ISSN 1330-9862
(FTB-2128)

original scientific paper

Succinic Acid Synthesis by Ethanol-Grown Yeasts

Svetlana V. Kamzolova, Alsu I. Yusupova, Emiliya G. Dedyukhina, Tatiana I. Chistyakova, Tatiana M. Kozyreva and Igor G. Morgunov*

G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, pr-t Nauki 5, Pushchino, RU-142290 Moscow Region, Russia

Received: July 4, 2008
Accepted: January 26, 2009

Summary

The synthesis of succinic acid in ethanol-containing media has been tested in 32 yeasts of different genera (*Debaryomyces*, *Candida*, *Pichia*, *Saccharomyces*, *Torulopsis*). The capability of succinic acid synthesis was revealed in 29 strains, from which two most effective producers were selected. When grown in a fermentor under high aeration in mineral medium with pulsed addition of ethanol, the strain *Candida catenulata* VKM Y-5 produced succinic acid up to 5.2 g/L with mass yield of 32.6 % and energy yield of 14.8 %; the other strain, *Candida zeylanoides* VKM Y-2324, excreted 9.4 g/L of succinic acid with mass and energy yields of 39 and 17.8 %, respectively. It was indicated that succinic acid formation in the yeasts was accompanied by the synthesis of considerable amounts of malic acid, which was apparently due to a high activity of the glyoxylate cycle. Growth characteristics of both strains were studied in dependence on the concentrations of ethanol, zinc ions and nitrogen in the medium.

Key words: succinic acid, *Candida catenulata*, *Candida zeylanoides*, ethanol

Introduction

Succinic acid (SA) is a key organic acid in metabolic pathways of organisms. The commercial demand for SA is expanding because of its use as an effective starting material for the synthesis of 1,4-butanediol, adipic acid, tetrahydrofuran, γ-butyrolactone, *N*-methylpyrrolidone, and linear aliphatic acids (*1*). SA and its derivatives are widely used in industries producing biodegradable plastics, pharmaceutical products and cosmetics like surfactants, detergents or ion chelators (*1,2*). It is applied in medicine as an antistress, antihypoxic and an immunoactive agent (*3*). In the production of jellies, jams, ciders and wines, SA is recommended as an acidulant/pH modifier, as a flavouring and as an antimicrobial agent (*1*).

Currently, SA is produced petrochemically from butane through maleic anhydride (*1*). However, much attention has recently been focused on the microbiological production of SA using microorganisms as an alternative to chemical synthesis. Microbiological SA production has been observed using anaerobic cultivation of rumen bacteria or mutant strains of *Anaerobiospirillum succiniciproducens* (*4–7*), *Actinobacillus succinogenes* (*8*) and *Escherichia coli* (*9,10*) on carbohydrate-containing media. In the literature, several pathways for the SA synthesis in anaerobic microorganisms have been considered: *via* the reductive branch of the tricarboxylic acid cycle (TCA), through fermentative oxidation in the TCA cycle, and through the glyoxylate cycle (GC) (*1,10*), which was activated under aerobic conditions (*11*).

There are only a few studies on the SA synthesis by aerobic microorganisms. In particular, the process of SA production was proposed with the use of mutant strain of baker's yeasts *Saccharomyces cerevisiae* (*12*) and with fungus *Penicillium simplicissimum* (*13*). The synthesis of SA under aerobic conditions was also revealed in yeast *Candida brumptii* IFO 0731 grown in hydrocarbon-containing media (*14*) and in ethanol-grown *Candida* sp. (*15*).

*Corresponding author; Phone: ++7 4967 730 742; Fax: ++7 495 956 3 370; E-mail: kamzolova@rambler.ru

Ethanol as the substrate for the microbial production of SA possesses some advantages over other possible carbon sources. First, ethanol can be produced from renewable resources (sugar cane, sugar beet, corn, or lignocellulose). Second, this substrate facilitates the isolation and purification of SA because of the low content of by-products produced. Third, the products manufactured from ethanol are permissible for usage in the food industry and medicine. Many yeasts of genera *Candida*, *Hansenula*, *Rhodosporidium* and *Endomycopsis* are able to assimilate ethanol with high rates of conversion and produce valuable metabolites (citric, *threo*-D(*S*)-(+)-isocitric and α-ketoglutaric acids) (*15*).

In this paper, the screening of SA-producing yeasts of different genera (*Debaryomyces*, *Candida*, *Pichia*, *Saccharomyces*, *Yarrowia* and *Torulopsis*) grown in ethanol-containing media under aerobic conditions was performed and the effect of medium composition on the growth of selected strains was studied. The choice of yeast organisms for biosynthesis of SA was motivated by higher biomass accumulation, faster carbon conversion and product formation, as well as greater tolerance to metal ions, thus allowing the use of less refined substrates.

Materials and Methods

Organisms

Screening of SA producers was carried out among 32 natural yeast strains belonging to the genera *Debaryomyces*, *Candida*, *Pichia*, *Saccharomyces*, *Yarrowia* and *Torulopsis*, which were obtained from the All-Russia Collection of Microorganisms (VKM) and the collection of the Laboratory of Aerobic Metabolism of Microorganisms of the Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Russia. The strains were maintained at 4 °C on agar slants with *n*-alkanes as the carbon source.

Chemicals

All chemicals and enzymes were purchased from Sigma-Aldrich (USA) or Boehringer Mannheim (Germany). Ethanol was purchased from the Kazan Ethanol Processing Company (Russia) and used as a carbon source.

