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Biotechnological Potential of *Yarrowia lipolytica* Grown under Thiamine Limitation

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Summary

During the cultivation of a thiamine-auxotrophic yeast strain *Yarrowia lipolytica* VKM Y-2412 on ethanol, the growth limitation by thiamine leads to the production of α-ketoglutaric acid. The α-ketoglutaric acid synthesis has been studied in dependence on pH, oxygen supply and ethanol, zinc and iron concentrations. Under optimal conditions, *Y. lipolytica* produced 88.7 g/L of α-ketoglutaric acid. The culture broth containing α-ketoglutaric acid was subjected to chemical treatment with hydrogen peroxide, which led to the formation of succinic acid in significant quantities (71.7 g/L). Further direct esterification of succinic acid with excess absolute ethanol yielded diethyl succinate. Biomass of *Y. lipolytica*, a superproducer of α-ketoglutaric acid, was characterized by a high content of protein and essential amino acids, free amino acids, and unusually large amount of γ-aminobutyric acid. The unique amino acid composition of the producer makes it possible to use this biomass as a component of parenteral nutrition mixtures and as a basis for neuroleptics.

Key words: microbial production, succinic acid, α-ketoglutaric acid, *Yarrowia lipolytica*, γ-aminobutyric acid, diethyl succinate

Introduction

Recent years have seen increased interest in the studies of unconventional yeast *Yarrowia lipolytica* (syn. *Candida lipolytica*). This is due to its distinction from the well-studied yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* in terms of its phylogenetic evolution, physiology and genetics (*1*), and to its extensive use in biotechnology. An international centre for coordination of research in this field has been organized; international conferences have been held since 1995. Significant progress has been achieved in the field of molecular biology and gene engineering experimental biology of *Y. lipolytica*; by 2004, the effort of several European research groups had succeeded in completing the genome study of the yeast, and its genetic map was compiled (*2*). The international *Y. lipolytica* research community conducts studies of the unique ability of the yeast to synthesize organic acids (citric, *threo*-D(S)-(+)-isocitric, pyruvic, α-ketoglutaric and succinic acids) in significant amounts (*3–22*), polyols like erythritol (*23,24*), and tailor-made lipids of very high-added value (*25*).

The physiological basis for the formation of citric and isocitric acids in *Y. lipolytica* grown on *n*-alkanes (*3–5*), glucose (*4,5–10*), glycerol (*11–17*) or vegetable oil (*18–20*) has been sufficiently well studied and described (*1,21, 22*). An indispensable condition for superproduction of these acids by *Y. lipolytica* is the limitation of cell growth by a mineral component of the medium, in particular, nitrogen, at excess carbon source. The production of the

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acids begins after the exhaustion of nitrogen from the medium and the cell transition into the growth decline phase, and is continued in the stationary phase by non-growing cells. Natural yeast strains grown in media with *n*-alkanes, oil or ethanol synthesize both citric and isocitric acids simultaneously in approximately equal amounts; predominantly citric acid is observed to be produced during the growth on glucose or glycerol.

The aim of this work is to show, using the *Y. lipolytica* yeast as an example, how the same producer can be made to synthesize some practically important compounds (α-ketoglutaric acid, succinic acid, diethyl succinate and γ-aminobutyric acid) under the conditions of thiamine deficiency. Ethanol was chosen as a carbon source since it has a number of advantages over other carbon sources. Firstly, it can be produced from renewable raw materials; secondly, ethanol as a chemically pure product contains almost no harmful impurities. For this reason, the use of ethanol as a raw material can simplify and make cheaper the isolation and purification of the target product. Of great importance is the fact that ethanol-based products can be used in food and medical industries.

Materials and Methods

Microorganism

The strain *Y. lipolytica* VKM Y-2412 (All-Russian Collection of Microorganisms, Pushchino Biological Research Center, Pushchino, Russia) was used. The strain was maintained at 4 °C on agar slants with *n*-alkanes as a carbon source.

Chemicals

All chemicals and enzymes were of the highest purity, commercially available and provided by Sigma-Aldrich (St. Louis, MO, USA). Ethanol was purchased from the Kazan Ethanol Processing Company (Kazan, Russia) and used as a carbon source.

