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EXPERIMENTAL ARTICLES

"Oxygen Regulation" of the Respiratory Chain Composition in the Yeast *Debaryomyces hansenii* under Multiple Stress

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Abstract—It was shown that two stress factors, hypoxia and hyperosmotic shock, if applied simultaneously to the yeast *Debaryomyces hansenii*, display an antagonistic mode of interaction, which results in an increased degree of halophily of this microorganism under microaerobic conditions. Studies of the effects of respiration inhibitors (sodium azide and salicyl hydroxamic acid, SHA) and of the pattern of changes in the composition of the respiratory chain of *Debaryomyces hansenii* under the stated stress conditions led to the suggestion of three (or four) chains of electron transfer functioning simultaneously in the cell: the classical respiratory chain involving cytochrome-*c* oxidase, an alternative respiratory chain involving a cyanide- and azide-resistant oxidase, and additional respiratory chains involving oxidases resistant to salt, azide and SHA. Thus, the antagonistic mode of interaction between hypoxia and hyperosmotic shock results from the redirection of the electron flow from the salt-susceptible respiratory systems to the salt-unsusceptible ones encoded by "the hypoxia genes" and activated (induced) under microaerobic conditions.

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Key words: oxygen regulation, osmotic shock, hypoxia, *Debaryomyces hansenii*

Any microorganism has specific regulatory systems, which form the complex of "global regulation" and, in particular, provide for protection from the stress impact of the environment. The comprehensive set of elements involved in the adequate stress response in yeasts accounts for the expression of more than 200 genes. Many of these systems are specific to certain types of stress factors. For example, the Hsfl system mediates the response to heat shock, whereas the Hogl system mediates the response to osmotic shock [1].

Lately, much attention has been drawn to the mechanisms of participation of oxygen in the regulation of metabolic processes. The essence of this phenomenon is that organisms undergo stress when transferred from aerobic (normoxia) to microaerobic (hypoxia) and, further, to anaerobic (anoxia) conditions, as well as when the environmental conditions change in the reverse order. Naturally, such processes are associated with the massive regulation of expression of many genes. These genes can be divided into two big groups, the "genes of aerobiosis," with maximal expression under aerobic conditions, and the "genes of hypoxia," with maximal expression under microaerobic or anaerobic conditions [2].

It is evident that the group of "genes of aerobiosis" comprises, first of all, the genes encoding components of the aerobic respiratory chain (for example, cytochromes), and other proteins participating in aerobic metabolism, including the enzymes which protect cells from reactive oxygen species (ROS). The group of "genes of hypoxia" is more difficult to determine since the above division is somewhat arbitrary. Thus, after transfer to microaerobic conditions, induction (or depression) of certain enzymes participating in aerobic metabolic pathways is observed, such as alternative oxidases–components of the aerobic respiratory chain, and reductases and desaturases participating in the biosynthesis of hemes, sterols, and unsaturated fatty acids. The enhancement of the expression of these genes possibly allows for a more effective use of limited amounts of oxygen [3].

With regard to yeasts, unfortunately, all these mechanisms have been studied only for *Saccharomyces cerevisiae* and for only one stress factor, namely oxygen. In natural environments, microorganisms are usually exposed to multiple stressors which can interact in various ways. For example, as we showed earlier, the microorganisms inhabiting oil fields are, in addition to oxygen deficiency, often exposed to high salt concentrations, and these stress factors (hypoxia and osmotic shock) can display an antagonistic mode of interaction [4]. The experimental data obtained indicate that salt (apart from purely osmotic effects) inhibits respiration and activity of enzymes protecting the cell from ROS. In the yeast strains capable of induction of an alternative respiratory system and additional amounts of protective salt-insusceptible enzymes under microaerobic conditions, these stressors were antagonistic (*Candida*

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lipolytica and, to a lesser extent, *Rhodo-torula aurantiaca*), which resulted in increased halophily (halotolerance) of these organisms under microaerobic conditions. In the yeast strains incapable of such induction of "hypoxia genes" (*Malassezia* sp.), these stressors displayed an additive mode of interaction [5].

