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EXPERIMENTAL ARTICLES

Effect of Vitamin Concentration on the Synthesis of Lactate, Ethanol, Pyruvate, and Ethyl Acetate in Cells of the Yeast *Dipodascus magnusii*

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Abstract—In the yeast *Dipodascus magnusii*, which is auxotrophic for thiamine and biotin, during cultivation on glucose with excessive thiamine concentration, pyruvate metabolism was shown to result in the synthesis of fermentation products, namely, ethanol and, to a lesser extent, lactate. Substantial synthesis of ethyl acetate was also observed under these conditions. Introduction of nicotinic acid (NA) into the medium resulted in time separation of ethanol and lactate production. It was shown that cultivation of the yeast under biotin deficiency resulted in nearly complete suppression of aerobic production of ethanol and cessation of ethyl acetate synthesis, whereas lactate synthesis was activated as early as in the first hours of cultivation. Upon introduction of NA under these conditions, lactate concentration sharply increased. These results show that the combination of thiamine and biotin with other vitamins can stimulate utilization of the pyruvate pool in yeasts towards formation of considerable amounts of lactate, which is typical only of cells of higher eukaryotes and bacteria.

Key words: yeasts, *Dipodascus magnusii*, fermentation, pyruvate, lactate, ethanol, ethyl acetate.

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Previously it has been noted that regulation of glucose degradation in yeasts and tumor cells is very similar in many aspects [1]. In both cases it results in excretion of fermentation end products: ethanol in the former and lactate in the latter case. The homology in glucose degradation between yeasts and tumor cells can be traced at the levels of the cell division cycle, transduction signal, and regulation of the key glycolytic enzymes. It was established that excretion of ethanol and lactate by yeasts and tumor cells, respectively, is a result of excessive enzyme activity at the point of pyruvate formation [1]. At the same time, the main difference between these processes is the inability of yeasts to produce noticeable amounts of lactate.

On the other hand, industrial application of lactate for the synthesis of the corresponding polymer caused significant interest in studying the possibility of its metabolic synthesis by yeast organisms, which are more acid-resistant than lactic acid bacteria. With this purpose, via deletion of the three genes encoding pyruvate decarboxylase and introduction of heterologous lactate dehydrogenase (EC 1.1.1.27), a strain of *Saccharomyces cerevisiae* was constructed. Incubation of this aerobic culture in a chemostat with glucose excess resulted in prompt appearance of lactate as the main fermentation product. At the same time, this strain did not grow under anaerobic conditions, and lactate formation under all cultivation conditions was lower than alcoholic fermentation in the wild type strain [2].

In this work we attempted to investigate the possibility of lactate synthesis by glucose-grown yeast *Dipodascus magnusii*, depending on the concentrations of thiamine and biotin, for which this culture is auxotrophic, and to study the effect of nicotinic acid on this process.

MATERIALS AND METHODS

Culture. The object of research was the yeast strain *Dipodascus magnusii* VKM Y-1072.

Medium and cultivation conditions. The yeast was cultivated in 750-ml flasks (50 ml of the medium) in a shaker and in 3-l fermentors (1.5 l of the medium) on Rider mineral medium with a mixture of trace elements according to Burkholder, pH 5.5, at 29°C. Concentrations of vitamins (biotin, thiamine, and nicotinic acid (NA)) varied depending on the goals and objectives of the experiment. Oxygen concentration in the fermentor was maintained at a level of pO_2 = 20% of saturation. Glucose (5%) was used as a carbon source. The initial concentration of NH_4^+ ions was 45 ± 5 mM.

Analytical methods. Glucose concentration was determined using a standard reagent kit (Diakon-DS).

