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**EXPERIMENTAL ARTICLES**

# Metabolic Characteristics of the Mutant *Yarrowia lipolytica* Strain 1 Producing α-Ketoglutaric and Citric Acids from Ethanol and the Effect of $[NH_4^+]$ and $[O_2]$ on Yeast Respiration and Acidogenesis

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**Abstract**—The comparative studies performed in this work showed that overproduction of α-ketoglutaric acid (KGA) and citric acid (CA) from ethanol by the mutant *Yarrowia lipolytica* strain 1 requires both a deficiency of thiamine and a relatively high concentration of ammonium ions in the medium, whereas CA overproduction requires an almost zero concentration of ammonium ions. The threshold value of the dissolved oxygen concentration in the medium, $pO_2$, for CA overproduction is considerably higher than for KGA overproduction. The respiration rate of CA-overproducing cells was 2–3.5 times higher than that of KGA-overproducing cells. The main terminal electron carrier functioning in the KGA-overproducing cells was cytochrome oxidase. In the CA-overproducing cells, the main terminal oxidase was presumably *o*-type cytochrome.

*Key words*: overproduction, organic acids, yeast, cytochromes, respiration.

Under certain cultivation conditions, the mutant *Yarrowia lipolytica* strain 1 grown on ethanol excretes some organic acids into the medium [1, 2]. It is believed that the main condition governing the overproduction of α-ketoglutaric acid (KGA) is the deficiency of thiamine in the medium, whereas citric acid (CA) is overproduced when there is a deficiency in nitrogen.

The aim of the present work was to comparatively study the physiological and metabolic changes occurring in yeast cells grown on ethanol under conditions promoting the overproduction of organic acids.

## MATERIALS AND METHODS

The mutant *Yarrowia lipolytica* strain 1 used in this study was obtained by treating the parent strain *Y. lipolytica* 704 with *N*-nitroso-*N*-methylurea [3].

The yeast was cultivated at 28°C in a 6-l ANKUM-2M fermentor in the medium described earlier [1]. The pH of the medium was maintained automatically at a level of 4.5 by adding a 20% solution of NaOH. The concentration of thiamine in the growth medium under KGA overproduction conditions was 3 and 200 μg/l under CA overproduction conditions (the latter value is the standard concentration of thiamine necessary for normal growth of *Y. lipolytica*). Ethanol was added in portions, according to its rate of consumption in the medium, so that the maximum ethanol concentration in the medium was always below 2.5 g/l. The concentration of dissolved oxygen in the medium, $pO_2$, was maintained automatically at a specified level by an oxystat system.

Yeast growth was estimated gravimetrically by weighing the dry biomass. The concentration of ammonium ions was determined as described earlier [1]. KGA, pyruvic acid (PA), and isocitric acid (ICA) in the culture liquid were assayed enzymatically [4, 5]. CA was assayed by the chemical method described by Zhabolovskaya *et al.* [6].

The respiration rate of intact yeast cells was measured using a Clark-type oxygen electrode with a temperature-controlled measuring cell (28°C).

The content of cytochromes in cells was determined from their redox difference absorption spectra using the following values of millimolar extinction coefficients: 24.0 for cytochrome *c*, 17.8 for cytochrome *b*, and 14.0 for the cytochrome complex $a + a_3$ [7].

The cell-free homogenate of yeast cells was prepared as described previously [2]. The activities of cytochrome oxidase (EC 1.9.3.1) and cytochrome *c* peroxidase (EC 1.11.1.5) were determined from cytochrome *c* oxidation rates [7, 8].

Protein was quantified by the method developed by Lowry *et al.* [9].

