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IMPACT OF EXOGENOUS AUTOREGULATORS OF INTERCELLULAR COMMUNICATION IN YEAST ON THE GROWTH OF SACCHAROMYCES CEREVISIAE

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Abstract

The study was aimed at growth capacity and growth of Saccharomyces cerevisiae population exposed to R-, S- and DL-isoforms of phenylethanol - autoinducer molecule of the yeast Saccharomyces. It is found that when impacting the cells, exogenous phenylethanol (PEL) of tration and growth phase inhibits the growth of the yeast culture S. cerevisiae, depending on its isoform, by aromatic alcohol. The maximum inhibition effect was shown for R-isoform of PEL in a concentration of 10 um when introducing it to the culture at the beginning of lag phase. In an exponentially growing culture, the exogenous R-PEL of the same concentration resulted in stopping the yeast growth and budding immediately after addition. R-PEL introduced into the culture at the beginning of phase of growth declining and stationary phase had no effect on the population size in comparison with that without PEL treatment, but it induced a cell morphogenesis characterized by the transition from unicellular in the chain forms. The filamentous growth was also characteristic for S. cerevisiae at cultivation on a limited nitrogen medium; it indicates similar mechanisms of action of exogenous and endogenous autoinducers secreted by a cell in response to nitrogen starvation.

Key words: S.cerevisiae, autoinducer molecules of yeast, phenylethanol (PEL), R-, S-, DL-isomers of PEL, morphogenesis, chain forms.

Introduction

One of the most important features of microbial cells is their ability to change the growth and division parameters under the control of ambient signals resulting from changes in the concentration of nutrients or autoregulatory factors in the culture medium [1-3]. The most important factors of control over the microbial population growth are low molecular thermostable extracellular metabolites that are responsible for density-dependent processes (quorum

sensing, QS) in a microbial population. The QS bacterial systems are considered as one of the mechanisms regulating microbial communication, which depends on a cell population density and secretion of the factors that induce the formation of biofilm, cell competence, bioluminescence, etc. [4, 5, 6].

Despite the vast amount of information about the molecules of bacterial quorum sensing, the most studied of which are acyl derivatives of L-homoserine lactones (HSL) [7,8], data on molecular communication of microscopic fungi were practically absent until the discovery of farnesol in the pathogenic fungus Candida albicans [9]. In the last decade it has been shown that, in addition to farnesol, the aromatic alcohol tyrosol also has QS molecular properties in C. albicans, and it controls growth of a culture, cell morphogenesis and biofilm formation [10].

The compounds similar to the QS molecules - phenylethanol, tryptophan - have also been described in the yeast Saccharomyces cerevisiae. As it has appeared, these aromatic alcohols are able to regulate the morphogenesis of yeast cells under conditions of nitrogen starvation [11]. Although fungi have many different known processes depending on the cell population density and potentially regulated by QS molecules, the inductor molecules themselves and their mechanisms of action still remain poorly understood [12]. The major obstacle to understand the ways the QS molecules regulate the intercellular communication processes of micromycetes is a lack of consensus on establishing their functional role. Based on analysis of the criteria characterizing the QS molecules of bacteria, an opinion has been formed that fungal autoinductor molecules, as well as bacterial, should be accumulated during the population growth in proportion to increase in the number of cells in a medium, with the consequences that lead to culture growth restriction on a particular stage of such culture development [13, 14]. Furthermore, after reaching a threshold level the QS molecules should induce a coordinated response across the cell population, rather than just evoke the processes of assimilation of the autoinductor molecule itself or its detoxification. The QS exogenous molecules of fungi should induce phenotypic manifestations of autoinducer’s action, while excluding those caused by other catabolic products.

Although the literature contains information about the similarity of functioning of the signal system controlled by tryptophol and phenylethanol in the yeast S.cerevisiae [11] and the QS described for Gram-negative bacteria [15], the role of autoinductor molecules of micromycetes in regulating the yeast population growth and development is unclear.

Therefore, one of the approaches to elucidate the role of factors of intercellular communication of the yeast in regulating their vital functions is to analyze the characteristics of growth of the culture exposed to the exogenous

autoinducers. The functional role of exogenous autoregulators should be determined according to the cellular physiological state, which predetermines the transition of a culture in a stationary phase, i.e. in a state combined with a proliferative rest and cessation of cell division.