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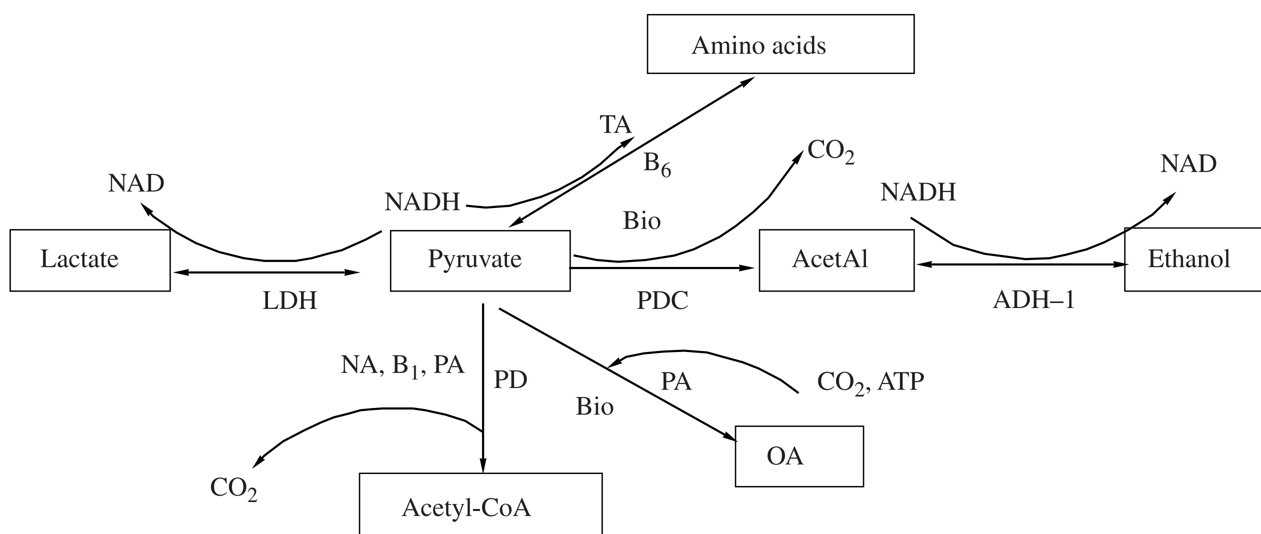

Fig. 1. Pyruvate metabolism in eukaryotic cells. PDC, pyruvate decarboxylase; TA, transaminases; PC, pyruvate carboxylase; PD, pyruvate dehydrogenase; LDH, lactate dehydrogenase; ADH-1, classical alcohol dehydrogenase; B_1, thiamine; B_6, pyridoxine; NA, nicotinic acid; Bio, biotin; PA, pantothenic acid; OA, oxaloacetate; AcetAl, acetaldehyde.

The method is based on oxidation of glucose by glucose oxidase with the formation of equimolar amounts of peroxide, which reacts with chromogenic compounds (λ 480–520 nm).

Ammonium content was measured by an ion-selective electrode (Orion).

Ethanol was detected polarographically by oxygen consumption in the presence of an aliquot of the culture liquid and methanol oxidase isolated from the yeast *Hansenula polymorpha* [3].

The content of ethyl acetate in the culture liquid was analyzed in a Pye-Unicam gas chromatographer with flame ionization detector, using a 2-m long glass column with internal diameter 2 mm, filled with Chromosorb W/AW-DMCS, 100–200 mesh (Fluka), covered with 20% neopentylglycol succinate. The column temperature was raised from 80 to 175°C at a rate of 6°C/min. The injector and detector temperatures were 140 and 180°C, respectively. Nitrogen was used as a carrier gas with the feeding rate of 20 ml/min.

For pyruvate measurement in the culture liquid, cells were separated from the medium by centrifugation (14000 *g*, 5 min); proteins were precipitated by addition of an equal volume of 6% perchloric acid. The precipitate was removed by centrifugation (14000 *g*, 15 min) and the supernatant was used for the analysis. Pyruvate concentration was determined by isocratic HPLC in an Aminex HPX-87H column, 300 × 7.8 mm (BioRad). H_2SO_4, 4 mM, was used as a mobile phase. Elution rate was 0.5 ml/min, temperature was 35°C, and detection was carried out at 206 nm. The calibration curve was plotted using a Bio-Rad standard kit.