Cultivation in fermentor

The yeasts were cultivated in a 10-litre ANKUM-2M fermentor (SKB, Pushchino, Russia) with an initial volume of 5 L. The medium contained (in g/L): $(NH_4)_2SO_4$ 12, $MgSO_4 \cdot 7H_2O$ 1.4, $Ca(NO_3)_2$ 0.8, NaCl 0.5, KH_2PO_4 2.0, K_2HPO_4 0.2, trace elements as described by Burkholder *et al.* (*26*) with slight modifications (in mg/L): I^- 0.1, B^{3+} 0.01, Fe^{2+} 0.05, Zn^{2+} 0.04, Mn^{2+} 0.01, Cu^{2+} 0.01, Mo^{2+} 0.01, and 2 μg/L of thiamine (as a source of vitamins). The fermentation conditions were maintained automatically at a constant level: temperature (28±0.5) °C; pH=(4.0±0.1) was adjusted with 20 % KOH, dissolved oxygen concentration (pO_2) was 60 % (from air saturation), and 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 the cells due to the total consumption of carbon sources. Cultivation was performed as indicated in the text.

Experiments in shake flasks

The effect of ethanol, zinc and iron, as well as pH of the medium and aeration on the cell growth of the yeast *Y. lipolytica* VKM Y-2412 (the growth experiments) and biosynthesis of α-ketoglutaric acid (the biosynthesis experiments) was studied in flask experiments.

Experiments on the effect of zinc, iron and pH of the medium on the cell growth were carried out as follows: cells in the active growth phase (12 h) were sampled from the fermentor, separated from the culture liquid by centrifugation, washed twice with 0.9 % NaCl and suspended in 50 mM phosphate buffer (pH=7.0). The cell suspension was put into flasks with 50 mL of medium and incubated on a shaker at 30 °C for 24 h with a pulsed addition of ethanol (0.1 mL per flask initially and by the 8th and 16th hour of incubation; in total 4.8 g/L). The medium contained (in g/L): $(NH_4)_2SO_4$ 1.0, $MgSO_4 \cdot 7H_2O$ 0.7, $Ca(NO_3)_2$ 0.4, NaCl 0.5, KH_2PO_4 1.0, K_2HPO_4 0.1, trace elements according to Burkholder (*26*), and 2 μg/L of thiamine. The initial concentration of cells in the growth experiment was maintained at no more than 0.2 g/L.

Experiments on the effect of zinc, iron and pH of the medium on the biosynthesis of α-ketoglutaric acid were carried out according to the following scheme: cells in the active acid-formation phase (72 h) were sampled from the fermentor, separated from the culture liquid by centrifugation, washed twice with 0.9 % NaCl and suspended in 50 mM phosphate buffer (pH=7.0). The cell suspension was added into flasks with 50 mL of medium without vitamins and nitrogen and incubated on a shaker at 30 °C for 24 h with a pulsed addition of ethanol (0.1 mL per flask initially and every four hours of incubation; in total 9.6 g/L). The initial concentration of cells in all variants of the acid-formation experiment was maintained at no more than 2.4 g/L.

When the effect of ethanol concentration was studied, ethanol was added into the medium initially in the concentrations from 1.6 to 40.0 g/L. In the experiments with the initial ethanol concentration of 1.6 and 3.2 g/L, the additional quantity of ethanol was supplemented into the medium up to 4.8 g/L in the growth experiments, and up to 9.6 g/L in the biosynthesis experiments.

In the aeration experiments, various aeration intensities were created by changing the volume of the medium in 750-mL flasks (50, 100, 150 and 200 mL). The oxygen solution rates in the culture were controlled by sodium sulphite method described by Pirt and Callow (*27*). In flasks containing 50, 100, 150, and 150 mL of the medium, the oxygen solution rate was 0.56, 0.30, 0.26, and 0.24 mmol/(L·min), respectively.