In accordance with the foregoing, the study was aimed at clarifying the physiological characteristics of the growth of S. cerevisiae under the influence of exogenous phenylethanol on the population of yeast in the various stages of its development.

Experimental part

Materials: As autoregulators, S-, R- and DL-isoforms of phenylethanol ($C_6H_5CH(CH_3)NH_2$, molecular weight of 121.18, purity of 98%) purchased from Sigma Aldridge, Germany were used (Figure 1). The studied phenylethanol concentration from 0.1 to 10 um was prepared by dissolving the preparations in distilled water.

The object of study was a strain of Saccharomyces cerevisisae 823 used for baker's yeast at the Buinsk sugar factory of the Republic of Tatarstan.

Methods: The yeast was grown at 30^0C in Sabouraud medium (agarized and liquid) [16], as well as on Rieder medium [17], complete (R1) and nitrogen-limited, containing the minimum number (3mkg/ml) of ammonium sulphate (R2) as a nitrogen source. The seed culture of yeast was prepared in the dense Sabouraud medium. After 18-hour incubation, the yeast was rinsed from the agar surface with a liquid medium and cultivated for 16-18 hours under stationary conditions. The resulting suspension was used as inoculum, which was introduced into a fresh liquid medium to the final concentration of 10 mln cells/ml.

The culture growth was controlled by counting the number of cells in a Goryaev chamber using a Carl Zeiss microscope. The culture growth dynamics was recorded by a value of the specific (μ) and the overall average growth rate ($V_{av.}$) of yeast [18].

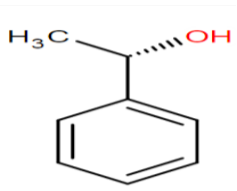
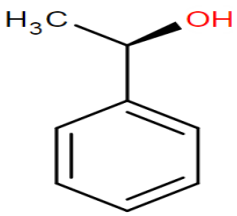
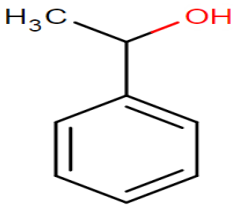
		
(S)-(–)-1-methylbenzylamine	(R)-(+)-1-methylbenzylamine	DL-(±)-methylbenzylamine
a	**b**	**c**

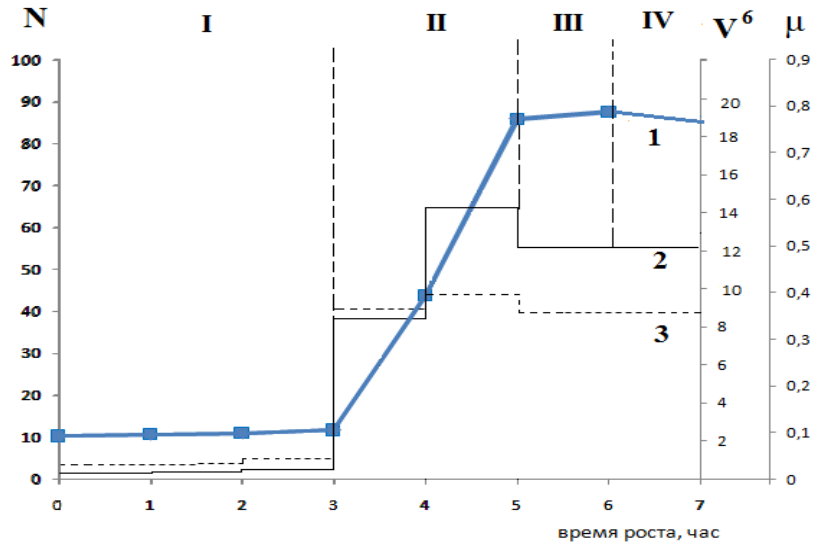
Figure 1. S-, R-, DL-isoforms of phenylethanol ($C_6H_5CH(CH_3)NH_2$), (a, b, c, respectively).