Cultivation

To select SA producers, strains were cultivated on an orbital shaker at (130±10) rpm and (28±1) °C for 48–72 h under nitrogen limitation in 20-mL flasks with 5 mL of the Reader medium containing (in g/L): $MgSO_4 \cdot 7H_2O$ 0.7, $Ca(NO_3)_2$ 0.4, NaCl 0.5, KH_2PO_4 1.0, K_2HPO_4 0.1, and Burkholder trace element solution with slight modifications (in mg/L): I^- 0.1, B^+ 0.01, Fe^{2+} 0.05, Zn^{2+} 0.04, Mn^{2+} 0.01, Cu^{2+} 0.01 and Mo^{2+} 0.01 (*16*). Concentration of $(NH_4)_2SO_4$ was 0.1 g/L to provide for nitrogen-limitation conditions. The final concentration of ethanol of 5 g/L was added into flasks periodically, as required. The mixture of vitamins contained (in mg/L): thiamine-HCl 0.5, biotin 0.02, pantothenate 0.5, inosite 10, nicotinate 1.0 and pyridoxine 0.5. Since growth was followed by a decrease in pH of the medium, in order to maintain the medium at pH=4.5–5.5, 10 % KOH was periodically added using pH paper strips.

To study the growth parameters and SA production, the selected strains *Candida catenulata* VKM Y-5 (syn. *Candida catenulata* Diddens et Lodder, *Candida brumptii* Langeron et Guerra) and *Candida zeylanoides* VKM Y-2324 were cultivated in 750-mL flasks with 50 mL of the medium, or in a 10-litre ANKUM-2M fermentor (SKB, Pushchino, Russia) with an operating volume of 5 L. The medium contained (in g/L): $MgSO_4 \cdot 7H_2O$ 1.4, NaCl 0.5, $Ca(NO_3)_2$ 0.8, KH_2PO_4 2.0, K_2HPO_4 0.2, and Burkholder trace element solution. The mixture of vitamins contained 1.0 mg/L of thiamine-HCL and 0.1 mg/L of biotin. Concentrations of ethanol, zinc ions, and nitrogen were varied as indicated in the text. Fermentation conditions were maintained automatically at the constant level: temperature (28±0.5) °C, pH=(5.5±0.1) was adjusted with 5–15 % KOH, dissolved oxygen fraction (pO_2) was 80 % (from air saturation), agitation was 800 rpm. Pulsed addition of ethanol was performed as the pO_2 value increased by 5 % indicating a decrease in respiratory activity of cells due to the total consumption of carbon sources. Cultivation was performed as indicated in the text.

Measurement techniques

Yeast growth was followed by measuring the absorbance of the culture at 540 nm with a Spekol 221 spectrophotometer (Carl Zeiss, Jena, Germany). The dry biomass was estimated from the absorbance of the cell suspension using a calibration curve.

Ethanol concentration was determined by gas-liquid chromatography on a Chrom-5 chromatograph (Laboratorni Pristoje Praha, Czech Republic) with a flame-ionization detector using a glass column (200×0.3 mm) packed with 15 % Reoplex-400 on Chromaton N-AW (0.16–0.20 mm) at a column temperature of 65 °C; argon was used as a carrier gas.

Concentration of ammonium was determined potentiometrically with an Ecotest-120 ionometer (Econix, Russia) using an Ekom-NH_4 electrode (Econix, Russia).

To analyze organic acids, the culture broth was centrifuged (8000×*g*, 20 °C, 3 min); then 1 mL of the supernatant was diluted with an equal volume of 8 % $HClO_4$ and the concentration of organic acids was measured by HPLC (LKB, Sweden) on an Inertsil ODS-3 reversed-phase column (250×4 mm, Elsiko, Russia) at 210 nm; 20 mM phosphoric acid were used as a mobile phase with the flow rate of 1.0 mL/min; the column temperature was maintained at 35 °C. Quantitative determination of organic acids was carried out using calibration curves constructed with the application of succinic, citric, *threo*-D(*S*)-(+)-isocitric, α-ketoglutaric, acetic, maleic, aconitic and fumaric acids (Boehringer Manheim, Germany) as standards. Additionally, SA was analysed enzymatically using biochemical kit (Boehringer Mannheim/R-Biopharm, Germany).

Mass cell yield ($Y_{X/S}$) (in %) was calculated as follows:

$$Y_{X/S} = X/S \cdot 100 \qquad /1/$$

where X is the total amount of biomass in the culture liquid at the end of exponential growth (in g/L), and S is the total amount of ethanol consumed (in g/L).

The SA mass yield (Y_{SA}) (in %) was calculated as follows:

$$Y_{SA}=SA/S\cdot 100 \qquad /2/$$

where SA is the total amount of succinic acid in the culture liquid at the end of fermentation (in g/L), S is the total amount of ethanol consumed during the cultivation (in g/L).

Energy yield of SA from ethanol (η_{SA}) estimates a fraction of energy content of the substrate (ethanol) which is incorporated into succinic acid. It was calculated on the basis of mass and energy balance theory (*17,18*).

Isocitrate lyase assay

Ethanol-grown cells were centrifuged (3000×*g*, 10 min, 4 °C), washed with 100 mM phosphate buffer (pH= =7.4), centrifuged at 3000×*g* (10 min, 4 °C) and used to prepare 10 % suspension in the same buffer (pH=7.4) containing 1 mM EDTA. Cells were disintegrated with Ballotini™ glass beads (BDH Chemicals Ltd, UK; *d*= =150–250 μ) on a planetary mill for 3 min at 1000 rpm (0 °C). The homogenate obtained was centrifuged (5000×*g*, 30 min, 4 °C), and the supernatant was used for determining the activity of isocitrate lyase (EC 4.1.3.1).

The activity of isocitrate lyase was measured using the method described by Dixon and Kornberg (*19*). The reaction mixture contained monopotassium salt of *threo*-D(*S*)-(+)-isocitric acid 4 mM, phenylhydrazine-HCl 8 mM, cysteine-HCl 4 mM, $MgCl_2$ 10 mM and potassium phosphate buffer 75 mM (pH=6.85).

The amount of enzyme catalyzing the conversion of 1 μmol of substrate per min was taken as the unit of enzyme activity (U). The enzyme activity was expressed as units per mg of protein (U/mg protein). Protein amount in the cell-free extract was determined by the Bradford method (*20*).

All the data presented are the means of three experiments and two measurements for each experiment. Standard deviations were also calculated (SD<10 %).

