of fatty acid composition of lipids was performed as described by Eroshin et al. [9].

2.3. Enzyme assays

All enzymatic assays were carried out at 28^0 C. Yeast cells were separated from a culture broth by centrifugation (4000 *g*, 10 min, 4^0C), washed twice with ice-cold 1 % NaCl, and disintegrated with ballotini glass beads in 100 mM phosphate buffer (pH 7.4) containing EDTA (1 mM) and phenylmethylsulphonyl fluoride (5 mM). The resulting suspension was centrifuged (5000 *g*, 20 min); and the supernatant was used for enzyme assays. The activities of alcohol dehydrogenase (EC 1.1.1.1), aldehyde dehydrogenase (EC 1.2.1.3), catalase (EC 1.11.1.6), 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) were analysed as described earlier [3, 4]. Protein concentration in the cell-free extract was determined as described by Bradford [13]. Enzyme activity was expressed in micromoles of product formed per minute per milligram protein (U/mg protein). All the data presented are the means of four measurements; standard deviations were calculated.

3. Results

3.1. Iron effect on the growth and citric acid production by glucose-grown C. oleophila

The effect of iron concentrations on yeast *C. oleophila* growth and citric acid production was studied in the broad range 20 µM - 1000 µM. Under all iron concentrations in the range from 20 to 1000 µM nitrogen was limiting-growth factor and nitrogen content in biomass of *C. oleophila* consisted of 4% (data not presented). Figure 1 illustrates the formation of biomass, citric acid and isocitric acid as a function of iron concentration in the medium as well as the experimental and calculated rate of biomass formation. Under conditions of nitrogen limitation, an increase in iron concentration from 20 to 250 µM resulted in an increase in the production of biomass from 6,8 to 16,9 g/L and decrease in citric acid production from 13.4 to 10.5 g/L. Further increase in iron concentration to 1000 µM not affected on the cell growth, whereby the citric acid production was decreased up to 4.8 g/L. Iron concentration in the range from 20 to 500 µM practically not influenced on isocitric acid production; the highest isocitric acid concentration of 1.86 g/L was found at high iron concentration of 500 µM. An increase in iron concentration from 20 to 1000 µM resulted in an decrease in the specific rate of citric acid synthesis from 0.058 to 0.0069 h^{-1} and volumetric production from 0.394 to 0.128 g/Lxh. The maximal product yield of 11% was determined at 20 µM, compared with only 3,6% at 1000 µM. The rate of biomass production as a function of iron concentration was described by Michaelis-Menten kinetics, with a Km constant of 0.0369 mM of iron and V_{max} of 0.5342 mM. The relationship between biomass production and iron concentration was found to be described by a parabolic equation of the form $(1/m_P) = (116.59)*(1/Fe)^{-0.4844}$. The dependence of biomass concentration upon iron concentration was found to follow a relationship of the form (1/biomass) = 0.0691(1/Fe)+1.8718.