The obtained results were subjected to statistical analysis using Microsoft Excel program. To assess a significant difference of the groups of data, an adequate level of significance p = 0.05 was accepted. The figures show 95% confidence intervals for true averages.

Results

Given that changes in the cellular physiological state emerging during a culture growth determine a shift of the microbial population growth stages [18], we registered primarily a batch culture growth dynamics in yeast S.cerevisiae cultured in the Sabouraud liquid medium [16] under the stationary conditions at an initial cell density of 10 mln/ml.

It has been found that the lag-phase of culture growth has continued for first three hours after inoculation, when the cell number remained at the initial level. In the period from 3 to 5 hours, the population saw an increased number of budding cells, and the total and specific growth rate of the culture reached a maximum, which is characteristic for the exponential growth phase. After 5-hour incubation, there was a reduction of the specific growth rate indicating that a phase of culture growth declining occurred, and after 7 hours both the specific and total growth rate decreased, and the culture entered a stationary phase (Figure 2).

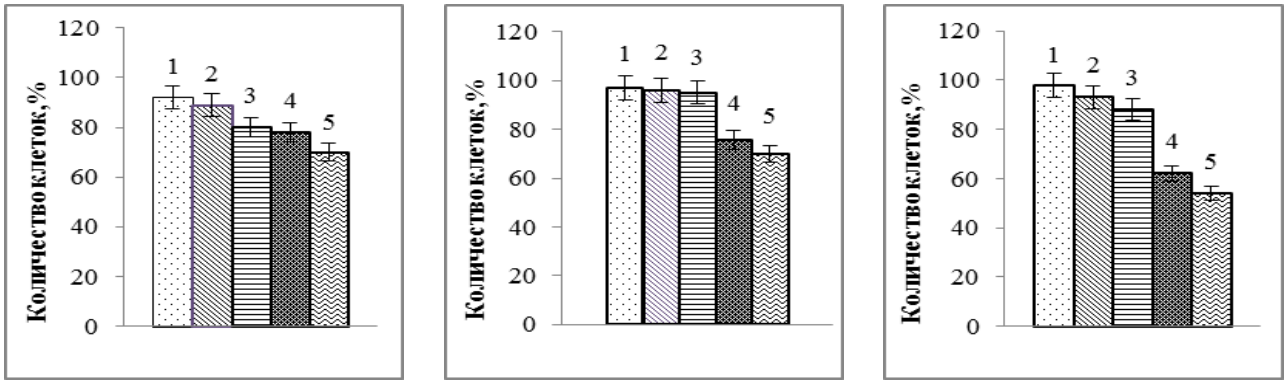


Время роста, час	Growth time, hour

Figure 2. Kinetics of S. cerevisisae culture growth in a Sabouraud liquid medium.1- cell number, mln/ml (N); 2 - total average culture growth rate ($V_{av.}$); 3 - specific culture growth rate (μ/h^{-1}); I - lag phase, II - exponential, III - growth declining phase, IV - stationary phase.

The action of R-, S-, DL-isoforms of PEL was tested at concentrations ranging from 0.1 to 10.0 um, which were selected on the basis of data about the content of such QS molecules in a culture liquid of S. cerevisisae within 5-8 um [11].

In the experiments, the isomers of PEL were introduced into a medium with the inoculum (early log phase), and the results of its impact on yeast growth were recorded according to the population size upon reaching a stationary phase (6 hrs). R-PEL had the most pronounced effect on the culture growth among three tested isoforms of PEL. At concentrations of 5 and 10 um, R-PEL inhibited the cell proliferation by 40-55% compared to untreated cells, whereas the same doses of S-PEL and DL-PEL suppressed the growth of yeast culture twice as weak as R-PEL (by 25-30% compared to untreated cells).