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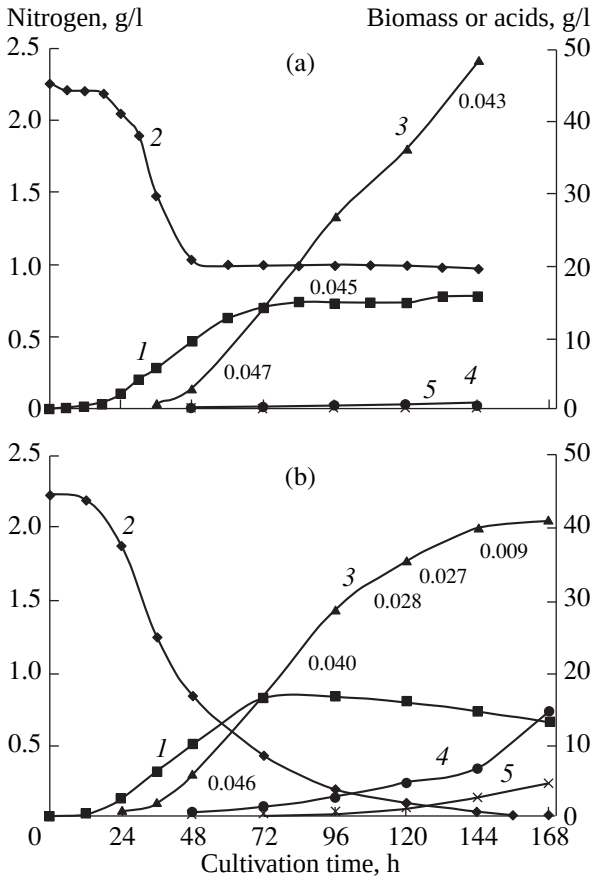


**Fig. 1.** Effect of nitrogen concentration on the accumulation of KGA in the medium under thiamine deficiency conditions: (a) after KGA began accumulating in the medium, the concentration of nitrogen was maintained at a level of 0.9–1.0 g/l; and (b) exhaustion of the nitrogen source was allowed in the medium. Curves *1*, *2*, *3*, *4*, and *5* show the dynamics of the biomass, nitrogen, KGA, CA, and isocitric acid, respectively. The figures under curve *3* show the specific rate of KGA synthesis (g KGA/(g biomass h)).

## RESULTS AND DISCUSSION

**Effect of the nitrogen source concentration on KGA and CA overproduction.** According to earlier findings, the major condition for CA overproduction by the yeast *Y. lipolytica* is the low concentrations of nitrogen sources in the medium, whereas KGA is overproduced under thiamine deficiency [1, 2]. The results obtained in the present study showed that thiamine deficiency is a necessary, but not sufficient, condition for KGA overproduction. Indeed, as can be seen from Fig. 1, a high rate of KGA accumulation in the medium under thiamine deficiency was observed only when the concentration of the nitrogen source in the medium was not lower than 0.9–1.0 g/l (Fig. 1a). As the nitrogen concentrations decreased, KGA overproduction tended to slow down (Fig. 1b). Simultaneously, citric and isocitric acids accumulated in the medium.

Nitrogen consumption during the overproduction of KGA drastically decreased (table). For instance, at vigorous aeration of the medium, the rate of nitrogen consumption decreased from a value of 4.88 mg/(g biomass h) typical of the period of active growth (24–36 h of growth) to a value of 0.26 mg/(g biomass h) typical of the period of KGA overproduction (48–144 h of growth). At poor aeration of the medium (low $pO_2$ values), the respective rates of nitrogen consumption were 6.0 and 0.46 mg/(g biomass h). It should be noted that the yeast growth rate during the period of KGA overproduction was low.

A comparison of the concerned parameters showed that the concentration of ammonium ions in the medium is utterly important in relation to overproduction of both KGA and CA (see table): it must be relatively high for KGA overproduction and close to zero for CA overproduction. The amount of CA accumulated in the medium was 2.5–3 higher than that of KGA. Correspondingly, the yield of CA with respect to the ethanol consumed was about twice higher than that of KGA. This suggests that, in the case of KGA overproduction, part of the carbon atoms of the consumed ethanol are used in assimilation processes closely related to nitrogen metabolism.