Results and Discussion

Screening of SA-producing yeasts

Screening of SA-producing yeasts was performed among 32 natural yeast strains belonging to the genera *Debaryomyces, Candida, Pichia, Saccharomyces, Yarrowia* and *Torulopsis*. It had been found earlier that the main condition for the production of citric, *threo*-D(*S*)-(+)-isocitric and other organic acids by yeasts includes limitation of cell growth by mineral components (nitrogen, sulphur, or phosphates) under the excess of carbon source (*15*). Therefore, in this study, yeast cultivation was performed under nitrogen limitation of cell growth. Biomass and the composition of the excreted products were determined only after complete consumption of ammonium sulphate from the medium and the transition of the culture from unlimited exponential growth phase to the growth retardation (at 48–72 h of cultivation). The data for acid production as the mass fraction of acid produced by 1 g of cells are presented in Table 1. Nitrogen deficiency in ethanol-containing medium resulted in the synthesis of a number of metabolites (SA, acetic, citric, *threo*-D(*S*)-(+)-isocitric, α-ketoglutaric, malic and fumaric acids, and others). The excretion of metabolites was found in all 32 tested strains. It should be noted that the excretion of organic acids into the medium was not revealed under unlimited growth (in the exponentional growth phase) in all studied strains. Among the studied yeasts, 18 strains produced lower mass fraction of SA (≤0.1 g/g cell), 9 strains produced SA from 0.1 to 0.5 g/g cell and two strains produced SA over 1.0 g/g cell. Earlier, the synthesis of SA under aerobic conditions had been revealed only in *Candida brumptii* IFO 0731 grown in hydrocarbon-containing media (*14*).

Based on the results in Table 1 and literature data, we can suggest that different kinds of yeast with various carbon sources indicate different response to the nitrogen limitation. In our case, of the 32 studied strains, 29 were capable of producing SA (0.07–1.5 g/g cell) when grown on ethanol-containing medium under nitrogen deficiency. Recent reports for similar types of fermentations have demonstrated that the nitrogen deficiency stimulated the biosynthesis of other secondary metabolites such as citric and *threo*-D(*S*)-(+)-isocitric acids and microbial lipids (*21–23*). It was revealed that the depletion of nitrogen induced repression of NAD(+)-dependent isocitrate dehydrogenase (*22,24*) due to the decrease in the content of AMP and increase in the NADH/NAD^+ ratio in the cells (*24*), and this event resulted, presumably, in the production of citric acid in view of the fact that citric acid formed in TCA can be excreted from the yeast cell rather than being metabolised through the TCA cycle. The widespread distribution of the SA synthesis among different species of yeasts that we observed seems to be due to the use of ethanol as a carbon source.

Possible metabolic pathways of SA production from ethanol by aerobic yeasts are shown in Fig. 1. The oxidation of ethanol to acetaldehyde is catalyzed by NAD-dependent alcohol dehydrogenase (EC 1.1.1.1), after which NAD-dependent aldehyde dehydrogenase (EC 1.2.1.2) catalyzes its oxidation to acetate and then acetate is transformed into acetyl-CoA, the main substrate of TCA (*25,26*). High activities of the TCA enzymes, including citrate synthase (EC 4.1.3.7), aconitate hydratase (EC 4.2.1.3), NAD-isocitrate dehydrogenase (EC 1.1.1.41) and low activity of succinate dehydrogenase (EC 1.3.99.1) are necessary for intensive SA production in view of the fact that SA formed in the TCA cycle can be excreted from the yeast cell rather than being metabolized through the TCA cycle. Moreover, the assimilation of ethanol involves the functioning of GC (*23*); in this case, unlike the TCA cycle, isocitrate is converted into glyoxylate and succinate by isocitrate lyase (EC 4.1.3.1.). The shortcut cycle is completed by the formation of malate from glyoxylate and acetyl-CoA.

Thus, the synthesis of SA in aerobic yeasts grown on ethanol can occur not only *via* the TCA but also through the GC that provides the metabolic flexibility of

Table 1. Acid production by different yeast strains in the medium containing ethanol as a carbon source under nitrogen limitation

Strain	$\frac{w(\text{acid})}{\text{g/g cell}}$						
	SA	Acetic acid	Citric acid	Isocitric acid	α-Ketoglutaric acid	Malic acid	Fumaric acid
Debaryomyces hansenii	0.10	0.1	0	0	0.1	0.1	0.10
Candida catenulata VKM Y-5	1.50	0.1	0.2	0.3	0.3	0.6	0.08
C. mycoderma VKM Y-240	0.10	0.1	0	0	0.3	0.1	0.08
C. rugosa VKM Y-67	0	0.1	0.1	0.01	4.2	0	0.07
C. paludigena VKM Y-2443	0.40	0.5	0	0	0.5	0.3	0.07
C. utilis VKM Y-74	0.42	0.1	1.0	0.1	0.3	0.45	0.10
C. utilis 766	0.10	0.1	0	0.1	0.5	0.1	0.12
C. zeylanoides VKM Y-6	0.50	0.45	0	0.1	0.5	0.5	0.13
C. zeylanoides VKM Y-14	0.10	0.1	0	0.1	0.4	0.1	0.10
C. zeylanoides VKM Y-2324	1.20	0.1	0	0.1	0.3	0.8	0.08
C. zeylanoides VKM Y-1543	0.10	0.1	0	0.1	0.3	0.4	0.07
C. zeylanoides VKM Y-2595	0.43	0.1	0	0.1	0.5	0.3	0.06
C. valida VKM Y-934	0	0.1	0	0.1	0.5	0.1	0.01
Kluyveromyces wickerhamii VKM Y-589	0.08	0.6	0.1	0	0.2	0.1	0.05
Pichia anomala VKM Y-118	0.10	0.1	0	0	0.4	0.1	0.07
P. besseyi VKM Y-2084	0.45	0.1	0.4	0.2	0.4	0.3	0.03
P. media VKM Y-1381	0.10	0.1	0.4	0.1	0.3	0.1	0.04
P. guilliermondii H-P-4	0.45	0.1	0.08	1.0	0.1	0.4	0.07
P. guilliermondii 916	0.09	0.5	0.34	1.1	0.15	0.1	0.05
P. inositovora VKM Y-2494	0.38	0.09	0.5	0.1	1.3	0.4	0.05
Saccharomyces cerevisiae VKM Y-381	0.08	0.5	0.3	0.1	0.3	0.1	0.06
Torulopsis candida 127	0.07	0	0.45	0.1	0	0.1	0.07
T. candida 420	0.10	0.4	0.3	0.1	0	0.1	0.08
Yarrowia lipolytica 12a	0.09	0.1	0	0	0.5	0.1	0.06
Y. lipolytica VKM Y-47	0.45	0.1	0.5	0.3	0.4	0.3	0.10
Y. lipolytica 69	0.50	0.1	1.2	0.4	0.3	0.4	0.12
Y. lipolytica VKM Y-57	0.07	0.1	0.1	0.1	0.5	0.1	0.06
Y. lipolytica 212	0.09	0.1	0.3	0.4	0.4	0.1	0.20
Y. lipolytica 374/4	0.08	0.1	0.1	0.01	0.4	0.1	0.02
Y. lipolytica 585	0	0.08	0.1	0.3	0.3	0.1	0.08
Y. lipolytica 695	0.10	0.1	0.5	0.1	0.4	0.1	0.10
Y. lipolytica 704	0.10	0	1.5	2.0	0.3	0.4	0.08