Variants of PEL concentrations

S-isoform DL-isoforms R-isoform

Количество клеток, %	Number of cells, %

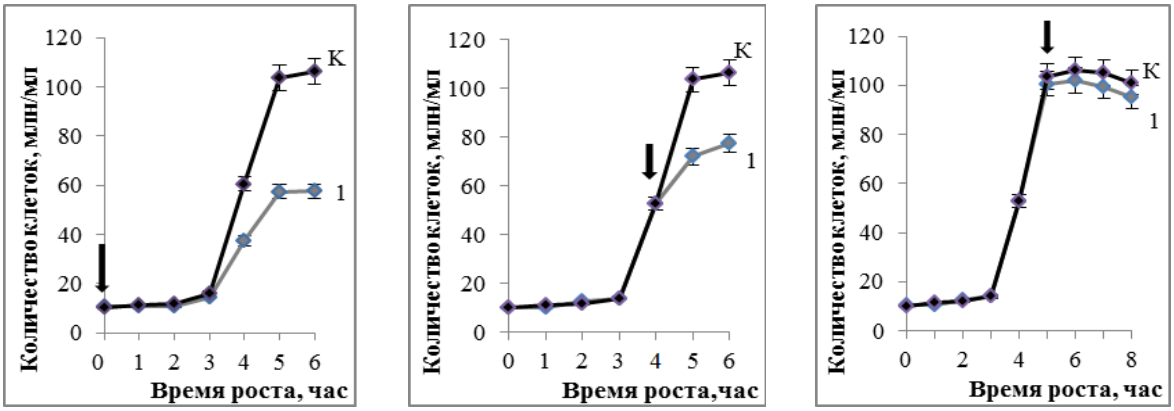
Figure 3. Changes in population size of S. cerevisisae (% of the control value taken as 100%) after 6-hour incubation in the presence of exogenous phenylethanol (S-, DL-, R- isoforms) added to a medium with the inoculum (early log phase). 1, 2, 3, 4, 5 - phenylethanol concentration: 0.1, 0.5, 1.0, 5.0, 10 um, respectively.

In connection with the obtained data, R-PEL in a concentration of 10 um was selected for further experiments with the introduction of autoregulators in different population growth phases.

R-PEL introduced in the early lag phase with the original cell number of 10 mln/ml for the first three hours of incubation, did not change the yeast growth parameters (Figure 4a). In the subsequent period, the inhibitory effect of PEL was demonstrated; it reached its maximum by 5 hours of culture growth (Figure 4a, Diagram 1), and the culture

entered the stationary phase with almost half the number of cells compared to the control culture growing without PEL.

The inhibition of yeast reproduction induced by PEL, which was added to the lag phase, amounted to 46% of the control yeast growth value. The effect of R-PEL introduced into a culture of the exponential growth phase (Figure 4b), emerged almost immediately after the addition of autoinducer, but the growth inhibition accounted for less than 30% (Figure 4b, Diagram 1). Study of the impact of exogenous R-PEL on the growth of culture S. cerevisiae in the growth declining phases (5h) and in early stationary phase (6h) showed no differences between the tested and control population sizes (Figure 4c).



Количество клеток, %	Number of cells, %
Время роста, час	Growth time, hour

Figure 4. Changes in the population of S. cerevisiae growing in the presence of R-PEL (10 um) added: in early lag phase (a), middle of exponential phase (b) and growth declining phase (c) of the culture. K-growth on a medium without PEL (control), 1 - growth on a medium with PEL. The arrow indicates the time R-PEL has been introduced in the culture.

Discussion

Eliminating the impact of exogenous PEL in connection with the culture growth and transition in a stationary phase is primarily related to accumulation, in a culture liquid, of the autoregulators synthesized by the cells themselves. It is known that a high density cell population is accompanied by 20-50 times higher level of extracellular PEL compared to its content in the population with a low original density (about 100 thous. cells/ ml) [11]. A common pool of the exogenous and endogenous signal molecule might have a critical level inhibiting cell proliferation processes, which

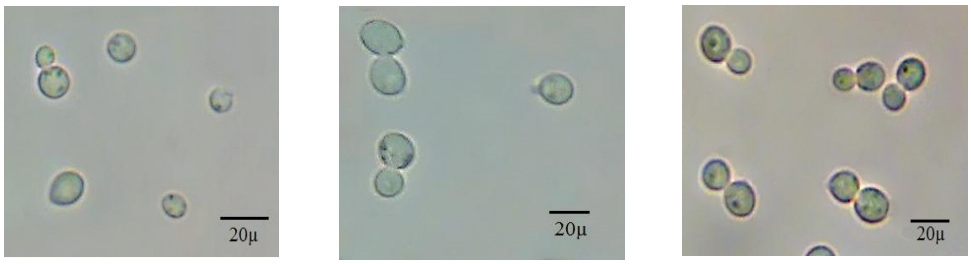
excess can no longer induce a further stop of the yeast budding. Since PEL is accumulated in an aging cell culture due to an endogenous synthesis, an addition of exogenous PEL does not lead to an increased effect.