**Effect of $pO_2$ on KGA and CA overproduction.** Earlier studies showed that the optimal $pO_2$ values for CA overproduction range from 20 to 50% oxygen saturation in the medium [2]. At $pO_2$ = 5–8% oxygen saturation, the accumulation of CA in the medium ceased after 50–60 h of cultivation (Fig. 2a). However, at $pO_2$ = 50–55% oxygen saturation, CA continued accumulating over a long time period (Fig. 2b). The biomass was greater at $pO_2$ = 5–8%.

Unlike with CA overproduction, low oxygen concentrations ($pO_2$ = 5–8%) in the medium not only failed to inhibit KGA overproduction, but even stimulated it. Moreover, the biomass was higher at low than at high oxygen concentration ($pO_2$ = 50%) (Figs. 2c, 2d).

Therefore, the overproduction of both KGA and CA depends on the $pO_2$ of the cultivation medium. The threshold value of $pO_2$ inhibitory to CA overproduction (5–8% oxygen saturation) is higher than the threshold value inhibitory to KGA overproduction, the latter being within 0.1–1% oxygen saturation (the respective experimental data are not presented). For the sake of comparison, the threshold oxygen concentration for the overproduction of isocitric acid by the wild-type *Y. lipolytica* strain is 28–30% [10].

**Cell respiration during KGA and CA overproduction.** Measurements showed that the dynamics of cell respiration during CA overproduction at low ($pO_2$ = 5–8%) and high ($pO_2$ = 50%) oxygen concentrations in the cultivation medium were almost identical: by the time CA began to accumulate in the medium, cell respiration had decreased to a certain value and then remained at an approximately constant level (Figs. 2a, 2b).

Under thiamine deficiency, which favors the overproduction of KGA, the dynamics of cell respiration at vigorous and poor aeration of the medium were practically the same as in the case of CA overproduction

Physiological and biochemical characteristics of the mutant *Y. lipolytica* strain 1 overproducing KGA and CA

| Parameter | KGA overproduction | | CA overproduction | |
|---|---|---|---|---|
| | $pO_2$, % | | $pO_2$, % | |
| | 50–55 | 5–8 | 50–55 | 5–8 |
| Final biomass, g/l | 12.0 | 15.0 | 9.0 | 12.0 |
| Total acid content after 6 days of cultivation, g/l | 42.0 | 50.0 | 120.0 | 60.0 |
| Acid yield with respect to the ethanol consumed, % | 40–44 | 42–44 | 80–85 | 40–42 |
| Specific production rate ($q_p$), g acid/(g biomass h) | 0.040 | 0.045 | 0.17 | 0.16 |
| Thiamine content, μg/g biomass | 0.25 | 0.20 | – | – |
| Initial [N] in the medium, g/l | 2.2 | 2.2 | 0.66 | 0.66 |
| Threshold [N] for overproduction, g/l | 1.0–1.3 | 1.0–1.3 | 0.05 | 0.05 |
| [N] at which overproduction slows down, g/l | 0.5 | 0.5 | | |
| Rate of N consumption during overproduction, mg/(g biomass h) | 0.26 | 0.46 | 0 | 0 |
| Respiration rate (nmol $O_2$/(min mg biomass)) during | | | | |
| growth | 1200 | 800 | 3000 | 2700 |
| overproduction | 300 | 200–300 | 550 | 600 |
| Inhibition of respiration by KCN during overproduction, % | 20–30 | 20–30 | 50–60 | 50–60 |
| Concentration of cytochromes during overproduction, nmol/g biomass | | | | |
| $a + a_3$ | 150 | 170 | 15 | 25 |
| *b* | 90 | 100 | 200 | 100 |
| *c* | 180 | 220 | 400 | 150 |

Note: [N] is the concentration of nitrogen source. "–" stands for "not measured."

(Fig. 2c, 2d). In all cases, cell respiration decreased by 1.5–2.5 times during the period of active accumulation of organic acids. The respiration of CA-overproducing cells was 2–2.5 times higher than that of KGA-overproducing cells. At $pO_2$ = 5–8% oxygen saturation, the respiration of KGA-overproducing cells was 1.5–2 times slower than at $pO_2$ = 50%.