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

cells in the formation of SA. The activity of isocitrate lyase, a key enzyme of the glyoxylate cycle, was studied in the selected SA-producing strains. It was revealed that the activities of isocitrate lyase in all strains capable of SA synthesis during their growth on the medium with ethanol was 6–10 times higher than that in glucose-grown strains (Table 2), when the accumulation of only trace amounts of SA was observed (≤0.05 g/g cell). Similarly, the activity of isocitrate lyase was increased considerably in citrate-producing yeast *Yarrowia lipolytica* grown on C_2 compounds (acetic acid, ethanol) (*23*,*27*) or on substrates leading to C_2 unit formation (*i.e.* lipids or *n*-alcanes) (*28*,*29*). Physiologically, the GC in yeast operates to maintain continuous resynthesis of oxaloacetate needed for the TCA. In yeast grown on glucose and other substrates for glycolysis, resynthesis of oxaloacetate may occur during carboxylation of pyruvate and phosphoenolpyruvate (*27*), and the functioning of GC is not necessary; it has been observed that during *Y. lipolytica* growth on glucose (*27*) or glycerol (*29*) the activity of isocitrate lyase is low. It should be noted that, in the case of citrate-producing and lipid-producing strains, the GC is also used for the formation of lipid-free material through the synthesis of protein. In the case of oleaginous microorganisms, the lipid turnover may occur through the reactions of GC, regardless of the carbon-source used for lipid production, since during lipid turnover the exogenous carbon-source is no longer used (*22*,*30*). It was observed that isocitrate lyase considerably increased during lipid degradation period (*31*).

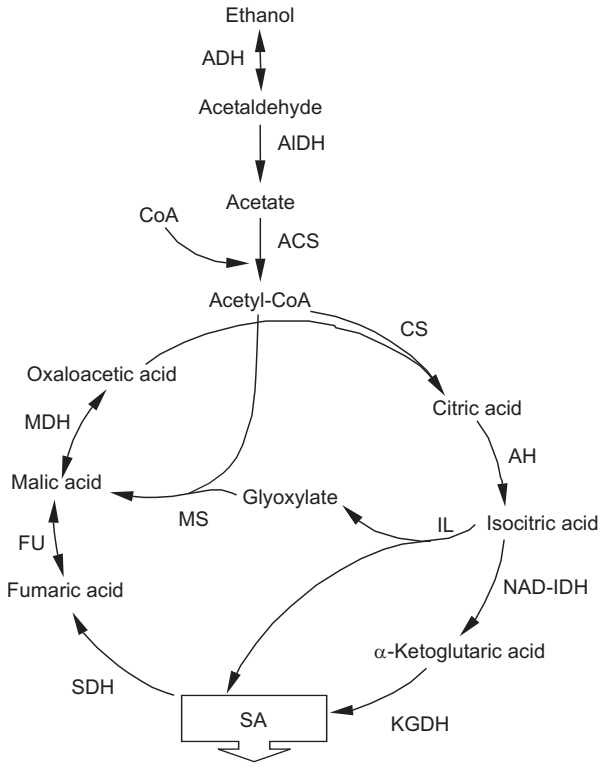

Fig. 1. Possible metabolic pathways of SA production from ethanol by aerobic yeasts
ADH – NAD-dependent alcohol dehydrogenase; AlDH – NAD-dependent aldehyde dehydrogenase; ACS – acetyl-CoA synthase; CS – citrate synthase; AH – aconitate hydratase; NAD-IDH – NAD-dependent isocitrate dehydrogenase; KGDH – α-ketoglutarate dehydrogenase; IL – isocitrate lyase, MS – malate synthase; SDH – succinate dehydrogenase; FU – fumarase; MDH – malate dehydrogenase

Two strains, *Candida zeylanoides* VKM Y-2324 and *Candida catenulata* VKM Y-5 (syn. *Candida brumptii*), which display the highest SA production (1.2 and 1.5 g/g cell, respectively), were chosen for further studies.

Determination of optimal conditions for the growth of Candida catenulata and C. zeylanoides

In the first series of experiments, the effect of ethanol concentration in the medium on the growth of selected SA-producing yeast strains was studied. The application of ethanol as a growth substrate shows certain difficulties in the cultivation of microorganisms due to its toxicity. High concentrations of ethanol affected the functioning of enzyme systems, inhibited cell growth and, in some cases, disrupted microbial cells. However, yeast cultivation in ethanol-containing medium under nitrogen limitation implies the excess of ethanol in the medium. That is why the study of cell resistance to ethanol is of great importance.