Lactate concentration was determined by spectrophotometry using a kit of reagents (Lachema). The detection was based on reduction of NAD to NADH in the course of lactate oxidation by lactate dehydrogenase (EC 1.1.1.27).

RESULTS AND DISCUSSION

Lactate accumulation is currently considered a metabolic dead end in the sense that it can be formed or utilized only through pyruvate. In the tissues of higher eukaryotes, its formation is determined mainly by pyruvate concentration and the degree of reduction of pyridine nucleotides (PN). Therefore, when performing experiments, we used the scheme of pyruvate metabolism in eukaryotic cells (Fig. 1). From the presented scheme it follows that the processes of lactate and ethanol synthesis are competitive with respect to NADH. At the same time, these processes determine the ability (or inability) of pyruvate dehydrogenase complex to direct pyruvate to either reduction to lactate or decarboxylation to acetaldehyde, or to the Krebs cycle (under the excess of pyruvate formed by glycolysis or transamination/deamination of amino acids).

The Effect of Glucose and Thiamine Concentrations on Aerobic Fermentation

Thiamine pyrophosphate is the cofactor of pyruvate dehydrogenase complex (Fig. 1); hence, we have performed studies of the glycolytic part of glucose metabolism depending on thiamine concentration.

Significant amounts of major fermentation products, ethanol and lactate, and their source (pyruvate) were revealed in the culture liquid at cultivation of the yeast under study at 5% initial glucose concentration and 1 mg/l thiamine concentration (Fig. 2). The maximal concentrations of fermentation end products were

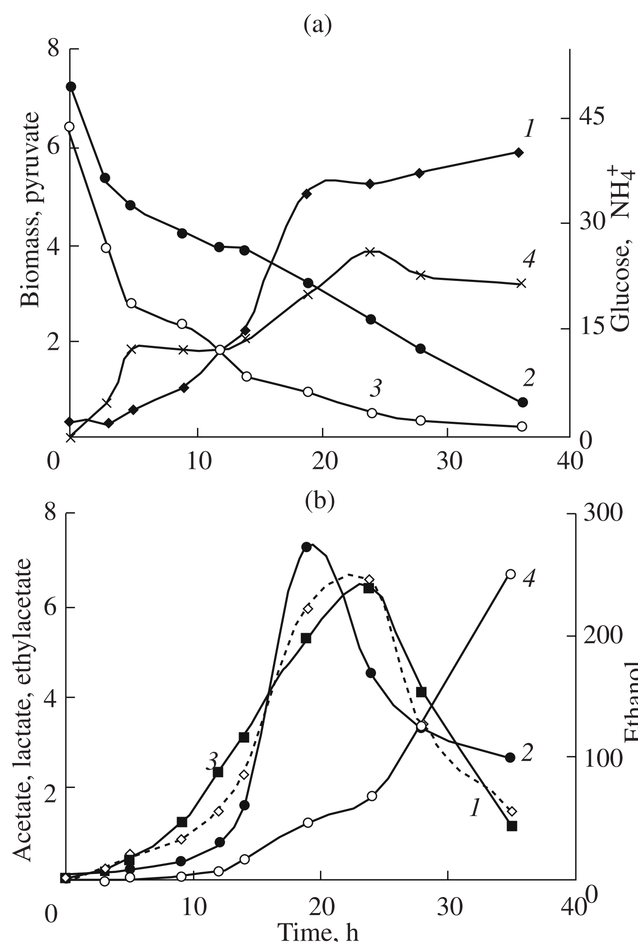

Fig. 2. Effect of thiamine (1 mg/l) on the formation of glycolysis and fermentation products in *D. magnusii* cells. Biotin concentration: 10 μg/l. a: *1*, biomass, g/l; *2*, glucose, g/l; *3*, [NH_4^+], mM; *4*, pyruvate, mM; b: *1*, ethanol, mM; *2*, lactate, mM; *3*, acetate, mM; *4*, ethyl acetate, mM.

obtained when the culture passed over to the stationary growth phase due to nitrogen source depletion.