For a more detailed study of the changes in the system of aerobic respiration under combined stress action (hypoxia + osmotic shock), the results of which are presented in this article, the yeast strain *Debaryomyces hansenii* was chosen, since many of its physiological functions are well-studied [6].

MATERIALS AND METHODS

The study was conducted with the yeast strain *Debaryomyces hansenii* (VKM Y-116). Cultivation was performed in 100-ml flasks containing 20 ml of the glucose–peptone–yeast extract (GPY) broth on a shaker (150 rpm) at 25–27°C [5]. During aerobic cultivation, the flasks were closed with cotton plugs. For the creation of microaerobic conditions, the flasks were corked with rubber stoppers with inserted 0.8 mm syringe needles with loose cotton pellets. Prior to microaerobic cultivation, the medium in the flasks was bubbled with argon. In this case, oxygen content in the gas phase during cultivation did not exceed 1–2% (by volume). Different salt concentrations in cultivation media were created by the addition of NaCl (wt/vol). In the base medium ("zero" salt level), NaCl content was approximately 0.3% (40–50 mM) due to impurities in organic substrates (yeast extract and casamino acids).

Relative optical density of cell cultures (D, light absorption + light scattering) was measured on a KFK-2-UHL 4.2 nephelometer at 540 nm.

Oxygen consumption was determined by the amperometric method [7] on a LP-7 polarograph with silver chloride and platinum electrodes. The working volume of the cell was 2 ml. For the preparation of yeast suspensions, cultures from the late exponential growth phase were used (2 to 4 days of incubation). Suspensions were prepared directly before measurement. The cells were harvested by centrifugation at 7 000 rpm and resuspended in 50 mM potassium phosphate buffer containing NaCl (or KCl) in the concentration corresponding to that of the cultivation medium. Measurements were taken in potassium phosphate buffer containing 1% glucose, at 6% (about 1M), 15% (about 2.5 M) NaCl, or without addition of salt. If the salt content of the incubation medium differed from that of the cultivation medium, the suspensions were left for 15 minutes for the balancing of ion fluxes. Sodium azide and salicylhydroxamic acid (SHA) were added to the final concentrations of 2 and 4 mM, respectively.

The differential spectra of cytochromes (dithionite-reduced form minus hydrogen peroxide-oxidized or air-oxidized form) were recorded on a computerized HITACHI-557 double-beam spectrophotometer at room temperature. The scanning speed was 60 nm/min. The suspensions containing 0.05–0.2 g of cells per ml (converted to dry biomass) were supplemented with 20% sucrose in order to decrease light scattering.

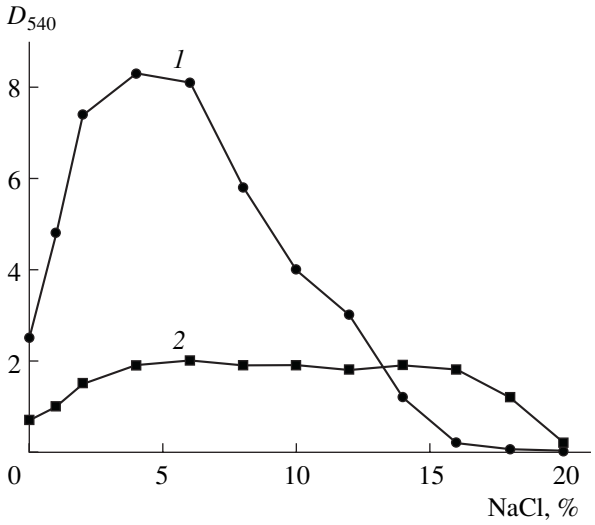

Fig 1. The relation between growth of *Debaryomyces hansenii* under (*1*) aerobic and (*2*) microaerobic conditions on NaCl concentration.

Low-temperature difference spectra were obtained using a modified spectrophotometer on the basis of Unicam SP.1800, which was equipped with a special Dewar flask placed closely to the photomultiplier. Suspensions were supplemented with glycerol to the concentration of 50% and frozen in liquid nitrogen. Then the suspensions were thawed until clarification and frozen in liquid nitrogen once more. The scanning speed was 60 nm/min.