This conclusion is consistent with the published data [19, 20] about correlation of high production rates of quorum-sensing molecules, phenylethanol and tryptophol, and expression of genes ARO8, ARO9 and ARO10 in charge of their synthesis in a phase of active population growth. However, by analogy with endogenously produced PEL the exogenous PEL is accepted by the proliferating culture as a signal of a stressful situation, in the process of adaptation to which regulons of a stationary phase are triggered in the yeast cells; such regulons are responsible for declining the cell growth and division [2].

The functional role of yeast QS molecules is also linked to their involvement in the apoptosis of S. cerevisiae [21]. One of the reasons why the unicellular organism's apoptosis occurs are the genetic alterations, which critical value depends on the cell population density, thus, pheromones and ammonia can act as communication molecules of S. cerevisiae [22, 23]. As shown by Severin and Hyman, high concentrations of the α-mating factor induce apoptosis in yeast cells, and the gradient of ammonia concentration formed over an entire period of colony growth induces death of old cells located in the center of the colony and stimulates the growth of cells located at the periphery towards the center increasing in such way the colony diameter [22]. The signal role of PEL in communication systems aimed at suppressing the survival and segregation of yeast cells stored for a long period in stationary cultures also leads to apoptosis [23]. Depletion of the reserves of nutrients in a medium, especially those of nitrogen, results in changing the yeast growth and morphogenesis parameters [11, 24, 25]. The nitrogen deficiency causes morphogenesis of yeast Saccharomyces due to phenylalanine biosynthesis stimulating the synthesis of cell surface GPI-receptor [26] - Flo11p gene product - in charge of the intercellular adhesion, yeast-to-substrate adhesion, hypha and biofilm formation [27]. The yeast Candida shows that unlike the lag and exponential growth phase medium containing in excess the nitrogen and carbohydrate sources necessary for a cell growth, unipolar yeast growth is induced in a nitrogen-limited medium of stationary phase, and newly emerging buds are not separated, they produce the chains similar to pseudohyphae [28]. According to the data [29], baker's yeast can be a good model for the formation of fungal biofilm.

We analyzed morphological features of the growth of yeast S. cerevisiae in a medium without PEL in terms of a population growth phase. It has appeared that in a lag-phase (0-3 hours) when the nutrients are contained in a medium in sufficient quantity, the population represents a single cell suspension (Figure 5.a). In the exponential phase culture (3-5 hour) under the conditions of a medium optimal for the growth a number of budding cells prevails (Figure 5.b),

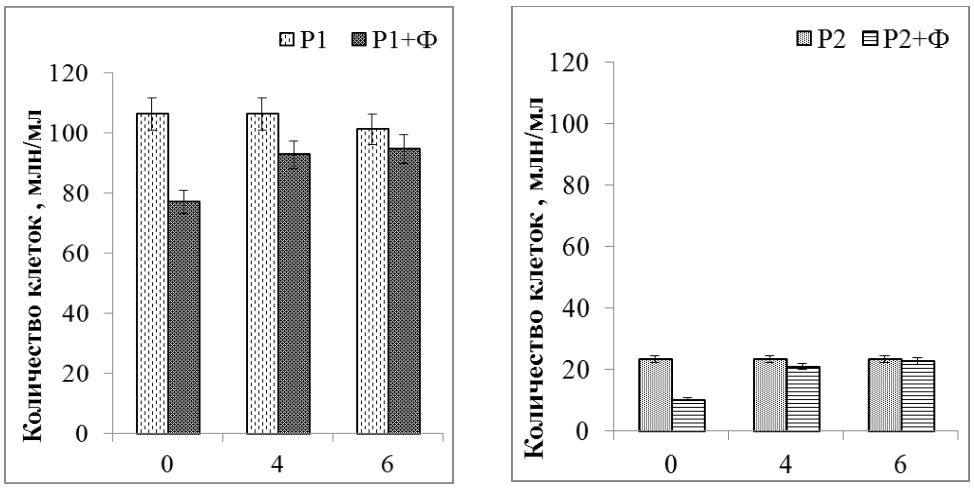
while in the growth declining and stationary phases (5-8 hours) the cells appear that are connected as chains (Figure 5.b), which is apparently a consequence of a lack of nutrients in the culture medium.