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 (Laboratorní Přístroje, Prague, 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 organic acids was determined using high-performance liquid chromatograph (HPLC; Pharmacia LKB, Uppsala, Sweden) with an Inertsil ODS-3 reversed phase column (250×4 mm, Elsiko, Moscow, Russia) at 210 nm; 20 mM phosphoric acid was used as a mobile phase with the flow rate of 1.0 mL/min; the column temperature was maintained at 35 °C. Quantitative determination of organic acids was carried out with the calibration curves plotted using α-ketoglutaric, succinic, citric, *threo*-D(S)-(+)-isocitric, acetic, maleic and fumaric acids (Sigma-Aldrich) as standards.

For the amino acid assay, the biomass was freeze-dried. A volume of 2 mL of 80 % ethanol acidified with 0.1 M HCl was added to 20–30 mg of dry biomass and held for 24 h at room temperature. The extract was centrifuged, the residue was discarded and the supernatant was assayed on a Biotronik LC2000 amino acid analyzer (Biotronik GmbH, Maintal, Germany) for free amino acids by the method of Moore *et al.* (*28*).

For the bound amino acid assay, the biomass residue after the extraction of free amino acids with ethanol was additionally washed with 80 % ethanol and dried at 65–70 °C. The sample (10–15 mg) was hydrolyzed with 6 M HCl at 110 °C for 24 h. Excess HCl was removed from the extract using a rotary evaporator. A volume of 2 mL of 0.2 M sodium citrate buffer (pH=2.2) was added to the dry residue. The amino acid content was determined using a Biotronik LC2000 automatic amino acid analyzer.

Calculation of fermentation parameters

To take into account the dilution of the medium due to the addition of KOH solution for maintaining the constant pH value, the total amount of α-ketoglutaric acid in the culture broth was used for calculations of the mass yield of α-ketoglutaric acid (Y_{KGA}) and volumetric α-ketoglutaric acid productivity (Q_{KGA}).

The mass yield of biomass ($Y_{X/S}$), expressed in g of biomass per g of ethanol consumed, was calculated from the equation:

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

The mass yield of α-ketoglutaric production (Y_{KGA}), expressed in g of α-ketoglutaric acid per g of ethanol consumed, was calculated from the equation:

$$Y_{KGA} = \frac{P}{S} \qquad /2/$$

while the volumetric α-ketoglutaric acid productivity (Q_{KGA}), expressed in g/(L·h), was calculated from the equation:

$$Q_{KGA} = \frac{P}{V \cdot t} \qquad /3/$$

where X is the amount of biomass in the culture liquid at the end of cultivation (g), P is the total amount of α-ketoglutaric acid in the culture liquid at the end of cultivation (g), S is the total amount of ethanol consumed (g), V is the initial volume of culture liquid (L), and t is fermentation time (h).

Statistical analysis

All the presented data are the mean values of three experiments and two measurements for each experiment; standard deviations were calculated (S.D.<10 %).

Results and Discussion

α-Ketoglutaric acid production

The yeast *Y. lipolytica* is not capable of synthesizing the pyrimidine moiety of the thiamine molecule. During the cultivation of *Y. lipolytica* under the conditions of thiamine deficiency, when thiamine is proven to be a growth-limiting factor, cells convert the source of carbon into an incomplete oxidation product, α-ketoglutaric acid (*4*, *29–40*). The acid is excreted into the medium and can be accumulated in large amounts. The regulatory mechanism of the TCA cycle key enzymes involved in the processes of α-ketoglutaric acid superproduction in the *Y. lipolytica* yeast has also been studied (*29*,*33–36*). Under thiamine deficiency, the activity of α-ketoglutarate dehydrogenase-containing thiamine pyrophosphate as a cofactor has been shown to sharply decrease; as a result, a major part of α-ketoglutaric acid formed is excreted from the cell into the culture liquid.