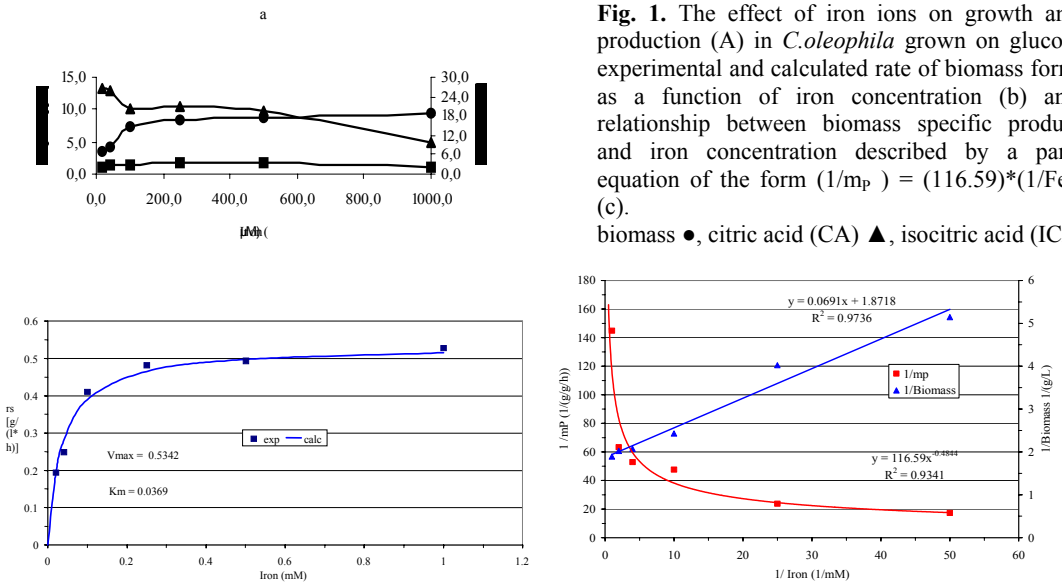

Fig. 1. The effect of iron ions on growth and CA production (A) in *C.oleophila* grown on glucose (a), experimental and calculated rate of biomass formation as a function of iron concentration (b) and the relationship between biomass specific productivity and iron concentration described by a parabolic equation of the form $(1/m_P) = (116.59)*(1/Fe)^{-0.4844}$ (c).
biomass ●, citric acid (CA) ▲, isocitric acid (ICA) ■

3.2. Iron effect on the growth and citric acid production by ethanol-grown Y.lipolytica

The effect of iron concentrations on *Y.lipolytica* growth and citric acid production was studied in the range of concentrations of iron from 0.89 to 178.6 μM. Results are presented in Figure 2. The iron concentration of 0.89 μM limited cell growth; in this case, the level of biomass was low (3.9 g/L) and citric acid synthesis was completely suppressed. At the other iron concentrations, the growth of *Y. lipolytica* was limited by nitrogen. Under conditions of nitrogen limitation, an increase in iron concentration from 1.79 to 12.5 μM resulted in an increase in the production of citric acid from 5.5 to 18.2 g/L; further increase in iron concentration from 12.5 to 62.5 μM practically not affected on the citric acid production and increase in iron concentration from 62.5 to 98.2 μM resulted in a sharp decrease in citric acid production to 3.0 g/L, and biomass level reduced to 4.7 g/L. At iron concentration of 178.6 μM, biomass comprised 3.1 g/L, whereby the citric acid production was completely inhibited. It should be noted that an increase in iron concentration from 1.79 to 62.5 μM resulted in an increase in the production of isocitric acid from 5,23 to 10,26 g/L. At iron concentrations in the range from 1.79 to 5.36 μM, the citric acid/isocitric acid ratio was 1:1; an increase in iron concentration up to 62.5 μM caused a shift towards citric acid production (citric acid/isocitric acid ratio became 2:1). Further increase in iron concentration up to 98.2 μM resulted in a predominant production of isocitric acid (citric acid/isocitric acid ratio was 1:3). Under conditions of nitrogen limitation, an increase in iron concentration from 1.79 to 62.5 μM resulted in an increase in the citric acid synthesis from 0.019 to 0.047 h^{-1} and volumetric production from 0.055 to 0.219 h^{-1}; The product yield reached the maximum of 58% at 62.5 μM iron.

To elucidate the role of iron ions in metabolism of *Y. lipolytica*, the activities of enzymes involved in primary ethanol oxidation, citric acid cycle and glyoxylate cycle were assayed. The activities of enzymes of *Y. lipolytica* at different iron concentrations are given in Table 1.

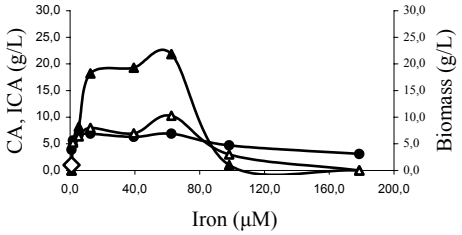

Fig. 2. The effect of iron ions on growth and citric acid production in *Y.lipolytica* grown on ethanol (biomass ●, citric acid (CA) ▲, isocitric acid (ICA) ■).

Iron concentrations in a range from 0.89 to 98.2 μM did not markedly influence activities of alcohol dehydrogenase, but affected significantly aldehyde dehydrogenase, aconitate hydratase, and catalase activities. Under iron deficiency, in the range of iron concentrations from 0.89 to 1.79 μM, aldehyde dehydrogenase, aconitate hydratase, and catalase activities were low (0.01, 0.04, and 185 U/mg of protein, respectively). An increase in iron concentration (up to 62.5 μM) resulted in increased activities of aldehyde dehydrogenase, aconitate hydratase, and catalase (by 8, 10 and 1.8 times, respectively). Moreover, under conditions of predominant production of isocitric acid, at high iron concentration (98.21 μM), the activity of aconitate hydratase was 0.68 U/mg protein as compared to 0.12 – 0.16 U/mg at the predominant production of citric acid (iron of 12.5 - 62.5 μM).