It should be noted that 2–4 mM pyruvate can always be detected in the medium prior to excretion of ethanol and lactate. Its real concentration is probably much higher, because pyruvate is known to be easily converted into methylglyoxal [4], which quickly polymerizes into a dimer. These findings demonstrate that the equilibrium in utilization of the pyruvate pool is greatly shifted towards the formation of ethanol (up to 300 mM) but not lactate (8–10 mM).

The study of the effect of glucose concentration on the concentration of pyruvate excreted during the first 7–9 hours of cultivation has shown that it is proportional to initial glucose concentration (data not presented). At the same time, ethanol and lactate concentrations depended on the concentrations of both carbon source and thiamine. At higher thiamine concentrations, the shift towards the formation of fermentation products considerably increased (data not presented). It should be noted that the "shift" of metabolism towards fermentation in the yeast *Endomyces magnusii* grown under aerobic conditions on fermented substrates (sucrose) under thiamine excess has been mentioned previously by other researchers [5, 6].

However, the spectrum of products excreted under these conditions is not confined to these two main fermentation products (Fig. 2b). It can be seen that ethanol accumulation in the medium is accompanied by increased discharge of acetate, which may be a derivative of both ethanol (aldehyde reaction) and pyruvate dehydrogenase complex (under ATP limitation). Moreover, biosynthesis of one of the volatile ethanol derivatives, ethyl acetate, begins in the medium, and its concentration increases as ethanol and acetate concentrations decline. Some researchers believe [7, 8] that this toxic compound is synthesized from ethanol and acetyl-CoA under ATP excess in the cells.

Thus, the results presented in this section show that thiamine and glucose concentrations significantly affect the processes of glycolysis and fermentation. At low concentrations of glucose (0.5–1%) and thiamine (50–200 μg/l), lactate was not found and ethanol was present in micromolar amounts [9], but their concentrations abruptly increased under the cultivation conditions described above.

The Effect of Nicotinic Acid Concentration on Fermentation

Nicotinic acid (NA) is another vitamin that affects glycolysis (fermentation) and pyruvate decarboxylase functioning (Fig. 1). The facts available in the modern scientific literature demonstrate a significant increase of the cellular NAD(H) pool upon introduction of this vitamin. For example, the study of lactate formation by the wine yeast strain *S. cerevisiae* K1-LDH, with the gene of lactate dehydrogenase of *Lactobacillus plantarum* introduced into its genome, showed that NA was the limiting factor in this process [10]. An increase of NA concentration in batch culture or in the medium used for chemostat cultivation resulted in a higher yield of lactate.

Cultivation of *D. magnusii* on 5% glucose in the presence of 1 mg/l NA (Fig. 3) essentially changed both the growth dynamics and the processes of glycolysis and fermentation. The growth dynamics under these conditions was of diauxic character. The first transition to the stationary phase was associated with accumulation and consumption of ethanol; the second one was associated with consumption of lactate, pyruvate, and other metabolites. It should be noted that the content of biomass in this variant of cultivation was also 2.5 times higher than in the absence of NA.

NA introduction also resulted in separation of the processes of ethanol and lactate production in time (Fig. 3). In the first hours of cultivation, there was a

sharp acceleration of aerobic alcoholic fermentation; only afterwards was lactate produced. However, the presence of NA did not reduce the degree of aerobic alcoholic fermentation as compared with growth under thiamine excess (Fig. 2). The dynamics of pyruvate production changed as well: after the first typical discharge of pyruvate into the medium, its concentration increased sharply in the subsequent period, which coincided with the maximal concentration of lactate produced. It should be noted that the dynamics of acetate and ethyl acetate accumulation in the medium changed under these cultivation conditions (Fig. 3).

Thus, the results demonstrate a significant effect of NA on the formation of fermentation end products, ethanol and lactate.

The Effect of Biotin on Fermentation Processes

According to the scheme (Fig. 1), ethanol synthesis (via pyruvate decarboxylase reaction) and Krebs cycle functioning (pyruvate dehydrogenase complex, α-ketoglutarate dehydrogenase, etc.) are affected by the presence of biotin. Biotin deficiency was supposed to significantly limit ethanol formation and pyruvate utilization via the pyruvate dehydrogenase complex and, consequently, result in increased lactate concentrations.