Graphical visualization of spectra was achieved with the Origin 7.5 software package using the "Smoothing, FTP filter" process. The maximums were determined with the help of the "Pick peaks" instrument. The figures represent the results of typical experiments.

RESULTS

As was shown earlier, some oil-oxidizing microorganisms isolated from formation waters of oil fields exhibit increased halophily (halotolerance) when cultivated in microaerobic conditions. This may explain their existence in natural ecotopes at salt concentrations exceeding those optimal for growth under aerobic conditions in a laboratory [8]. This pattern has also been established for a number of other microorganisms [5].

Most strains of *Debaryomyces hansenii* are typical moderate halophiles with optimal salt concentrations from 4 to 6% (0.8–1.0 M) [6]. Cultivation of this microorganism under microaerobic conditions resulted in a

The respiration rate of *Debaryomyces hansenii* and the influence of inhibitors on it

Experimental conditions*	Respiration rate, ng-atom O/min OD	Degree of inhibition with azide, %	Degree of inhibition with SHA, %	Degree of inhibition with azide + SHA in combination, %
Growth under aerobic conditions				
6 ⟶ 6	480	37.8	30	69.8
6 ⟶ 15	190	28.5	29	73.3
6 ⟶ 0	450	32.4	37.2	78
Growth under microaerobic conditions				
6 ⟶ 6	400	57.3	16	59.9
6 ⟶ 15	390	58.5	12.6	60.4
6 ⟶ 0	405	61	11.6	61.8
Growth under aerobic conditions				
0 ⟶ 0	340	34.7	44.3	73.4
0 ⟶ 15	90	23.5	41.5	68.8
15 ⟶ 15	250	37.7	27.5	71.5
15 ⟶ 0	510	48.6	22.3	71.3
Growth under microaerobic conditions				
0 ⟶ 0	240	60.3	10.3	65.8
0 ⟶ 15	230	60.3	10.5	58
15 ⟶ 15	390	59.8	11.5	56.8
15 ⟶ 0	450	60.7	5	63

* The first number corresponds to the NaCl concentration in the cultivation medium, the second number, to the NaCl concentration in the incubation medium during measurement of the respiration rate.
The table represents average data of the experiments run in three to four replicates.

significant extension of the range of NaCl concentrations at which growth was possible (Fig. 1). Under oxygen-limited conditions, there was a decrease in the growth rate and maximal biomass gain. However, these parameters practically did not change at different salt concentrations up to 18% (about 3.0 M) NaCl, whereas, under aerobic conditions, growth became significantly slower at 10–12% (about 1.5–2 M) NaCl.

Measurements of *Debaryomyces hansenii* respiration rates were conducted for the cultures grown under aerobic and microaerobic conditions, without NaCl, at optimal salt concentrations (6% NaCl), and at unfavorable salt concentrations (15% NaCl) (table).

Cells grown at optimal salt concentrations (6%) under aerobic conditions exhibited high respiration rates (table, variant 6 ⟶ 6). Excess of NaCl (15%) in the incubation medium resulted in inhibition of respiration (variant 6 ⟶15), as for other microorganisms studied previously [5]. Cultivation without addition of NaCl (variant 0 ⟶ 0) resulted in a slight decrease in the respiration rate. These cultures were susceptible to the excess of NaCl (variant 0 ⟶ 15). The respiration rate of the cultures grown at excess salt concentrations (15%) was characterized by the parameters close to those of the variant 6 ⟶ 0 when measured in the incubation medium without addition of salt (variant 15 ⟶ 0), but it decreased significantly when measured in the incubation medium with 15% NaCl (variant 15 ⟶15).

Cultivation under microaerobic conditions resulted in a slight inhibition of respiration. However, the level of susceptibility of respiration to the excess of NaCl decreased significantly (for example, variant 6 ⟶15 for microaerobic conditions).

The data obtained indicated certain changes in the respiration system of the cultures exposed to both hypoxia and osmotic shock. For the determination of the nature of these changes, we used respiration inhibitors, sodium azide, the inhibitor of cytochrome-*c*-oxidase (this inhibitor provides more stable results than potassium cyanide), and SHA, the inhibitor of the alternative cyanide-resistant oxidase.