Yeasts *C. catenulata* and *C. zeylanoides* were cultivated at different ethanol concentrations (from 0.2 to 24.0 g/L). The data about the dependence of maximum specific growth rates (μ_{max}) of both yeast strains on ethanol concentration in the media are shown in Fig. 2. Yeast *C. catenulata* had the highest values of μ_{max} (0.15–0.18 h^{-1}) at the ethanol concentrations of 0.2–0.8 g/L. In the case of *C. zeylanoides*, the highest values of μ_{max} (0.339–0.345 h^{-1}) were revealed at the ethanol concentrations from 0.2 to 2.4 g/L. At the ethanol concentrations above 4 g/L, specific growth rates of these strains decreased considerably. For comparison, optimal concentration of ethanol for the growth of CA-producing yeast *Yarrowia lipolytica* was 2.5 g/L (*32*). Based on the obtained results, in further experiments, pulsed additions of ethanol into the medium for cultivation of *C. catenulata* and *C. zeylanoides* did not exceed 1.0 and 3.0 g/L, respectively.

It is known that yeasts grown in ethanol-containing media are characterized by increased requirements for zinc ions (*23,33*) since the first reaction of ethanol oxidation is catalyzed by zinc-containing enzyme, NAD-dependent alcohol dehydrogenase (EC 1.1.1.1).

The effect of zinc concentration on the yeast growth was studied within the range of 0.01–259 mg/L. Taking into account that the physiological effect of trace ele-

Table 2. SA production and isocitrate lyase activity in the yeast cultivated with ethanol or glucose as a carbon source

Strains	Ethanol		Glucose	
	w(SA)	Isocitrate lyase activity	*w*(SA)	Isocitrate lyase activity
	g/g cell	U/mg protein	g/g cell	U/mg protein
Candida catenulata VKM Y-5	1.50	0.640±0.100	0.05	0.036±0.006
C. paludigena VKM Y-2443	0.40	0.211±0.085	0.01	0.020±0.010
C. utilis VKM Y-74	0.42	0.423±0.090	0.02	0.019±0.005
C. zeylanoides VKM Y-6	0.50	0.650±0.120	0.01	0.050±0.008
C. zeylanoides VKM Y-2324	1.20	0.727±0.130	0.05	0.040±0.008
C. zeylanoides VKM Y-2595	0.43	0.560±0.120	0.02	0.030±0.010
Pichia besseyi VKM Y-2084	0.45	0.706±0.145	0.01	0.087±0.012
P. guilliermondii H-P-4	0.45	0.803±0.100	0.01	0.078±0.01
P. inositovora VKM Y-2494	0.38	0.700±0.100	0.01	0.078±0.015
Yarrowia lipolytica VKM Y-47	0.45	0.475±0.125	0.02	0.030±0.010
Y. lipolytica 69	0.50	0.500±0.087	0.02	0.028±0.011

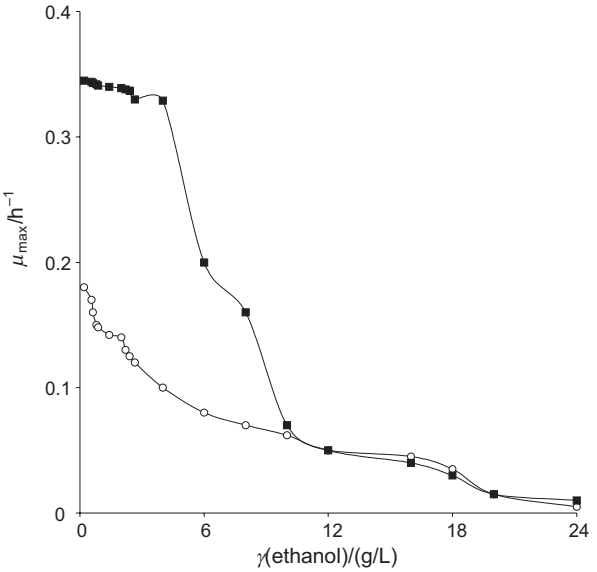

Fig. 2. Maximum specific growth rates of (○) *C. catenulata* and (■) *C. zeylanoides* as a function of ethanol concentration in the media

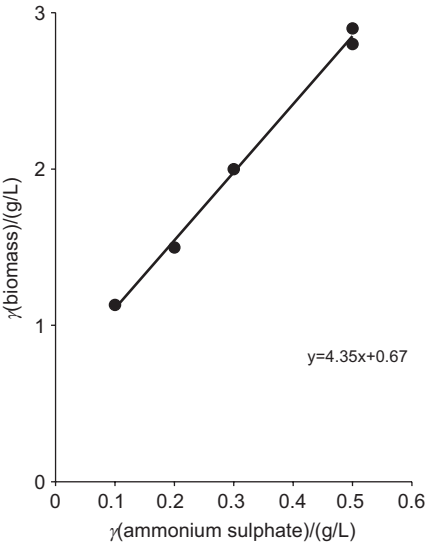

Fig. 3. The effect of ammonium sulphate concentration in the medium on the accumulation of *C. catenulata* biomass under nitrogen-limiting conditions

ments is established by their intracellular concentration rather than by their supply in the medium (*33*), the yeast growth was presented as the function of intracellular zinc content. It was revealed that the growth of *C. zeylanoides* was limited at mass fraction of Zn^{2+} of 0.02 mg/g. Based on literature data (*23*), it can be suggested that zinc limitation of cell growth was due to the limitation of Zn-dependent alcohol dehydrogenase, which resulted in an imbalance between alcohol dehydrogenase and aldehyde dehydrogenase activities, and hence in a decrease in acetyl-CoA supply, the main precursor of the TCA cycle intermediates, which is involved in the generation of energy. Biomass of *C. zeylanoides* was maximal at the mass fraction of Zn^{2+} of 0.4 mg/g and decreased twofold at the mass fraction of Zn^{2+} of over 26 mg/g. At the same time, the mass fraction of Zn^{2+} of 26 mg/g was optimal for the growth of *C. catenulata.* These findings indicate that zinc ions are of great importance for yeast growth on ethanol and their optimal concentrations are specific for individual yeast species.