This section of the work considers the possibility of producing α-ketoglutaric acid from ethanol using the yeast *Y. lipolytica* VKM Y-2412. The use of ethanol as a growth substrate poses certain problems since it is toxic for yeast cells. High concentrations of ethanol affect the functioning of the cell enzyme systems, inhibit the growth and in some cases cause the death of microbial cells. A particular feature of yeast growth in a medium with ethanol is excretion of acetate; a possible cause of this event is an imbalance among the activities of alcohol dehydrogenase, aldehyde dehydrogenase and alcohol oxidase. Zinc and iron, which are integral components of many enzyme systems involved in ethanol metabolism (alcohol dehydrogenase, aldehyde dehydrogenase, catalase, aconitase, cytochromes), have been shown to have a profound influence on yeast metabolism (*41*). The effect of oxygen concentration and pH on the synthesis of α-ketoglutaric acid in *Y. lipolytica* grown on *n*-alkanes, glucose and acetate has been shown (*31*,*32*). In this context, it was important to choose the optimal concentrations of ethanol, zinc and iron, as well as pH of the medium and the level of oxygen for the growth of the yeast *Y. lipolytica* VKM Y-2412 and biosynthesis of α-ketoglutaric acid.

The effect of zinc on *Y. lipolytica* growth was studied within Zn^{2+} concentration range of 0.01–10.0 mg/L. As it is seen in Fig. 1a, Zn^{2+} concentration is an efficient factor regulating the growth and synthesis of α-ketoglutaric acid. The concentration of Zn^{2+} lower than 0.01 mg/L limited the yeast growth (biomass was as low as 1.0 g/L) and α-ketoglutaric acid synthesis was insignificant (0.8 g/L). An increase in Zn^{2+} concentration from 0.01 to 0.30 mg/L increased biomass (up to 3.0 mg/L) and α-ketoglutaric acid synthesis (by 2.1 times).

The synthesis of α-ketoglutaric acid is maintained at a comparatively high level of 3.6–3.8 g/L with an increase in Zn^{2+} concentration up to 2.0 mg/L. An increased requirement for zinc under the cultivation on ethanol is,

possibly, due to the activation of alcohol dehydrogenase in yeast cells. At a concentration of Zn^{2+} greater than 5 mg/L, yeast growth and α-ketoglutaric acid biosynthesis were inhibited. All subsequent experiments were carried out at Zn^{2+} concentration of 0.3 mg/L.

The effect of Fe^{2+} on yeast growth was studied within the concentration range of 0.01–10.0 mg/L. As it is seen in Fig. 1b, the yeast *Y. lipolytica* VKM Y-2412 is sensitive to Fe^{2+} ions in the medium. The concentration of Fe^{2+} lower than 0.01 mg/L limited the cell growth and the accumulation of biomass was 0.95 g/L. Acetic acid (3.26 g/L) was accumulated in the culture liquid along with α-ketoglutaric acid (1.0 g/L). It seems that at high Zn^{2+} (0.30 mg/L) and low Fe^{2+} (0.01 mg/L) concentrations, there is an imbalance between the formation of acetic acid and its further conversion, and as a result, acetic acid is accumulated in the cell and excreted into the culture liquid. Acetic acid production from several *Y. lipolytica* strains has previously been reported during the cultivation of yeast on glucose-based media (*42*).

An increase in Fe^{2+} concentration from 0.01 up to 0.50 mg/L leads to an increase in biomass (2.6 times) and α-ketoglutaric acid biosynthesis (by 4.6 times). The synthesis of α-ketoglutaric acid is maintained at a high level of 3.5–4.8 g/L at 0.1–3.0 mg/L of Fe^{2+}. A further increase in the concentration of Fe^{2+} up to 10.0 mg/L led to a decrease in the biomass accumulation and α-ketoglutaric acid biosynthesis. All subsequent experiments were carried out at 0.3 mg/L of Zn^{2+} and 1.2 mg/L of Fe^{2+}.

The effect of ethanol on yeast growth was studied within the ethanol concentration range of 1.6–40.0 g/L. As seen from Fig. 1c, the yeast grew well and produced α-ketoglutaric acid at initial ethanol concentrations in the medium from 1.6 up to 7.2 g/L; the maximum biosynthesis of α-ketoglutaric acid was observed at the ethanol concentration of 1.6 g/L. An increase in the ethanol concentration to over 10.0 g/L led to a decrease in biomass accumulation and α-ketoglutaric acid biosynthesis. At the ethanol concentration of 40.0 g/L, the cell growth as well as the α-ketoglutaric acid synthesis and accumulation of acetic acid (6.5 g/L) were inhibited. In the subsequent experiments, ethanol was periodically added into the medium to maintain its concentration at 1.6 g/L.