These data suggest that the processes of KGA and CA overproduction are associated with considerable changes in the functioning of the respiratory chain, or the electron transfer system, of *Y. lipolytica*. In this respect, of great interest are the experimental results presented in Fig. 3.

When KGA began to accumulate in the medium at $pO_2$ = 50% (Fig. 3a), the rate of air supply into the fermentor reached 1.5–2.0 l/min and remained at this level over the entire period of KGA accumulation (Fig. 3b). As the nitrogen concentration in the medium decreased to a threshold level (Fig. 3a), the accumulation of KGA slowed down, while citric and isocitric acids began to accumulate. In this case, cell respiration intensified and the rate of air supply by the oxystat system increased to 10 l/min (Fig. 3b). Finally, all of the available carbon was consumed by cells for the synthesis of CA; therefore, ICA also ceased to accumulate in the medium (Fig. 3a).

**Changes in the electron transport system of *Y. lipolytica* associated with the overproduction of KGA and CA.** In the CA-overproducing cells, the concentration of the cytochrome complex $a + a_3$ drastically decreased and that of cytochromes *b* and *c* increased by about a factor of two (see table), so the proportion between these cytochromes also changed. Conversely, the concentration of cytochromes and the proportion between them in the KGA-overproducing cells changed insignificantly (see table).

After the exhaustion of the nitrogen source in the medium and the onset of the CA accumulation process, the activities of the enzymes involved in the oxidation of cytochrome *c* (table), i.e., cytochrome oxidase (CCO) and cytochrome *c* peroxidase (CCP), decreased to almost zero. This fact, combined with the relatively high respiration rate of CA-overproducing cells, suggests that cytochrome *c* in these cells is oxidized by a terminal electron carrier other than CCO or CCP.

Figure 4a presents the redox difference absorption spectra of the dithionite-reduced minus $H_2O_2$-oxidized cells of the mutant *Y. lipolytica* strain 1 grown under different cultivation conditions. The difference spectrum of the cells grown in complete (i.e., thiamine and nitrogen sufficient) medium (Fig. 4a, spectrum *1*) exhibited absorption peaks typical of the mitochondrial cytochromes $a + a_3$ (605 and 444 nm), *b* (562, 524, and 430 nm), and *c* (549, 519, and 419 nm). The CO-difference spectrum of dithionite-reduced CO-gassed minus dithionite-reduced cells (Fig. 4b, spectrum *1*) exhibited