The use of respiration inhibitors revealed a complicated pattern, which can not be interpreted unambiguously (table). However, taking into consideration the published data, our results can be best explained assuming the existence of at least three types of aerobic respiratory chains in *Debaryomyces hansenii* which can function simultaneously but at different rates depending on the cultivation conditions.

(1) Under aerobic conditions at optimal NaCl concentration (6%), both the respiratory chain involving cytochrome oxidase (azide- and cyanide-susceptible, or

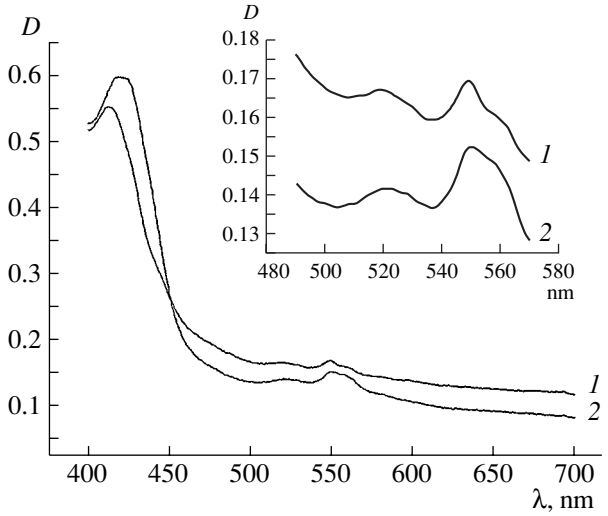

Fig 2. Difference spectra of cytochromes (in the range from 400 to 700 nm) of *Debaryomyces hansenii* grown at 6% NaCl under (*1*) aerobic and (*2*) microaerobic conditions. In the insert, the magnified spectra in the range of 490 to 570 nm are presented.

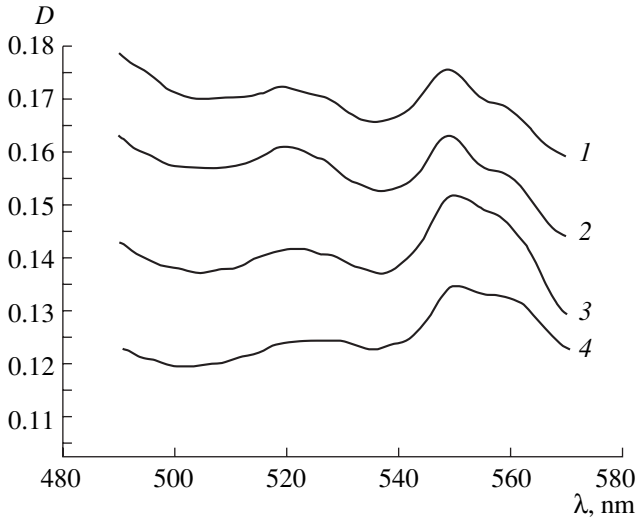

Fig. 3. Difference spectra of cytochromes (in the range from 490 to 570 nm) of *Debaryomyces hansenii* grown under (*1*, *2*) aerobic and (*3*, *4*) microaerobic conditions without addition of NaCl (*2*, *4*) and at 15% NaCl (*1*, *3*).

"classical" respiratory chain, CRC) and the azide- and cyanide-resistant (or alternative, ARC) respiratory chain function, which was indicated by the inhibition of respiration with azide by 30–40% and with SHA by 30–40%, respectively. Incomplete inhibition of respiration with two inhibitors used in combination (azide + SHA) indicated the possible existence of the third, supplementary, respiratory chain (SRC), which will be discussed further.

(2) Under hypoxia (cultivation in microaerobic conditions) at optimal NaCl concentration (6%), the share of CRC increased, and the share of ARC decreased, which was indicated by the inhibition of respiration with azide by about 60% and with SHA by 11–16%, respectively. Inhibition of respiration with two inhibitors in combination was still incomplete.

(3) Exposure to hypoosmotic shock (cultivation in the medium without NaCl) under aerobic conditions did not result in any significant changes in the susceptibility of the respiration chain to the inhibitors. Exposure to hypoosmotic shock under microaerobic conditions resulted in the primary functioning of CRC (inhibition of respiration with azide by 60%) and a slight decrease in the inhibitory effect of the combination of azide and SHA.