Table. Effect of iron on activity of the enzymes, respiratory and cytochrome system in *Y.lipolytica*

Index	Concentration of iron ions in the medium, μM							
	0.89	1.79	5.36	12.5	39.29	62.5	98.21	178.6
Enzymes (U/mg of protein)								
ADH	0.07	0.07	0.07	0.07	0.06	0.07	0.06	0.04
AlDH	0.01	0.01	0.05	0.08	0.08	0.08	0.03	0.03
Catalase	185	160	220	300	340	340	300	250
Citrate synthetase	1.3	1.6	1.7	1.8	1.7	1.8	1.7	1.6
Aconitate hydratase	0.04	0.06	0.05	0.16	0.12	0.40	0.68	0.68
NAD^+-IDH	0.02	0.06	0.05	0.05	0.05	0.08	0.01	0.01
$NADP^+$-IDH	0.03	0.04	0.05	0.05	0.08	0.08	0.08	0.06

ATP-citrate lyase	0.05	0,05	0,06	0,1	0,1	0,1	0,6	0,3
Isocitrate lyase	0.02	0.02	0.03	0.05	0.06	0.06	0.02	0.02
Respiration nmol O_2 (min/ mg biomass	140	530	670	670	675	680	600	200
Cytochromes (nmol/ g cell)								
a + a3	50.4	68.4	68.7	52.7	90.1	123.8	130.8	53.9
b	31.2	44.6	45.8	33.9	64.6	73.3	64.5	44.9
c	131.9	165.5	164.0	165.5	256.5	291.3	300.0	165.5

Moreover, the increase in iron concentration from 0.89 to 62.5 μM resulted in the increase in activities of enzymes of citric acid cycle, such as citrate synthease (by 38.5%) and NAD- isocitrate dehydrogenase (by 4 times) and activity of isocitrate lyase (by 3 times) – enzyme of glyoxylate cycle; activities of key enzymes of lipid synthesis, such as ATP-citrate lyase and NADP-dependent isocitrate dehydrogenase increased by 2 and 2,6 times, respectively.

It might be assumed that the changes in the activities of enzymes at different iron ions concentrations may affect the functioning of the mitochondrial electron transport chain, which determines the level of energy supply for various metabolic processes. The effect of various iron concentration on the respiratory activity, cytochrome content of *Y. lipolytica* is presented in Table. Under iron limitation the respiratory rate drastically decreased and consisted of 140 nmol O_2/(min mg biomass. Iron limitation resulted also in a decrease in the content of cytochromes: cytochrome *c* (131.9 nmol/g cells, cytochrome *b* (31.2 nmol/g cells and cytochrome $a+a_3$ (50.4 nmol/g cells. The increase in iron concentration from 0.89 to 62.5 μM resulted in the increase in respiratory rate (by 4,9 times) and content of cytochromes: cytochrome *c* (by 2,2 times), cytochrome *b* (by 2,3 times) and cytochrome $a+a_3$ (by 2,5 times).

4. Discussion

The present study deals with the important trace element iron, which is an integral component of many enzyme systems, such as aldehyde dehydrogenase, aconitate hydratase, catalase, succinate dehydrogenase, catalase and cytochromes. The response of citric acid metabolism to different iron concentrations was studied with ethanol-grown yeast *Y. lipolytica* and glucose-grown yeast *C. oleophila*; the emphasis was focused on the physiological parameters of yeast growth and CA overproduction. The effect of iron on citric acid accumulation by yeasts has been shown to appear in a variable profile when grown on different carbon sources – glucose and ethanol. Similarly to *A. niger* fermentation [17], in experiments with glucose-grown *C. oleophila*, it was indicated that iron promoted yeast growth; growth rate was described by Michaelis-Menten kinetics, with a Km constant of 0.0369 mM of iron (Fig.1) while the increase in iron concentrations in the range from 20 μM to 1000 μM decreased citric acid production and in this case, the significant quantities of glucose remained unconsummated (data were not shown). It seems, the increase in iron concentration resulted in the increase in citric acid cycle functioning and generation of ATP; a relatively high intracellular ATP concentration tended to inhibit phospho-fructokinase and glycolysis rate decreased [18, 19]. Earlier, it was found that 10 mM ATP inhibited phospho-fructokinase from *Y.lipolytica* [20]**.** These findings are in good agreement with results reported for humal cells cultivated on glucose [21]. As contrast, low iron concentration (20 μM) intensify the glycolysis rate, supplying surplus of energy, which is mainly used for the production of CA and its secretion by the active transport system instead of growth. Thus, anaerobic conditions are simulated even in the presence of oxygen (a kind of Crabtree effect).