Yeast growth and α-ketoglutaric acid production largely depend on aeration. As seen in Fig. 2a, at high aeration (0.56 mmol/(L·min)), the accumulation of biomass was 1.4 times lower as compared to the low oxygen conditions (0.24 mmol/(L·min)). α-Ketoglutaric acid was virtually not excreted under low aeration conditions; active acid formation was observed only at high aeration level. For the yeast *Y. lipolytica* 695 grown on glucose and acetate under thiamine limitation, the biomass was shown to be 1.5–2 times higher under low aeration conditions (pO_2 equal of 5–10 % from air saturation) than at intensive aeration (pO_2 equal of 60–90 % from air saturation); in contrast, the intensity of the α-ketoglutaric acid biosynthesis under low aeration conditions decreased significantly (two- to three-fold) (*31*).

One of the significant factors influencing the α-ketoglutaric acid biosynthesis is pH of the medium. As seen in Fig. 2b, the yeast *Y. lipolytica* is capable of growing

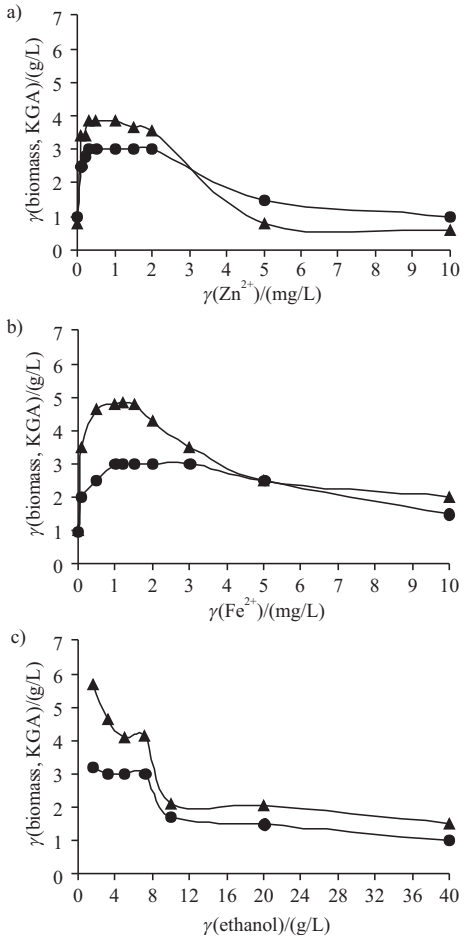

Fig. 1. The effect of: a) Zn^{2+}, b) Fe^{2+}, and c) ethanol on the growth (●) and α-ketoglutaric acid production (▲) by *Y. lipolytica* VKM Y-2412

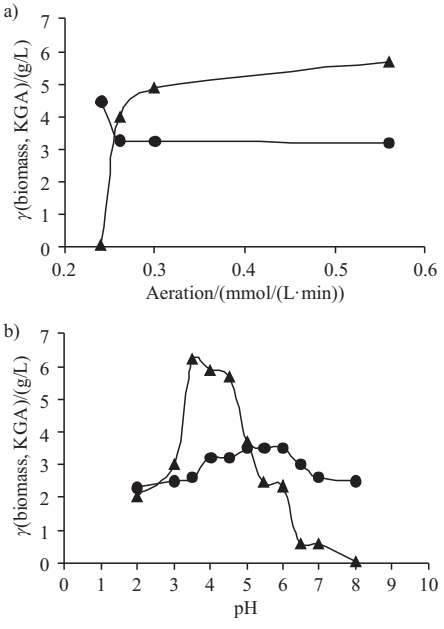

Fig. 2. The effect of: a) aeration and b) pH on the growth (●) and α-ketoglutaric acid production (▲) by *Y. lipolytica* VKM Y-2412