(4) Simultaneous exposure to hyperosmotic shock (cultivation at 15% NaCl) and hypoxia resulted in the distinct predominance of the CRC share (inhibition with azide by 60%).

Since the effects of the inhibitors are not strictly specific, it was necessary to obtain data on the content of the respiratory chain in the experiments described for a more accurate characterization of the respiratory mechanisms under the influence of various sets of stressors.

The analysis of the difference spectra of cytochromes revealed that the changes were most pronounced in the cultures grown under microaerobic conditions (hypoxia) in comparison with the aerobic variants (normoxia). This can be illustrated by the following examples.

Figure 2 shows difference spectra for the cells of *Debaryomyces hansenii* grown under aerobic and microaerobic conditions at optimal salt concentrations (6%). It can be seen that hypoxia resulted in shifts in the absorption spectra (peaks in the γ-maximum region) to the long-wave range, and in the changes in the ratio of cytochromes *b* and *c* (which is more evident in the insert).

Exposure to hypo- and hyperosmotic shock under aerobic conditions did not result in any changes in the cytochrome composition as compared with the optimal salt concentration (Fig. 3, curves *1*, *2*). Osmotic shock and hypoxia applied simultaneously caused the same effects as hypoxia itself (Fig. 3, curves 3, 4).

Since in the spectra taken at room temperature the absorption peaks of cytochromes *b* and *c* are superimposed, low-temperature difference spectra were used to reveal and identify the changed cytochrome components. The results are presented in Fig. 4.

It should be noted that the complete agreement in the numerical values of the absorption maximums of cytochromes with the classical samples is possible only after isolation of these proteins in a pure homogeneous form. If the spectra are recorded for whole-cell suspensions, different deviations in numerical values are observed depending on the degree of light scattering of the suspension and other factors. For example, in low-temperature spectra, a 1 to 4-nm shift of the absorption

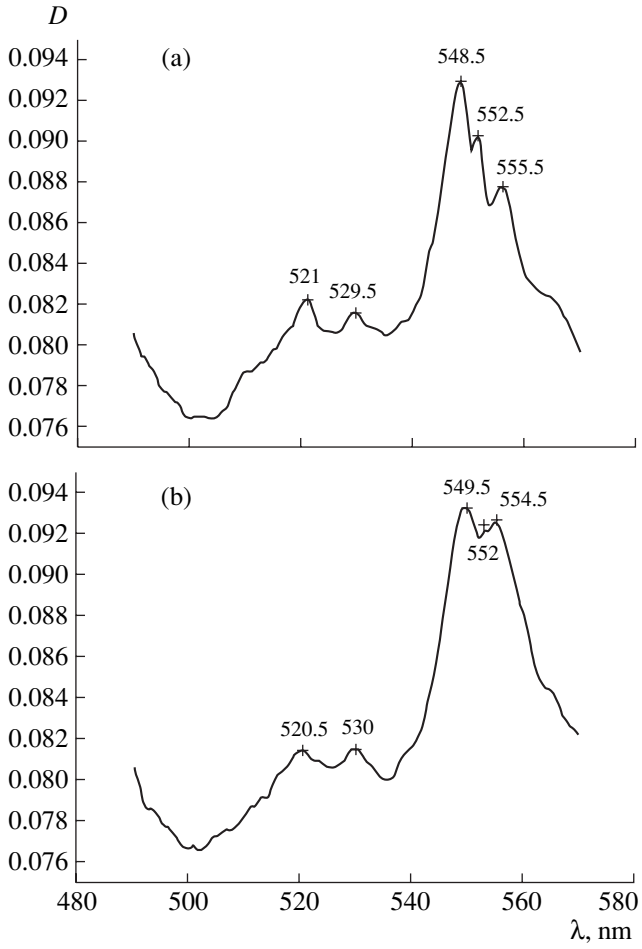

Fig. 4. Low-temperature difference spectra of cytochromes (in the range from 490 to 570 nm) of *Debaryomyces hansenii* grown under (a) aerobic and (b) microaerobic conditions at 6% NaCl.