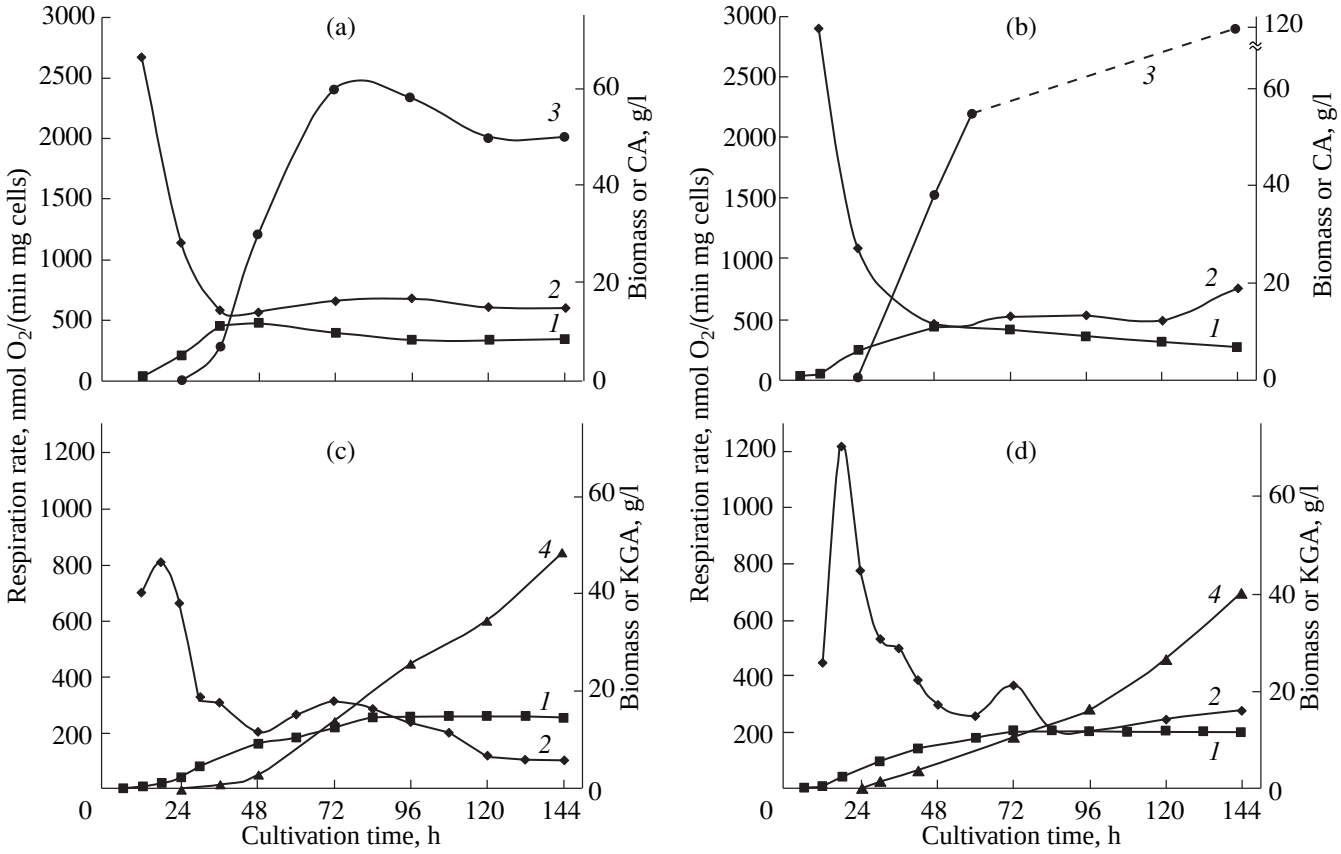


**Fig. 2.** Effect of $pO_2$ on growth, respiration, and the excretion of organic acids by *Y. lipolytica* cells grown under conditions favoring (a and c) CA overproduction ($pO_2$ = 5–8% oxygen saturation in the medium) and (b and d) KGA overproduction ($pO_2$ = 50–55% oxygen saturation). Curves *1*, *2*, *3*, and *4* show the dynamics of the biomass, respiration, KGA accumulation, and CA accumulation, respectively.

absorption peaks at 585–587 and 426–430 nm, which are typical of the cytochrome complex $a + a_3$, as well as at 540 and 399–404 nm, which may belong to a CO-binding pigment.

The redox difference spectrum of KGA-overproducing cells (Fig. 4a, spectrum *2*) was similar to that of cells grown in a complete medium (Fig. 4a, spectrum *1*) and differed from it only in the absence of an absorption peak at 420 nm. The CO-difference spectra of these two types of cells were almost identical (Fig. 4b, spectra *1* and *2*).

As for CA-overproducing cells (72 h of growth; 63 g/l of the CA accumulated in the medium), they lacked the cytochrome $a + a_3$ peak both in the redox difference spectrum (Fig. 4a, spectrum *3*) and in the CO-difference spectrum (Fig. 4b, spectrum *3*). Furthermore, instead of the commonly observed minimum at 445–450 nm (Fig. 4a, spectra *1* and *2*), which is typical of reduced flavoproteins, the redox difference spectrum of the CA-overproducing cells had a maximum at 469–476 nm (Fig. 4a, spectrum *3*). This maximum was clearly seen in cells actively synthesizing CA, when the cellular content of the cytochrome complex $a + a_3$ decreased (Fig. 5). In cells grown in a complete medium or under KGA overproduction conditions, the maximum at 469–476 nm was either absent or was masked by the minimum generated by reduced flavoproteins. We believe that the spectral maximum under discussion belongs to a hemoflavoprotein such as yeast hemoglobin or the ferredoxin–ferredoxin reductase complex.