It is known that when microorganisms are cultivated under growth limitation conditions, there is a direct correlation between the concentration of limiting component in the medium and the cell density (*34*). To study the cell requirements for nitrogen, the yeast *C. catenulata* was cultivated in flasks in the media with different concentrations of ammonium sulphate, which was the growth-limiting component. The obtained linear correlation between the concentration of ammonium sulphate in the medium and the biomass level (Fig. 3) allowed us to calculate the concentration of ammonium sulphate in the medium required to obtain desirable biomass amount by using the following equation of linear regression:

$$Y=4.35X+0.67 \qquad /3/$$

where Y is the biomass (g/L), and X is the concentration of ammonium sulphate in the medium (g/L).

Taking into account the results obtained in flask experiments, a batch cultivation of *C. catenulata* and *C. zeylanoides* was performed in a 10-litre fermentor with an operating volume of 5 L under high aeration (pO_2=80 % of air saturation) and at the constant pH=(5.5±0.1). The applied medium differed from the Reader medium in the increased content of $ZnSO_4 \cdot 6H_2O$ (2.678 g/L for *C. catenulata* and 0.033 g/L for *C. zeylanoides*). Concentration of ammonium sulphate for yeast cultivation was 6 g/L to obtain a biomass of 20 g/L. Pulsed additions of ethanol into the medium for cultivation of *C. catenulata* and *C. zeylanoides* did not exceed 1.0 and 3.0 g/L, respectively.

Biosynthesis of succinic acid by C. catenulata and C. zeylanoides cultivated in ethanol-containing media in a fermentor

Time courses of yeast growth, ammonium consumption, and SA synthesis are shown in Fig. 4. By the end of growth phase, biomass of *C. catenulata* and *C. zeylanoides* reached up to 20 g/L. According to Eq. 3, the expected biomass in a fermentor should be 26.8 g/L, whereas the cultivation of both strains in the the medium containing 6 g/L of ammonium sulphate resulted in the formation of only 20 g/L of cells. A possible explanation of this discrepancy may be the fact that the medium dilution in a fermentor was rather high due to the addition of a large volume of KOH solution for maintaining the constant pH value during the growth of cells and SA production.

Intense excretion of SA was observed in the stationary phase and reached 5.2 g/L in *C. Catenulata* and 9.4 g/L in *C. zeylanoides* in 68 h of cultivation. As compared to literature data, the SA production by hydrocarbon-grown yeasts was 23.6 g/L in the mutant strain *C. brumptii* IFO 0731 in 9 days of cultivation (*12*).

Our data on the efficiency of the growth and SA synthesis by *C. catenulata* and *C. zeylanoides* are given in

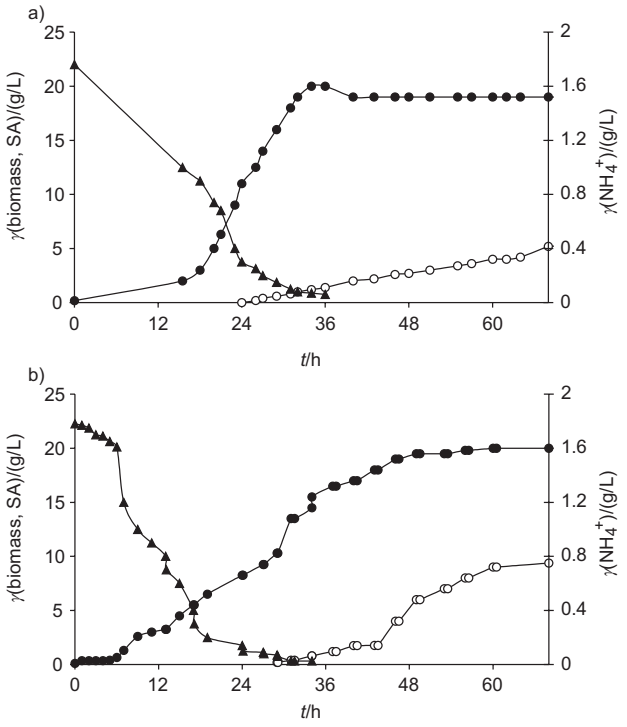

Fig. 4. Time courses of (●) growth, (▲) ammonium consumption, and (○) SA synthesis by (a) *C. catenulata* and (b) *C. zeylanoides*

Table 3. The mass cell yield ($Y_{X/S}$) was 67 % in *C. catenulata* and 63 % in *C. zeylanoides*. The maximum specific growth rate (μ_{max}) calculated from the linear segment in the semilogarithmic plot of the growth curve amounted to 0.16 h^{-1} for *C. catenulata* and 0.31 h^{-1} for *C. zeylanoides*; these values are comparable with literature data obtained with ethanol-grown *Y. lipolytica*, a producer of citric acid (*32*).

As seen from Table 3, in both strains the excreted by-products included malic acid as the major component, and α-ketoglutaric and *threo*-D(S)-(+)-isocitric acids as minor components. It should be noted that *C. catenulata* and *C. zeylanoides* produced SA and malic acid approximately at the same level, which is indicative of a high activity of the GC.

Table 3. Growth parameters and organic acid production by *C. catenulata* and *C. zeylanoides*

Parameters	*C. catenulata*	*C. zeylanoides*
$Y_{X/S}$/%	67.0	63.0
μ_{max}/h^{-1}	0.16	0.31
γ(succinic acid)/(g/L)	5.2	9.4
γ(malic acid)/(g/L)	4.0	8.8
γ(*threo*-D(*S*)-(+)-isocitric)/(g/L)	1.0	1.7
γ(α-ketoglutaric acid)/(g/L)	1.3	0.8
Y_{SA}/%	32.6	39.0
η_{SA}/%	14.8	17.8

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

The SA mass yield (Y_{SA}) was 32.6 % for *C. catenulata* and 39.0 % for *C. zeylanoides*. According to literature data, the value of Y_{SA} in *C. brumptii* IFO 0731 grown in *n*-paraffin-containing medium was 67 % (*14*).

However, since carbon substrates are characterized by different energy capacities, it is inappropriate to compare the SA mass yields from different substrates. It is more correct to compare the energy yields of SA (η_{SA}) in different strains.

The value of η_{SA} estimates a fraction of energy content of the substrate (ethanol), which is incorporated into SA, and it was calculated on the basis of mass and energy balance theory (*17,18*). Quantities that characterize mass and energy balance of cell metabolism are based on the generalized unit of reductivity, 'redoxon', which is an electron that can be transferred to oxygen; a former variant of this unit was available electron (*18*). By definition, energy yield of the product is a fraction of the total amount of substrate redoxons (available electrons), which is incorporated into the product.