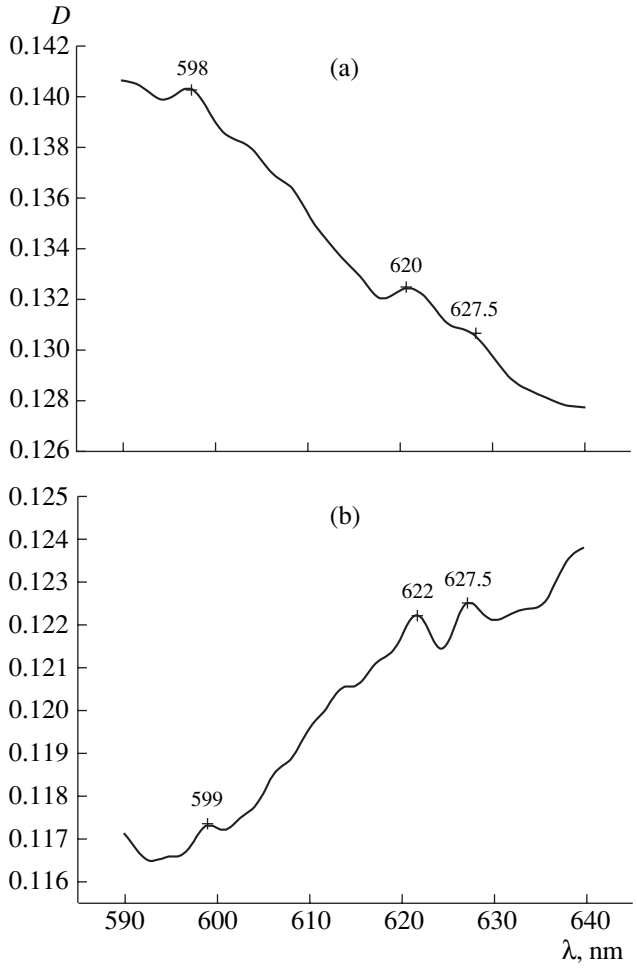

Fig. 5. Difference spectra of cytochromes (in the range from 590 to 640 nm) of *Debaryomyces hansenii* grown under (a) aerobic and (b) microaerobic conditions at 6% NaCl.

peaks to a short-wave range is observed. We tried to use native cells without any treatment (including mitochondria extraction), which is likely to cause the loss of labile components. That is why in the course of further discussion we will operate with the rounded values of absorption peaks.

Thus, in the cells grown under aerobic conditions at the optimal salt concentration (6%), the following types of cytochromes were revealed (Fig. 4a):

(1) Classical cytochrome *c* with the α-band absorption maximum at about 550 (548.5–549.5) nm, and cytochrome c_1 with the α-band maximum at about 553 nm. Both cytochromes had the β-band maximums at about 520 nm and the γ-band maximums at about 415 nm.

(2) A *b*-type cytochrome with the α-band maximum at about 555 nm. Cytochrome c_5 with a similar maximum is described in literature, but up to now it has been found only in *Azotobacter* [9]. In addition, the β-band of this cytochrome had a maximum at 530 nm (and the γ-band, at 430 nm, Fig. 2, *2*), which is characteristic of *b*-type cytochromes. Thus, the given component can be regarded as a classical *b*-type cytochrome being the part of the *bc1* complex. The jut in the region of 560 nm most probably corresponds to another type of cytochrome *b*.

Cultivation under hypoxia resulted in a noticeable shift in the ratio of cytochromes *b* and *c* (Fig. 4b), primarily due to a sharp increase in the relative level of cytochrome b_{555}.

The cytochrome oxidase part of the respiratory chain is best characterized by the difference spectra in the region of 590 to 640 nm (Fig. 5). The difference spectra of the cultures grown under both aerobic and microaerobic conditions had three peaks in the α region, at about 600, 620 and 630 nm. A 600 nm maximum is likely to correspond to the mitochondrial cytochrome *c* oxidase aa_3, although in the yeast *C. parapsilosis*, a cytochrome oxidase with the spectrum analogous to the bacterial cytochrome a_1 with the α-band maximum at 590 nm was described [10].