The CO-difference spectrum of the CA-overproducing cells had maxima at 399–404, 418–420, 537–540, and 570 nm (Fig. 4b, spectrum *3*).

The aforementioned differences in the redox and the CO-difference spectra of cells grown under different conditions can be explained as follows. In the cells grown in a complete medium and under KGA overproduction conditions, electrons are obviously transferred via the phosphorylating respiratory chain with the cytochrome $a + a_3$ complex as the terminal oxidase. In the CA-overproducing cells, which are distinguishable by intense respiration and their low content of the cytochrome $a + a_3$ complex, the role of terminal oxidase must be played by another electron carrier.

As was mentioned above, the CO-difference spectrum of CA-overproducing cells has spectral maxima at 399, 420, 540, and 570 nm. The maxima at 420, 540,

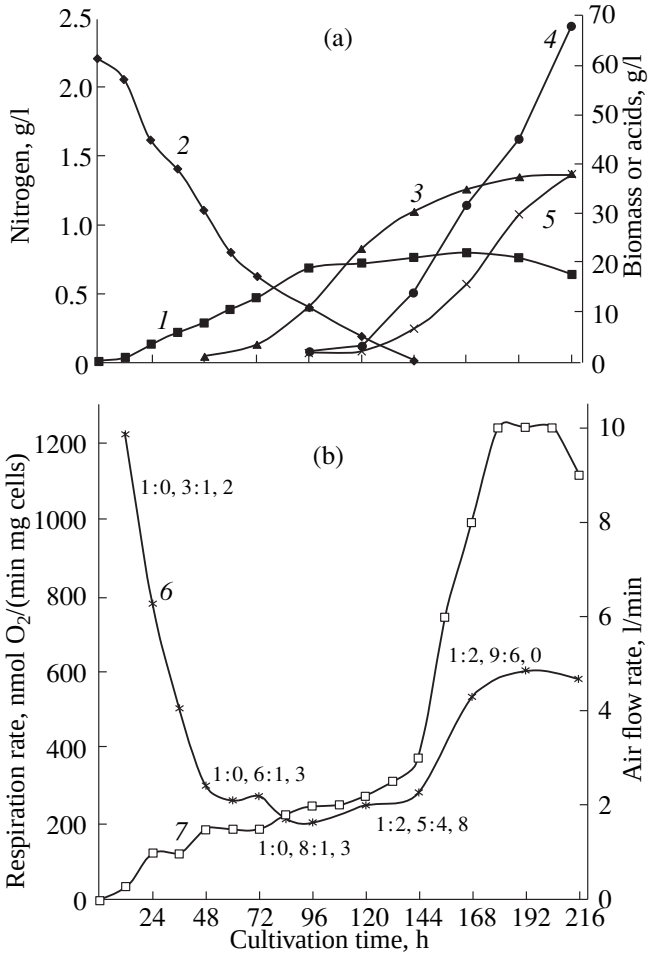


**Fig. 3.** Respiration dynamics, air supply into the fermentor, and the accumulation of organic acids by *Y. lipolytica* cells grown under thiamine deficiency at $pO_2$ = 50–55% oxygen saturation: (a) curves *1*, *2*, *3*, *4*, and *5* show the dynamics of the biomass, nitrogen, KGA, CA, and isocitric acid, respectively; and (b) curves *6* and *7* show the dynamics of cell respiration and the intensity of aeration of the cultivation medium, respectively. Numerals at curve *6* indicate the proportions between cytochromes ($a + a_3 : b : c$), where the concentration of the cytochrome $a + a_3$ complex is taken as unity.