In the case of *Y. lipolytica* grown on ethanol, it was found that intensive citric acid production required increased iron concentrations (12.5 - 62.5 μM). We suggested that increased iron requirements are due to the presence of few enzymatic reactions sensitive to iron ions. A possible sequence of metabolic steps in ethanol oxidation in yeast *Y.lipolytica* can be considered as follows: the oxidation of ethanol in yeast to acetaldehyde is catalyzed by NAD-dependent alcohol dehydragenase. Then, NAD-dependent aldehyde dehydragenase catalyzes its oxidation to acetate and then acetate is transformed into acetyl-CoA. The present results indicated that the low iron concentration (0.89 - 1.79 μM) suppressed the activity of

aldehyde dehydragenase (Table). As seen from Table, the increase in iron concentration resulted in increased in aldehyde dehydragenase activity. Marked changes in ferroprotein, catalase, enzyme involved also in ethanol oxidation [22], was also observed at low iron concentrations. The catalase activity under iron-limitation decreased twofold leading to intracellular accumulation of H_2O_2, which may be an inhibitor of all cell functions. It seems, the imbalance between the enzyme systems involved in primary ethanol oxidation (alcohol dehydrogenase/aldehyde dehydrogenase) resulted in the limited availability of acetyl-CoA, the main substrate of citric acid cycle cycle. The limited availability of acetyl-CoA resulted in the reduction of citrate synthase, aconitate hydratase, NAD-isocitrate dehydrogenase reactions and enzymes of glyoxylate cycle. It should be noted that low iron ions concentration resulted in a dramatical decrease in the activities of isocitrate lyase, the key enzyme of glyoxylate cycle and the limitation of the reaction of the cleavage of isocitrate to succinate and malate is the main source of these substrates in mitochondria during the dysfunctioning of citric acid cycle caused by the oversynthesis of citric acid (blocking at the level of isocitrate dehydrogenase). As contrast, the increased iron (in a range of 12.5–62.5 μM) stimulated citrate synthase, aconitate hydratase and the consecutive steps of citric acid and glyoxylate cycles. Thus, the experiments performed showed that the effect of iron ions on the citric acid production could be related to changes in the activities of both primary ethanol oxidation and citric acid cycle. It might be assumed that the changes in the activities of citric acid and glyoxylate cycle enzymes under low iron ions concentrations affected the functioning of the mitochondrial electron transport chain in cells at different iron supply. respiration chain rate**.** It was found that cell respiration and cytochromes content drastically decreased at low iron supplementation (0.89 - 1.79 μM). At increased iron concentration, the cytochromes content was twice as great at low iron, thus, probably providing an efficient functioning of the mitochondrial respiration chain. Thus, at increased iron (in a range of 12.5–62.5 μM) ensuring CA production, TCA cycle, especially CS showed high activity. As reported earlier [2, 14] the high activity of CS, as distinct from other TCA enzymes including AH, NAD-ICDH, and NADP-ICDH, is necessary for intensive CA production in view of the fact that CA formed in the TCA cycle can presumably by excreted from the yeast cell rather that being metabolized though the TCA cycle. In the present study, it was found that, indeed, the TCA cycle has operated and generated intermediates of the cycle including citrate and NADH. A relatively high intracellular NADH concentration tended to inhibit the activities of NAD- and NADP-ICDH [23], which were 22- and 18-fold lower that the CS activity; therefore, the bulk of CA and insignificant amount of ICA were excreted into the medium. It should be noted that at low iron concentration (0.89 - 1.79 μM), TCA cycle flux significantly can be decreased.

To conclude, the concentration of iron ions is a significant factor in the microbiological production of CA by yeast. The cultivation of yeast on ethanol require increased iron ions concentration (in the range 12.5–62.5 μM) as compare to glucose-growing cells (20 μM) . For *C.oleophila*, grown on glucose the high CA production was achieved with the lowest of the investigated iron concentration of 20 μM. A further increase in CA production can be achieved by adjusting the iron content to very low concentrations. The application of different carbon substrates (glucose, ethanol or n-paraffines) order the chosen the concentration of iron ions stimulating not only TCA cycle, and hence citrate secretion, but affecting the regulation of metabolism through the primary steps of utilization of carbon sources.

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