The value of η_{SA} was calculated using elementary composition of SA and ethanol:

$$\eta_{SA}=(\gamma_{SA}\delta_{SA})/(\gamma_S\delta_S)Y_{SA} \qquad /4/$$

where δ_S and δ_{SA} are mass fractions of carbon in ethanol (S) and succinic acid (SA), γ_S and γ_{SA} are reductance degrees, the number of redoxons per 1 carbon atom in ethanol (S) and succinic acid (SA), respectively.

For the substance (individual compound or a mixture) having the elementary composition calculated per atom of carbon of $CH_pO_nN_q$, the reductance degree (γ) was calculated as follows:

$$\gamma=4+p-2n-3q \qquad /5/$$

where the number of equivalents of available electrons (redoxons) is 4 for carbon, 1 for hydrogen, –2 for oxygen, and –3 for nitrogen (*17,18*).

The elementary composition of SA is $C_4H_6O_4$ or $CH_{6/4}O$ after the calculation per 1 carbon atom, from which the reductance degree (γ_{SA}) is 4+1.5–2=3.5. The mass fraction of carbon in the molecule of SA (δ_{SA}) is 0.406. Therefore, the value of $\gamma_{SA}\delta_{SA}$ is 1.42. Correspondingly, the value of $\gamma_S\delta_S$ for ethanol is 3.12. Thus, the energy yield of SA from ethanol can be calculated as $(1.42/3.12)\cdot Y_{SA}$. Therefore, the values of η_{SA} were 14.8 and 17.8 % for *C. catenulata* and *C. zeylanoides*, respectively. To compare, the value of η_{SA} for hydrocarbon-grown *C. brumptii* IFO 0731 was calculated on the basis of the data presented in the literature (*12*). Taking into account that the value of $\gamma_{SA}\delta_{SA}$ comprised 1.42 and the value of $\gamma_S\delta_S$ for *n*-paraffin, which contained mainly C_{16}–C_{18} alkanes, averaged to 5.1, the value of η_{SA} for hydrocarbon-grown *C. brumptii* IFO 0731 was calculated as $(1.42/5.1)\cdot 67=18.7$ %. Thus, the value of η_{SA} for ethanol-grown strain *C. zeylanoides* (17.8 %) was comparable with that obtained by hydrocarbon-grown *C. brumptii* IFO 0731. Energy capacity of the compounds (*Q*) can be calculated from their elementary composition, since the heat evolved per equivalent of available electrons transferred to oxygen was shown to be about 112.97 kJ

(*18*,*35*). The calculated energy capacities of ethanol, glucose, and hydrocarbons comprise about 29, 15, and 48 kJ/g, respectively. Thus, it can be stated that the maximum theoretically possible mass yield of SA (Y_{SA}) from ethanol should be almost twofold higher than that from glucose and about 1.6 times lower than that from hydrocarbons.

Conclusions

To conclude, the studied yeast strains *C. catenulata* and *C. zeylanoides* grown aerobically in ethanol-containing media produced SA up to 5.6 and 9.4 g/L, respectively. The SA mass yield (Y_{SA}) was 32.6 and 39.0 % for *C. catenulata* and *C. zeylanoides*, respectively. It can be assumed that physiological and genetic manipulations undertaken to reduce the synthesis of by-products would increase the SA production by the yeast strains.

Acknowledgement

We gratefully acknowledge financial support from the Russian Foundation for Basic Research (Grant no. 09-04-00809-a).