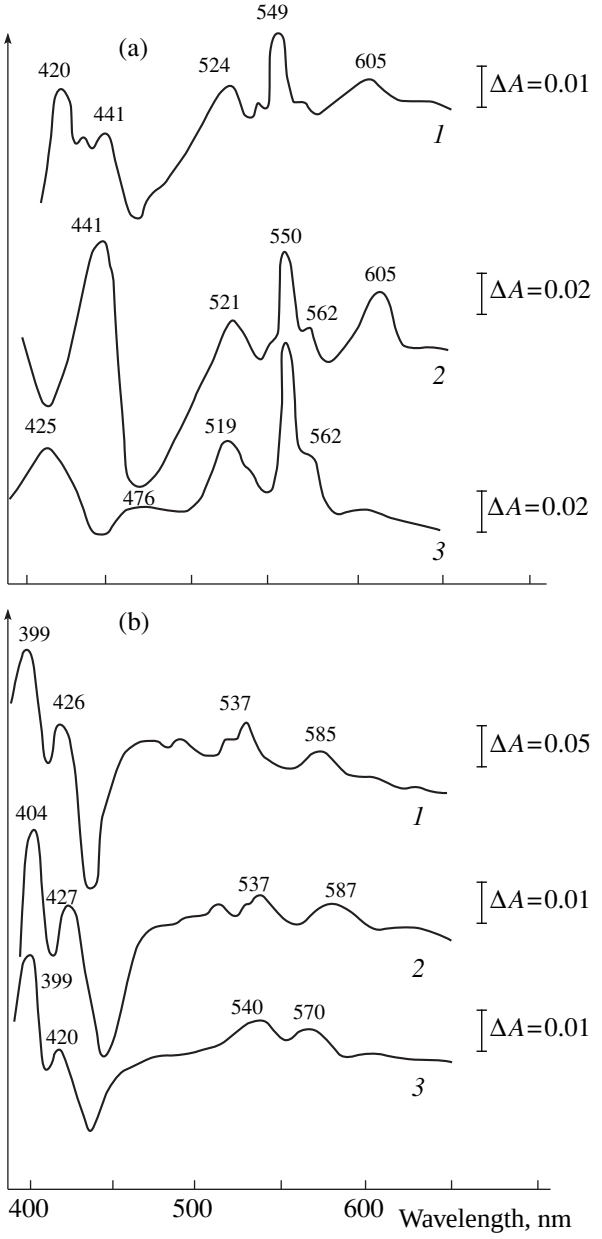


**Fig. 4.** The absorption spectra of *Y. lipolytica* cells grown under different cultivation conditions: (a) the redox difference absorption spectra of dithionite-reduced minus $H_2O_2$-oxidized cells and (b) the CO-difference spectra of dithionite-reduced CO-gassed minus dithionite-reduced cells. Curves *1* represent the spectra of cells grown in a complete medium for 24 h; curves *2* represent the spectra of cells grown under KGA overproduction conditions (72 h of cultivation; 27 g/l of accumulated KGA); and curves *3* represent the spectra of cells grown under CA accumulation conditions (72 h of cultivation; 63 g/l of accumulated CA).

and 570 nm are observed only in the CA-overproducing cells and may belong to a CO-binding pigment, such as CCP, yeast hemoglobin [11–13], and the CO-binding component of cytochrome *o*, which is believed to be a precursor of cytochrome $a + a_3$ or its substituent under certain cultivation conditions [14–16]. It was found that cytochrome *o* can accept electrons at the levels of both coenzyme *Q* and cytochrome *c*. The γ band at 399–404 nm in the CO-difference spectra may belong to a siroheme-containing enzyme [17], ferredoxin or flavodoxin.

Of these CO-binding pigments, cytochrome *o* is the most likely potential candidate for the role of the terminal oxidase that substitutes cytochrome oxidase in the CA-overproducing cells, since neither hemoglobin nor ferredoxin-containing enzymes can reduce $O_2$ to $H_2O$. Earlier, Baroncelli *et al.* detected cytochrome *o* in *Candida (Yarrowia) lipolytica* cells grown on *n*-alkanes [18].