within a broad range of pH; the maximum growth rate was at pH=4.0–6.5. Lowering the pH to 2.0–3.5 led to a decrease in the accumulation of biomass by approximately 25 %. The optimum pH for growth does not coincide with the optimal conditions for α-ketoglutaric acid biosynthesis; intensive formation of α-ketoglutaric acid was observed at pH=3.5–4.5. According to literature data, the optimum pH for α-ketoglutaric acid production from *n*-alkanes was in the range of 3.5–4.5 (*5*,*32*). In the medium with glycerol, the optimal pH for α-ketoglutaric acid production was 5.5 (*40*). By contrast, the optimum pH range for citric acid production (4.5–6.0) corresponded to the optimum pH values (4.5–6.0) for the growth of *Y. lipolytica* in the media containing ethanol (*41*), glucose (*42*) or glycerol (*11*,*13–15*,*17*).

As the result of the studies, we selected conditions providing for the largest synthesis of α-ketoglutaric acid in the medium with ethanol: low aeration in the growth period and high aeration in the α-ketoglutaric acid production phase; the optimum pH of the medium was 4.5 in the growth phase and 3.5 in the stage of α-ketoglutaric acid synthesis. Ethanol was periodically added to keep constant the concentration of 1.6 g/L; concentrations of Zn^{2+} and Fe^{2+} were increased up to 0.3 and 1.2 mg/L, respectively. Optimization of the cultivation conditions led to an increase in the α-ketoglutaric acid production from 3.8 up to 6.2 g/L.

The data on the growth dynamics and α-ketoglutaric acid production are given in Fig. 3. In the first 48 h, cells multiplied and biomass increased up to 10.0 g/L,

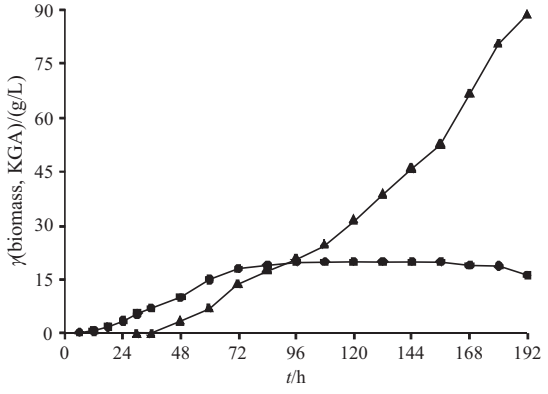

Fig. 3. Time courses of growth (●) and α-ketoglutaric acid production (▲) by *Y. lipolytica* VKM Y-2412

after which the culture passed into the growth decline phase (up to 96 h) caused by the depletion of thiamine in the medium. At this stage, the synthesis of α-ketoglutaric acid started. Intensive formation of α-ketoglutaric acid was observed in the stationary phase (after 96 h). By the end of cultivation (192 h), α-ketoglutaric acid was accumulated in the culture liquid in the amount of 88.7 g/L with an insignificant content of the other acids. The level of biosynthesis was on average 0.76 g/(L·h) with the yield of the product from the ethanol introduced into the medium of 70 %. For comparison, some relevant methods for the synthesis of α-ketoglutaric acid are summarized in Table 1 (*33*,*43–47*). *Y. lipolytica* is able to synthesize α-ketoglutaric acid from purified *n*-paraffin (*43–45*) and ethanol (*33*) in significant amounts. Flask experiments using substrates from renewable feedstock indicate that triglycerides (sunflower and rapeseed oil) (*46*) and glycerol (*47*) are potentially efficient carbon sources for α-ketoglutaric acid production by *Y. lipolytica*.

Chemically assisted microbial production of succinic acid from α-ketoglutaric acid

Recently, we have shown that decarboxylation of α-ketoglutaric acid in the presence of hydrogen peroxide leads to the formation of succinic acid (*38*) at a concentration of 63.4 g/L. However, with respect to consumed ethanol the yield was low (58 %). In this work, it was of interest to study the possibility of practical decarboxylation of α-ketoglutaric acid in a culture liquid of an 8-day *Y. lipolytica* culture to succinic acid in the presence of H_2O_2.