References

1. J.G. Zeikus, M.K. Jain, P.E. Elankovan, Biotechnology of succinic acid production and markets for derived industrial products, *Appl. Microbiol. Biotechnol. 51* (1999) 545–552.
2. T.M. Carole, J. Pellegrino, M.D. Paster, Opportunities in the industrial biobased products industry, *Appl. Biochem. Biotechnol. 115* (2004) 871–885.
3. N.I. Fedotcheva, A.P. Sokolov, M.N. Kondrashova, Nonenzymatic formation of succinate under oxidative stress, *Free Radic. Biol. Med. 41* (2006) 56–64.
4. R. Datta, Process for the production of succinic acid by anaerobic fermentation. *US patent 5,143,833* (1992).
5. M.V. Guettler, M.K. Jain, D. Rumbler, Method for making succinic acid, bacterial variants for use in process and methods for obtaining variants. *US patent 5573931* (1996).
6. N.S. Samuelov, R. Datta, M.K. Jain, J.G. Zeikus, Whey fermentation by *Anaerobiospirillum succiniciproducens* for production of a succinate-based animal feed additive, *Appl. Environ. Microbiol. 65* (1999) 2260–2263.
7. P.C. Lee, W.G. Lee, S.Y. Lee, H.N. Chang, Y.K. Chang, Fermentative production of succinic acid from glucose and corn steep liquor by *Anaerobiospirillum succiniciproducens*, *Biotechnol. Bioprocess Eng. 5* (2000) 379–381.
8. M.V. Guettler, D. Rumler, M.K. Jain, *Actinobacillus succinogenes* sp. nov., a novel succinic-acid-producing strain from the bovine rumen, *Int. J. Syst. Bacteriol. 49* (1999) 207–216.
9. H. Lin, G.N. Bennet, K.Y. San, Fed-batch culture of a metabolically engineered *Escherichia coli* strain designed for high-level succinate production and yield under aerobic conditions, *Biotechnol. Bioeng. 90* (2005) 775–779.
10. H. Wu, Z.M. Li, L. Zhou, Q. Ye, Improved succinic acid production in the anaerobic culture of an *Escherichia coli pflB ldhA* double mutant as a result of enhanced anaplerotic activities in the preceding aerobic culture, *Appl. Environ. Microbiol. 73* (2007) 7837–7843.
11. G.N. Vemuri, M.A. Eiteman, E. Altman, Effects of growth mode and pyruvate carboxylase on succinic acid production by metabolically engineered strains of *Escherichia coli*, *Appl. Environ. Microbiol. 68* (2002) 1715–1727.
12. Y. Arikawa, T. Kuroyanagi, M. Shimosaka, H. Muratsubaki, K. Enomoto, R. Kodaira, M. Okazaki, Effect of gene disruptions of the TCA cycle on the production of succinic acid in *Saccharomyces cerevisiae*, *J. Biosci. Bioeng. 87* (1999) 28–36.
13. M. Gallmetzer, J. Meraner, W. Burgstaller, Succinate synthesis and excretion by *Penicillium simplicissimum* under aerobic and anaerobic conditions, *FEMS Microbiol. Lett. 210* (2002) 221–225.
14. M. Sato, T. Nakahara, K. Yamada, Fermentative production of succinic acid from *n*-paraffin by *Candida brumptii* IFO 0731, *Agr. Biol. Chem. 36* (1972) 1969–1974.
15. R.D. Mandeva, I.T. Ermakova, A.B. Lozinov, Metabolite excretion by yeasts of the genus *Candida* in media lacking sources N, P, S, or Mg and having different carbon sources, *Mikrobiologiya* (Moscow), *50* (1981) 62–68 (in Russian).
16. P.R. Burkholder, J. McVeigh, D. Moyer, Studies on some growth factors of yeasts, *J. Bacteriol. 48* (1944) 385–391.
17. I.G. Minkevich, Mass-energy balance for microbial product synthesis – Biochemical and cultural aspects, *Biotechnol. Bioeng. 25* (1983) 1267–1293.
18. L.E. Erickson, I.G. Minkevich, V.K. Eroshin, Application of mass and energy balance regularities in fermentation, *Biotechnol. Bioeng. 67* (2000) 748–774.
19. G.H. Dixon, H.L. Kornberg, Assay methods for key enzymes of the glyoxylate cycle, *Biochem. J. 72* (1959) 3P.
20. M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem. 72* (1976) 248–254.
21. E. Kim, Effect of thiamine on the by-products formation by *Yarrowia lipolytica*, *Biotechnol. Bioprocess. Eng. 4* (1999) 185–188.
22. S. Papanikolaou, S. Sarantou, M. Komaitis, G. Aggelis, Repression of reserve lipid turnover in *Cunninghamella echinulata* and *Mortierella isabellina* cultivated in multiple-limited media, *J. Appl. Microbiol. 97* (2004) 867–875.
23. T.V. Finogenova, S.V. Kamzolova, E.G. Dedyukhina, N.V. Shishkanova, A.P. Il'chenko, I.G. Morgunov, O.G. Chernyavskaya, A.P. Sokolov, Biosynthesis of citric and isocitric acids from ethanol by mutant *Yarrowia lipolytica* N 1 under continuous cultivation, *Appl. Microbiol. Biotechnol. 59* (2002) 493–500.
24. I.G. Morgunov, N.Y. Solodovnikova, A.A. Sharyshev, S.V. Kamzolova, T.V. Finogenova, Regulation of NAD(+)-dependent isocitrate dehydrogenase in the citrate producing yeast *Yarrowia lipolytica*, *Biochemistry* (Moscow), *69* (2004) 1391–1398.
25. G. Barth, W. Künkel, Alcohol dehydrogenase (ADH) in yeast. II. NAD^+- and $NADP^+$-dependent alcohol dehydrogenases in *Saccharomycopsis lipolytica*, *Z. Allg. Mikrobiol. 19* (1979) 381–390.
26. G. Barth, C. Gaillardin: *Yarrowia lipolytica*. In: *Non Conventional Yeasts in Biotechnology: A Handbook*, K. Wolf (Ed.), Springer-Verlag, Berlin, Germany (1996) pp. 313–388.
27. I.T. Ermakova, N.V. Shishkanova, O.F. Melnikova, T.V. Finogenova, Properties of *Candida lipolytica* mutants with the modified glyoxylate cycle and their ability to produce citric and isocitric acid. I. Physiological, biochemical and cytological characteristics of mutants grown on glucose or hexadecane, *Appl. Microbiol. Biotechnol. 23* (1986) 372–377.
28. T.V. Finogenova, N.V. Shishkanova, I.T. Ermakova, I.A. Kataeva, Properties of *Candida lipolytica* mutans with modified glyoxylate cycle and their ability to produce citric and isocitric acid. II. Synthesis of citric and isocitric acid by *C. lipolytica* mutants and peculiarities of their enzyme systems, *Appl. Microbiol. Biotechnol. 23* (1986) 378–383.

29. S.V. Kamzolova, T.V. Finogenova, I.G. Morgunov, Microbiological production of citric and isocitric acids from sunflower oil, *Food Technol. Biotechnol. 46* (2008) 51–59.

30. S. Papanikolaou, I. Chevalot, M. Komaitis, I. Marc, G. Aggelis, Single cell oil production by *Yarrowia lipolytica* growing on an industrial derivative of animal fat in batch cultures, *Appl. Microbiol. Biotechnol. 58* (2002) 308–312.

31. J.E. Holdsworth, M. Veenhuis, C. Ratledge, Enzyme activities in oleaginous yeasts accumulating and utilizing exogenous or endogenous lipids, *J. Gen. Microbiol. 134* (1988) 2907–2915.

32. S.V. Kamzolova, T.I. Chistyakova, E.G. Dedyukhina, N.V. Shishkanova, T.V. Finogenova, Effects of temperature, pH and ethanol concentration on the maximal specific growth rate and biomass composition of *Yarrowia lipolytica*, mutant N 1, *Microbiologiya, 65* (1996) 176–180 (in Russian).

33. E.G. Dedyukhina, V.K. Eroshin, Essential metal ions in the control of microbial metabolism, *Process Biochem. 26* (1991) 31–37.

34. S.J. Pirt: *Principles of Microbe and Cell Cultivation*, Blackwell Scientific Publications, Oxford, UK (1975).

35. I.G. Minkevich, V.K. Eroshin, Productivity and heat generation of fermentation under oxygen limitation, *Folia Microbiol. 18* (1973) 376–385.