In the CA-overproducing cells, in which the tricarboxylic acid (TCA) cycle is blocked at the level of NAD-dependent mitochondrial isocitrate dehydrogenase, energy is mainly produced through the phospho-

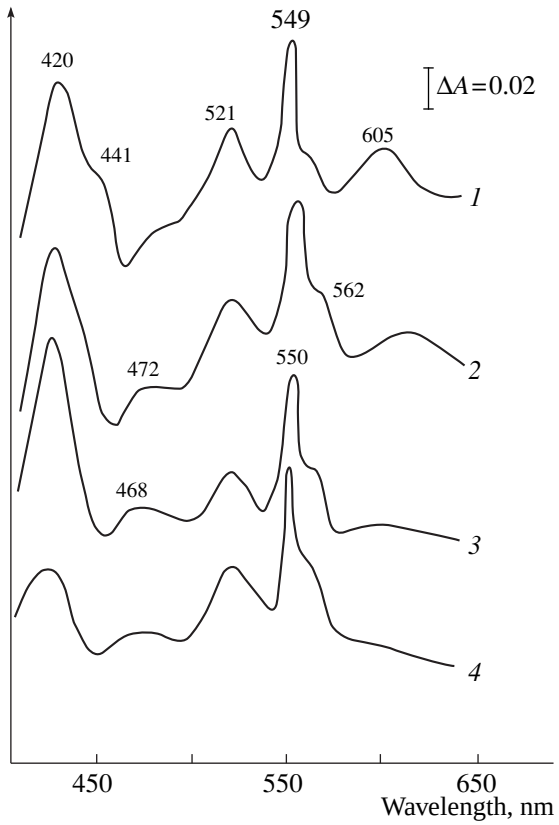


**Fig. 5.** Changes in the redox difference absorption spectra of the dithionite-reduced minus $H_2O_2$-oxidized *Y. lipolytica* cells grown under CA overproduction conditions: curves *1*, *2*, *3*, and *4* correspond to 12, 48, 60, and 72 h of cultivation, respectively.

rylative oxidation of the NADH formed during the oxidation of ethanol and acetaldehyde by alcohol and aldehyde dehydrogenases. The lowering of the concentration of the cytochrome $a + a_3$ complex in the CA-overproducing cells is conditioned by their low requirement for ATP and reduced pyridine nucleotides. In such cells, cytochrome *c* is probably oxidized by cytochrome *o* in a nonphosphorylative way.

As for the KGA-overproducing cells, in which the TCA cycle is inhibited at the level of α-ketoglutarate dehydrogenase and, hence, only one site of NAD(P)H formation in the TCA cycle is blocked, they retain a normally functioning nitrogen metabolism and other assimilation processes (see table). The transition of cells to a state favorable to KGA overproduction induces only small changes in the concentration of cytochromes and in the proportions between them, which is an indication that the phosphorylating respiratory chain in the KGA-overproducing cells is not altered. In this case, cytochrome oxidase functions as the main terminal oxidase (Fig. 4).

In spite of insignificant changes in the respiratory chain of the KGA-overproducing cells, their respiration is much slower than that of the CA-overproducing cells (see table). This fact can be accounted for by a lower degree of coupling between respiration and phosphorylation in the CA-overproducing cells as compared to the KGA-overproducing cells. The NADH formed by alcohol and aldehyde dehydrogenases in the CA-overproducing cells undergoes uncoupled oxidation.

Another reason for the slow respiration of the KGA-overproducing cells may be their active nitrogen metabolism, which is clearly seen from the high rates of ammonium consumption by these cells (see table). Glutamate dehydrogenase, the main enzyme of nitrogen metabolism, requires both ammonium ions and NAD(P)H. Therefore, we cannot exclude the possibility that most of the reduced pyridine nucleotides formed in the KGA-overproducing cells are used for the synthesis of glutamic acid rather than oxidized by the respiratory chain.

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