Indeed, cultivation of the studied yeast with 2 μg/l biotin resulted in a drastic decrease of the level of ethanol production and cessation of ethyl acetate synthesis (Fig. 4). At the same time, lactate synthesis commenced even in the first hours of cultivation and increased in the subsequent period.

Although growth rate and glucose uptake were reduced under biotin deficiency, the consumption of nitrogen (NH_4^+) was not less than in other experimental variants (Figs. 2, 3, 5). Moreover, the excretion of pyruvate increased sharply after nitrogen source depletion. The concentration of acetate under these conditions changed insignificantly and remained constant during the whole cultivation process. The insignificant level of alcoholic fermentation under these conditions and the lack of coincidence of the dynamics of ethanol and acetate accumulation suggest that the pyruvate dehydrogenase complex is a source of acetate in this case.

Thus, it is obvious that the decrease of decarboxylation caused by biotin deficiency shifts the equilibrium in pyruvate utilization towards lactate formation; i.e., formation of ethanol in this case is insignificant.

The Effect of Biotin Deficiency in the Presence of Na on Excretion of the Products of Glycolysis and Fermentation

The results obtained demonstrated that biotin deficiency suppressed alcoholic fermentation and stimulated lactate formation. The same effect was achieved by addition of NA. We have tested the concurrent effect of these two factors on metabolism of the yeast under study (Fig. 5). It can be seen that under biotin deficiency but in the presence of NA, lactate concentration significantly exceeded ethanol concentration, which was lower by an order of magnitude than for cultivation with excess thiamine or thiamine + NA (Figs. 2, 3). The maximal accumulation of lactate during cultivation occurred prior to the maximal ethanol concentration. At the same time, intensive synthesis of ethyl acetate was observed under these conditions; as mentioned above, such synthesis did not occur under biotin limitation (Fig. 4). The low ethanol concentration in the medium typical of the so-called "respiratory yeasts" [11], to which the studied culture belongs, may be associated with consumption of high amounts of alcohol and acetate (or acetaldehyde) for the synthesis of such compounds as ethyl acetate, butyl acetate, etc. [7, 8]. We believe that these eukaryotic cells preserve the aerobic type of metabolism via the binding of a considerable part of fermentation end product.