a **b** **c**

Figure 5. Morphological changes in the growth of yeast S.cerevisiae in a medium without PEL in various phases of culture growth: (a) - lag phase, (b) - middle of exponential phase and (c) - stationary phase of growth.

Results of studying the growth of S.cerevisiae in the presence of PEL demonstrated that the nature of the yeast reproduction suppression is virtually identical on a complete Rieder medium (R1) and a nitrogen-limited Rieder medium (R2), although with a nitrogen deficiency the total number of cells showed a 5-fold decrease compared to a "rich" medium (Figure 6a, b).



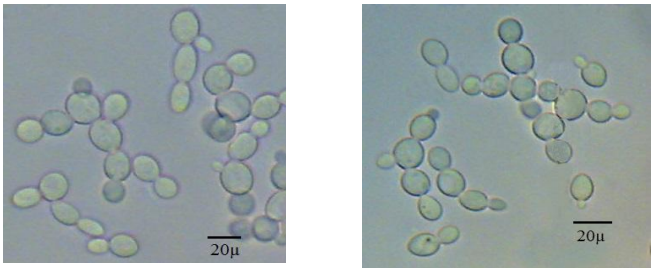
Culture time, h Culture time, h

a **b**

Количество клеток, млн./мл	Number of cells, mln/ml
P1	R1
P1 + Ф	R1 + P
P2	R2
P2 + Ф	R2 + P

Figure 6. Growth of S. cerevisiae in the presence of 10 um of exogenous PEL (+P) added in a lag phase of 10 pM on a complete Rieder medium R1 (a) and nitrogen-depleted Rieder medium R2 (b)

However, chain forms (Figure 7) prevailed in the morphology of yeast on a nitrogen-depleted medium and on both media (R1 and R2) in the presence of PEL; it indicates that processes induced by a deficit of nitrogen and PEL are similar.



a **b**

Figure 7. Chain forms of S.cerevisiae cells in a population after 8-hour growth on media R1 (a) and R2 (b) in the presence of R-PEL added in the early lag phase of culture growth

Thus, we have shown that yeast has the same answer regarding the depletion of nutrients in a medium and the appearance of autoregulatory QS molecules, in particular R-PEL: limitation on cell growth and activation of dimorphic switch resulting in a slow vegetative growth. Filamentous growth of yeast is considered an important adaptive response when thanks to branched pseudohyphae the starving micromycete colonies gain an advantage in the absorption of culture medium's nutrients at a low level of biomass [28]. In the presence of the same concentration of exogenous PEL on a nitrogen-limited medium, the growth is inhibited by more than 50%, whereas on a rich medium - by only 25% (Figure 6). The activation of endogenous PEL biosynthesis might increase its concentration in a medium at a nitrogen deficiency.

Consequently, similar to endogenous PEL, the exogenous one regulates the number of S.cerevisiae population and induces switching of the growth of single yeast cells to filamentous. The effect of exogenous PEL is practically not seen in introducing it into the culture of a stationary phase of growth, because it has already switched on filamentous growth induced by endogenous aromatic alcohols - signal molecules of the QS system. Despite the fact that the fungal QS compounds are only at the initial stage of studying, it is clear that they, as regulators of dimorphic growth, make an important contribution to the mechanisms of virulence and invasion of pathogenic microscopic fungi, for example, Candida albicans, Magnaporthe grisea, Ustilago maydis capable of causing systemic fungal infections [30, 31]. Targeted regulation of the level of signal aromatic alcohols of fungi will allow to partially suppress their

development. And in developing a strategy for prevention and treatment of fungal infections, a dimorphic switch of micromycete growth and development itself can be an attractive target.

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