Maximums at 620 and 630 nm are harder to identify. When registering the difference spectra of the yeast cytochromes, researches rarely use the range beyond 610 nm, because the α-band maximum of the classical cytochrome oxidase is at ca. 605 nm. According to the data described in literature, the following cytochromes have spectral characteristics close to those we found in *D. hansenii*: cytochrome-*bd* ubiquinole oxidase of *E. coli* (α-maximum at ca. 630 nm) [11], and the cytochrome cd_1 (the α-maximum at ca. 620 nm) found in a number of denitrifiers, including *Paracoccus denitrificans* close to mitochondria in the respiratory chain composition [12].

Although the exact nature of the cytochrome components revealed in *D. han-senii* can be determined only after isolation and studying of the hemes of these cytochromes, the preliminary analysis of the spectra shows that, under hypoxia, the ratio of the components with the maximums at 620 and 630 nm changes significantly, the level of the latter increasing (Fig. 5).

DISCUSSION

Analysis of the data obtained and the data described in literature leads to the following general conclusion.

Nowadays, more and more evidence is being gathered in favor of the theory that the regulation of the process of electron transfer under the influence of the environment changes outside the so-called "optimal" range (in other words, under the influence of stressors) is realized not by complete switching from one electron transport chain to another (as was simplistically believed earlier), but by changes in the input ratio of simultaneously functioning respiratory mechanisms.

A particularly controversial subject of discussion has been the mechanisms and the physiological role of cyanide-resistant respiration. For a long time, this type of respiration was viewed as an alternative to the classical respiration mechanism induced in response to stress conditions. Some authors still hold to this point of view [13]. There are data indicating that electron transport through the ARC pathways (from ubiquinone to oxygen) is not coupled with the accumulation of energy [14], and serves only for the regulation of the degree of reduction of the intermediate carriers. On the other hand, it is proven that ATP is formed in the process of the ARC functioning, although the ARC probably has only one coupling site [15]. A considerable contribution to the study of mechanisms regulating the scale and rate of alternative respiration, as well as the description of the mechanisms of interaction with the classical respiration, was made by Akimenko et al. [16, 17].

It is becoming more and more evident that the ARC is widespread in yeasts, and in the latter case in the yeast species incapable of aerobic fermentation [18]. Both CRC and ARC can be constitutive and function in parallel, although they react differently to the ambient conditions, oxygen partial pressure, in particular [19].

The multiplicity of electron transport pathways in the respiration systems of yeasts is well demonstrated by the example of *C. parapsilosis* [20]. This yeast has four simultaneously functioning chains of electron transfer involving three types of terminal oxidases, namely an alternative cyanide-resistant oxidase, a classical cytochrome *c* oxidase, and a supplementary cytochrome *c* oxidase inhibited only by high concentrations of cyanide (10 mM).

Taking into consideration all these data, we can assume the following scheme of electron transfer and the effects of the stressors and inhibitors under study on electron transfer in *D. hansenii* (Fig. 6). We consider it necessary to assume such a scheme, as it is useful for planning further experiments, although it remains largely speculative.

Presumably, three (or more likely four) chains of electron transfer function in this organism, as was revealed by the inhibition analysis (table). All these chains use electrons from the common pool of reduced ubiquinone(s).

CRC includes classical mitochondrial cytochromes c_{550}, $c1_{552}$, and, presumably, b_{555}. This chain is terminated with the $aa3_{600}$-type cytochrome *c* oxidase susceptible to low concentrations of azide.

ARC is also of a classical type and does not include cytochromes. Ubiquinole oxidase is usually the component susceptible to SHA.

The rest of electron flow is insusceptible to SHA and azide and can be used by one (or two) SRC. The composition of SRC remains least defined, but one pathway is most likely to include cytochrome b_{555} instead of cytochrome b_{560}, and a component similar to cytochrome $cd1_{620}$ acts as a terminal oxidase.

The other pathway is determined by the presence of the component with the absorption peak at 630 nm, which can indicate the involvement of a bd_{630} type cytochrome (receiving electrons directly from the reduced ubiquinone pool) which is also practically insusceptible to SHA and azide.