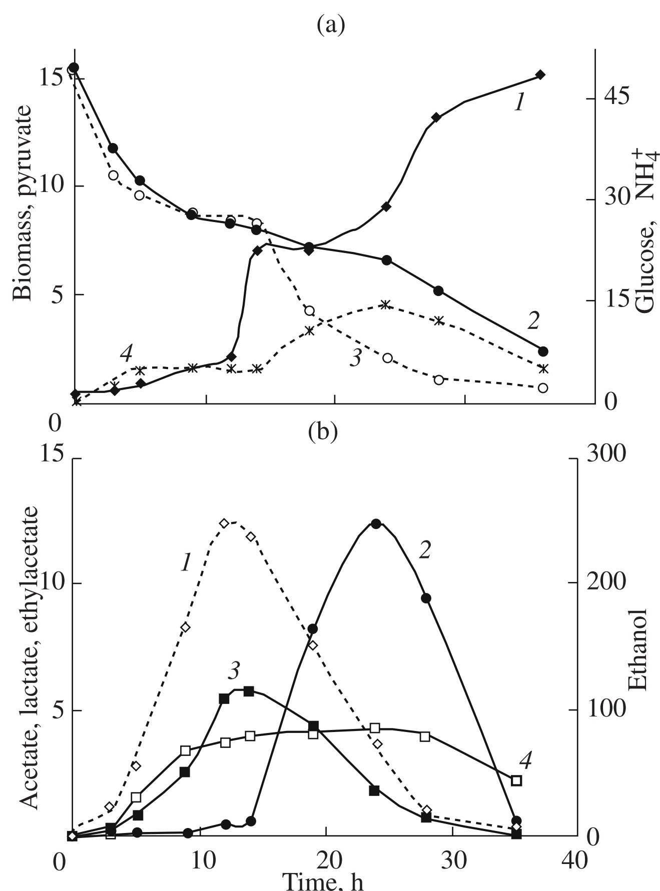

Fig. 3. Effect of nicotinic acid (500 μg/l) on the formation of glycolysis and fermentation products in *D. magnusii* cells. Biotin concentration: 10 μg/l; thiamine concentration: 200 μg/l. a: *1*, biomass, g/l; *2*, glucose, g/l; *3*, [NH_4^+], mM; *4*, pyruvate, mM; b: *1*, ethanol, mM; *2*, lactate, mM; *3*, acetate, mM; *4*, ethyl acetate, mM.

It should be noted that the dynamics of yeast growth under biotin deficit but in the presence of NA, as well

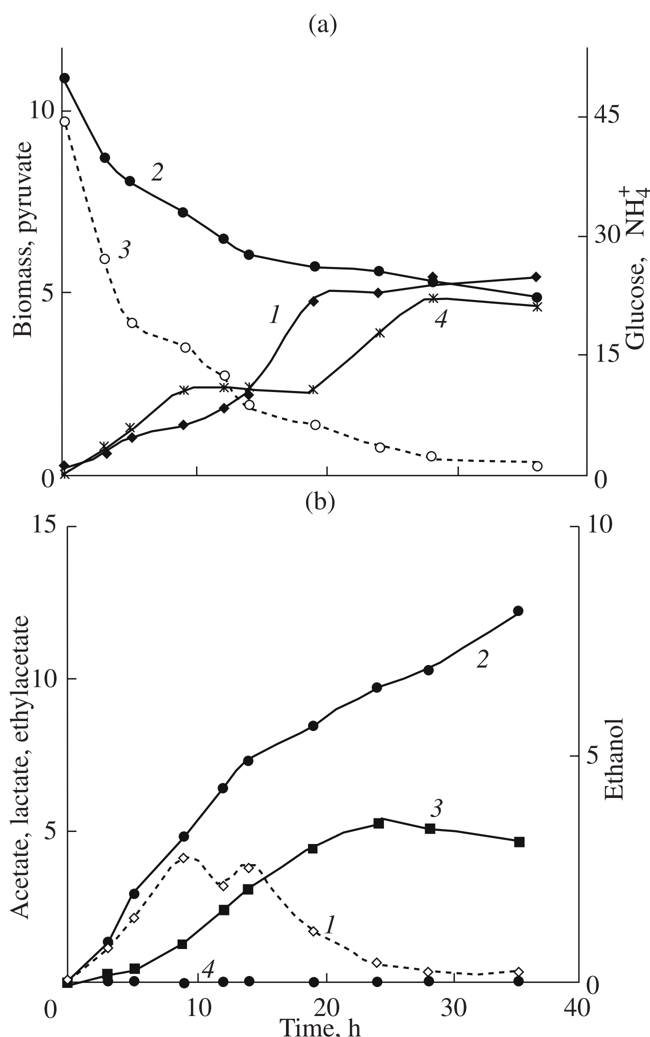

Fig. 4. Effect of biotin deficiency (2 μg/l) on the formation of glycolysis and fermentation products at cultivation of *D. magnusii* yeasts on 5% glucose. Thiamine concentration: 200 μg/l. a: *1*, biomass, g/l; *2*, glucose, g/l; *3*, [NH_4^+], mM; *4*, pyruvate, mM; b: *1*, ethanol, mM; *2*, lactate, mM; *3*, acetate, mM; *4*, ethyl acetate, mM.