A culture liquid sample containing α-ketoglutaric acid at a concentration of 88.7 g/L (608 mM) was incubated for 1 h in the presence of various concentrations of H_2O_2. The HPLC chromatograms of the standard solutions of succinic acid and α-ketoglutaric acid as well as the culture liquid samples before and after incubation for 1 h in the presence of H_2O_2 are given in Fig. 4. As it can be seen, the culture liquid before incubation exhibits only the peak of α-ketoglutaric acid (elution time 8.33 min), whereas during the incubation of the culture liquid filtrate with 2560 mM H_2O_2, the culture liquid is found to exhibit only the peak of succinic acid (elution time 9.73 min), *i.e.* we observed the complete oxidation of 88.7 g/L (608 mM) of α-ketoglutaric acid to succinic acid, whose concentration reached 71.7 g/L (608 mM), and the yield of the ethanol introduced into the medium during α-ke-

Table 1. Efficiency of α-ketoglutaric acid (KGA) synthesis by yeast

Organism	Substrate	Cultivation mode	γ(KGA) / g/L	Y_{KGA} / %	Reference
Candida lipolytica	*n*-paraffins	batch	48.0	60.0	(*43*)
C. lipolytica	*n*-paraffins	batch	185.0	80.0	(*44*)
Yarrowia lipolytica H222-27-11	*n*-paraffins	fed-batch	195.0	90.0	(*45*)
Y. lipolytica N 1	ethanol	fed-batch	49.0	42.0	(*33*)
Y. lipolytica H222-27-11	sunflower oil	batch	35.0	38.0	(*46*)
Y. lipolytica H222-27-11	rapeseed oil	batch	34.0	37.0	(*46*)
Y. lipolytica WSH-Z06	glycerol	batch	39.2	39.2	(*47*)
Y. lipolytica VKM Y-2412	ethanol	fed-batch	88.7	70.0	present work

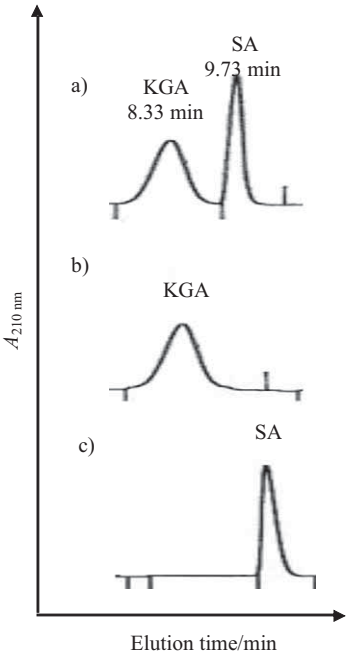

Fig. 4. HPLC spectra of: a) a mixture of standards of α-ketoglutaric acid (KGA) and succinic acid (SA), b) culture liquid before treatment with H_2O_2, and c) culture liquid 1 h after the treatment with H_2O_2

toglutaric acid production was 70 % (Table 1). The obtained results on succinic acid concentration and yield are better by 13.2 and 20.6 %, respectively, than those we published earlier (*38*). Succinic acid was isolated from the culture liquid filtrate in a crystal form as described earlier (*38*) with a purity of 99.9 %.

It should be noted that the microbiological production of succinic acid has received increasing interest because of its potential as a platform chemical for the production of various value-added derivatives. Succinic acid and its derivatives have numerous applications, including surfactants, detergents, electroplating, food, pharmaceutical, antibiotics, amino acids and vitamins (*48*,*49*).

The results of the efficient succinic acid production by yeast *Y. lipolytica* obtained in the present work confirm that the application of yeast organisms has a considerable promise for the industrial succinic acid production. The invented process leads to the accumulation of succinic acid in the concentrations corresponding to the best data reported in literature. For comparison, the anaerobic bacterium *Actinobacillus succinogenes* 130Z, which is able to accumulate a significant amount of succinic acid (66.4 g/L), excretes considerable amounts of byproducts, including acetic, formic, propionic, and pyruvic acids at concentrations of 12.0, 8.7, 2.5, and 4.3 g/L, respectively (*50*). *Anaerobiospirillum succiniciproducens* strains cultivated in glucose-containing media accumulated succinic acid at concentrations of 25–55 g/L with a ratio of succinic acid to other acids no greater than 5:1 (*51*).