Under aerobic conditions at optimal salt concentration (6%), the relative share of these systems is approximately equal (table), which allows using a wide range of electron donors. Hypoosmotic shock has little influence on respiration and on the relative ratio of the components of the respiratory chains. This could be because the background salt content in the cultivation medium (about 0.3%) is enough to support the basic physiological needs. Hyperosmotic shock under aerobic conditions also does not significantly change the relative share of these systems in respiration (table, Fig. 3).

Under microaerobic conditions (hypoxia), the respiration systems characterized by a high affinity to oxygen should be activated. The share of CRC increases, which is visible not only from an increase in the degree

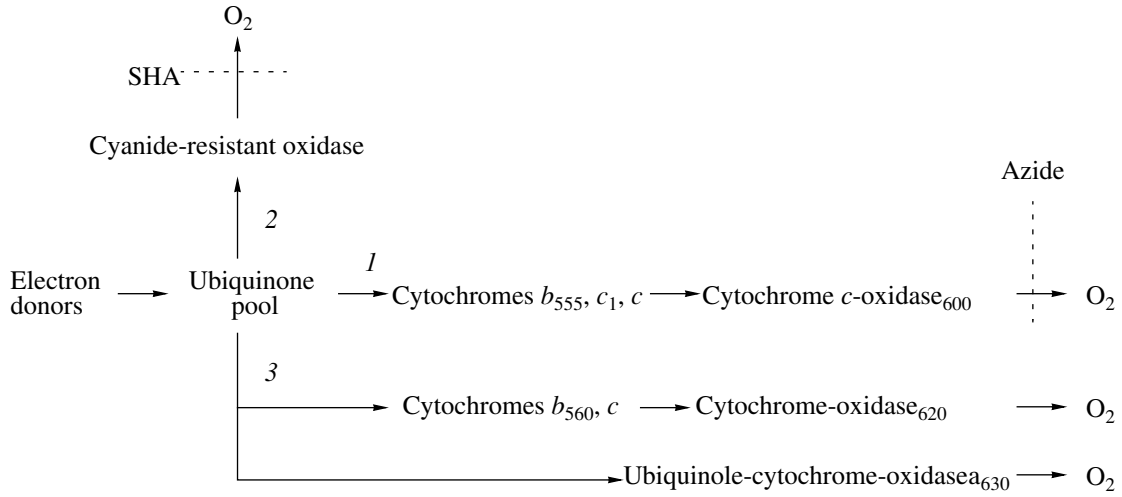

Fig. 6. Scheme of electron transfer in *Debaryomyces hansenii*: (*1*) classical respiratory chain, CRC; (*2*) cyanide-resistant respiratory chain, ARC; (*3*) supplementary respiratory chains, SRC. Dotted line indicates sites of action of inhibitors.

of inhibition with azide, but also from a sharp increase in the relative content of cytochrome b_{555} (table, Fig. 4b). On the other hand, SRC can be induced (or activated), especially the one which directly uses electrons from the reduced ubiquinone pool, as under hypoxia the degree of reduction of this component increases (Fig. 5). As these systems have low susceptibility to NaCl, microorganisms can handle the effects of hyperosmotic shock, which is expressed as an increase in the degree of halophily reported previously [5].

Thus, we can formulate a more defined conclusion, which confirms and extends the earlier conclusions on the reasons of antagonism between hypoxia and hyperosmotic shock. Apart from osmotic effects, high salt concentration (hyperosmotic shock) inhibits respiration and the synthesis of enzymes protecting the cell from reactive oxygen species [4, 8], which causes oxidative shock. Microaerobic conditions (hypoxia) result in a decrease of the partial pressure of oxygen and activation (induction) of "genes of hypoxia" encoding the components of respiratory chains and protective enzymes which are less susceptible to salt [5]. As a result, hypoxia lessens the harmful effects of hyperosmotic shock and increases the degree of halophily (halotolerance) of microorganisms. This fact is of high ecophysiological importance, as it explains the frequent occurrence of aerobic slightly halophilic microorganisms in environments with low partial pressure of oxygen and high salt content (for example, in stratal waters of oil fields).

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