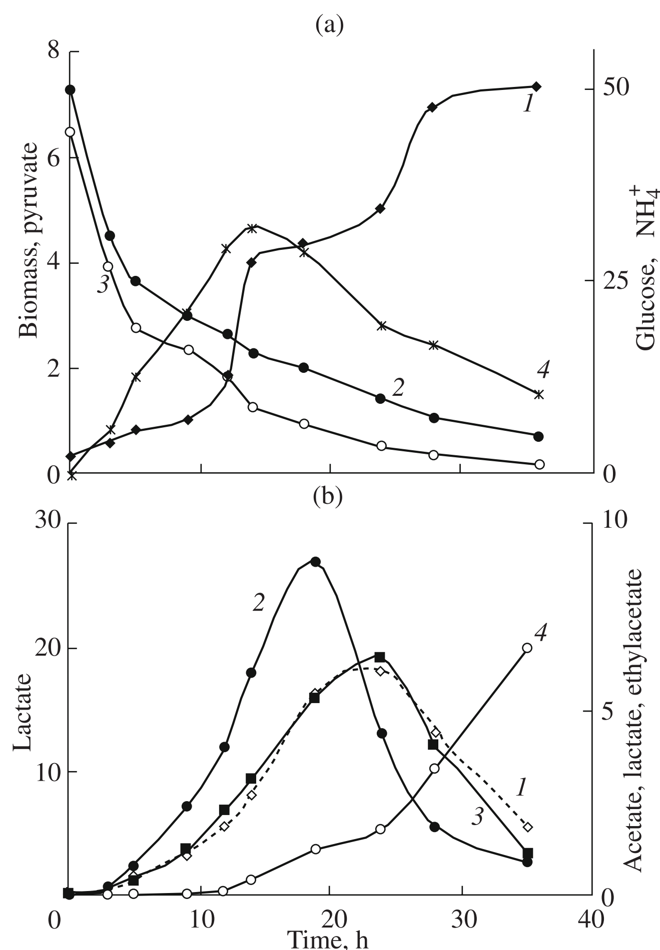

Fig. 5. Effect of biotin deficiency (2 μg/l) and nicotinic acid (500 μg/l) on the formation of glycolysis and fermentation products at cultivation of *D. magnusii* yeasts on 5% glucose. Thiamine concentration: 200 μg/l). a: *1*, biomass, g/l; *2*, glucose, g/l; *3*, [NH_4^+], mM; *4*, pyruvate, mM; b: *1*, ethanol, mM; *2*, lactate, mM; *3*, acetate, mM; *4*, ethyl acetate, mM.

as in the case of growth under thiamine excess + NA, was of diauxic character. However, in this case, the first transition was associated with consumption of the major accumulated product, lactate, and the second one, with consumption of other metabolites (data not presented).

Thus, it was established that under certain conditions glucose-grown yeasts formed a significant amount of lactate (30–40 mM); under biotin deficiency, accompanying ethanol was practically not produced. Concentrations of lactate produced were comparable to or much higher than those observed in the higher eukaryotes under conditions of lactoacidosis (5–6 mM) [12]. It is believed that increased lactate production in the latter case may result from tissue hypoxia, alkalosis, and transamination of catecholamine and alanine to pyruvate. However, while reciprocal relations only between pyruvate and lactate exist in higher eukaryotes (tumor cells, diabetes, etc.), in the yeast this tandem is supplemented by ethanol. The processes of ethanol and lactate formation are competitive, because both of these products are formed from pyruvate (Fig. 1), lactate as a result of its reduction by NADH and ethanol as a result of its decarboxylation to acetaldehyde followed by reduction of the latter by NADH. Thus, the former case involves consumption of reduced pyridine nucleotides, while the latter, on the contrary, involves their synthesis. Changing the vitamin concentrations makes it possible to regulate the processes of synthesis of the end products of fermentation and glycolysis (of pyruvate) in yeasts. Obviously, such regulation may occur also in cells of higher eukaryotes.

Our studies have shown that not only an appropriate change of the yeast genome [2], but also regulation of

metabolism by vitamins may result in a significant shift of fermentation (glycolysis) towards lactate formation. Moreover, our experiments show that biotin deficiency, which leads to practically complete suppression of aerobic ethanol production (the Crabtree effect), makes it possible to achieve a homofermentative process of lactate production. However, lactate concentration under these experimental conditions is significantly lower than that obtained by lactic acid fermentation in *Lactobacillus* sp.

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