Diethyl succinate

Besides its direct applications, succinic acid produced by *Y. lipolytica* yeasts can be used in the form of diethyl succinate as flavouring for food products and as a perfume component (flower fragrance).

Diethyl succinate was obtained by direct esterification of succinic acid, produced by the method described in the previous section, with excess absolute ethanol. Sulphuric acid (3 % of succinate) was used as a catalyst. Water formed was distilled as an azeotropic mixture with toluene. Upon elimination of the solvents, diethyl succinate was distilled at a reduced pressure. Boiling temperature was 105 °C at 1999.8 Pa.

Characteristics of amino acid composition of the superproducer biomass

As seen in Table 2, the production of α-ketoglutaric acid by *Y. lipolytica* cells grown on ethanol under thiamine deficiency is accompanied by the formation of a large pool of amino acids (314.2 mg per g of dry mass) in which glutamic acid, asparagine, lysine, leucine and valine prevailed (up to 50 % of the total amount of amino acids). The pool of free acids is 4.8 % of the total amount of amino acids in the cells. It should be noted that γ-aminobutyric acid, which is the only amino acid not occurring in proteins, makes significant part of the free acid pool (37.3 %).

Table 2. Amino acid (AA) content of *Y. lipolytica* biomass

AA	Bound AA	Free AA	Total amount of bound and free AA
	mg per g of dry mass	mg per g of dry mass	mg per g of dry mass
glutamic acid	45.9±0.1	0.3±0.1	46.2
asparagine	35.3±3.5	0.9±0.4	36.1
lysine	28.3±4.1	1.2±0.0	29.6
leucine	27.3±0.8	1.8±0.3	29.1
valine	21.6±2.6	0.6±0.3	22.2
threonine	19.4±1.8	0.3±0.1	19.7
serine	18.1±1.6	0.3±0.1	18.4
tyrosine	17.8±2.9	0	17.8
alanine	17.5±0.8	2.0±1.5	19.5
isoleucine	17.2±1.7	0.4±0.2	17.6
phenylalanine	16.5±3.4	0	16.5
glycine	16.3±0.8	0.6±0.4	17.0
histidine	8.6±1.3	0.3±0.1	8.9
methionine	7.1±0.5	0.1±0.1	7.2
cysteine	3.1±0.0	0.5±0.3	3.6
γ-aminobutyric acid	0	5.6±0.2	5.6

γ-Aminobutyric acid is known to be the main inhibitory neurotransmitter of the nervous system, whose receptors are widespread in the brain structures, virtually in all neuronal groups, in contrast to glutamate and aspartate, which belong to excitatory neurotransmitters. Disrupted biosynthesis and secretion of γ-aminobutyric acid in humans leads to various disorders, including epilepsy and Alzheimer's disease.

The function of γ-aminobutyric acid has been less studied in plants and microorganisms. It has been found that γ-aminobutyric acid is produced in yeast cells in response to various stresses, such as hypoxia, exposure to cold, oxidative stress (*52*), as well as under thiamine deficiency (*53*). The unique amino acid composition of the producer makes it possible to use this biomass in parenteral nutrition mixtures and as the basis for neuroleptics.

Conclusions

It was shown in this work that some valuable metabolites (such as α-ketoglutaric acid, succinic acid, diethyl succinate, biomass enriched with protein and essential amino acids), which can be used in food and medical industries, were successfully produced under the conditions of thiamine (a growth-limiting factor) deficiency. The model organism used for that purpose was *Yarrowia lipolytica*, because it is considered a nonpathogenic organism and, therefore, metabolites produced by this culture may be classified as Generally Recognized As Safe (GRAS) by the Food and Drug Administration (FDA, USA).

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