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EXPERIMENTAL ARTICLES

Study of the Content of Inorganic Polyphosphates in *Saccharomyces cerevisiae* Grown on Different Carbon Sources with Different O_2 Concentrations in the Medium

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Abstract—The content of different fractions of inorganic polyphosphates (polyP) was studied in *Saccharomyces cerevisiae* VKM Y-1173 growing on a complete medium with glucose under hypoxia and active aeration as well as on ethanol. The highest growth rate was observed for aerobic fermentation, while the yield of biomass was maximal for cultivation on ethanol. In the mid-log growth phase, the amount of polyP was maximal in the cells grown on glucose under hypoxia and minimal on ethanol. In this latter case, the content of different polyP fractions changed unevenly: polyP3, polyP4, and polyP1 decreased by approximately 60%, 45%, and 30%, respectively; the salt-soluble polyP2 remained at almost the same level; while polyP5 abruptly increased 10- to 15-fold. These findings demonstrate that the metabolic pathways for polyP fractions are different. A significant drop in the amount of the main polyP fractions accompanied by a decrease of the polyP average chain length in the presence of carbon and P_i sources in the medium is evidence of active involvement of polyP as additional energy sources in the flows of energy in actively growing yeast cells.

Key words: inorganic polyphosphates, carbon source, catabolite repression, aeration, yeast, *Saccharomyces cerevisiae.*

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Inorganic polyphosphates (polyP) are linear polymers consisting of orthophosphoric acid residues that are linked by a phosphoanhydride bond. They have been found in all examined organisms, including animals and humans [1–3].

Studies of recent years have revealed new, previously unknown functions of these compounds in cell metabolism. In addition to storing phosphorus and energy easily utilized by cells in conditions of their limitation, polyP proved to play a key role in maintenance and functioning of yeast cell wall structure, formation of transport channels in the membranes, regulation of the pool of metal cations, enzyme activity, and gene expression. These polymers also play a role in stress response and survival of microorganisms [2–4].

PolyP have been found in all yeast cell organelles: cell envelope, nuclei, vacuoles, mitochondria, and cytosol. Their metabolism is closely associated with the main processes occurring in these structures [1–3, 5].

Yeast growth phase, carbon sources, and P_i concentration in the medium have a significant influence on polyP metabolism, accumulation and uptake.

The cells of *Saccharomyces cerevisiae* accumulate these polymers until the mid-log growth phase; subsequently, their content usually drops and increases again in the beginning of the stationary phase. The increase of polyP content in the mid-log growth phase is accompanied by a sharp decrease of their mean chain length [6]. Upon the transfer of cells to phosphate-free medium, polyP are quickly utilized and their content and chain length decrease [7].

The available data demonstrate that the polyP content in *Candida intermedia* and *Torulopsis famata* was 1.5-fold higher when paraffins are used as substrates as compared with the growth on glucose [8]. The same was observed in *C. guillermondii*, although polyP5, unlike other fractions, increased on glucose but not on paraffins [9].

The content of polyP detected by ^{31}P-NMR (localized mostly in the vacuoles) [2, 10, 11] was higher in *S. cerevisiae* grown on glucose under aeration as compared with anaerobic conditions [12]. The tendency for increase of the content of vacuolar polyP under aeration was confirmed in a later work. It was also shown that the amount of this polyP was the highest under growth on lactate [11].

On the transfer from P_i-deficient to P_i-containing medium in the presence of glucose, the cells of *S. cerevisiae* and *Kluyveromyces marxianus* formed more acid-insoluble polyP than when growing on ethanol [13].

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The effect of carbon sources and/or aeration was generally considered for total polyP or for individual polyP fractions. It is known, however, that different polyP fractions of the yeast not only have different localization within yeast cells but are also coupled with different metabolic pathways. It has been shown, for example, that the biosynthesis of high-polymer alkali-soluble polyP is closely associated with the synthesis of cell wall mannoproteins [1, 2, 14]. Metabolism of the salt-soluble polyP fraction is associated with RNA synthesis [1–3, 15]. Low-molecular acid-soluble polyPs found in the cytosol, nuclei, vacuoles, and mitochondria also seem to be associated with the main metabolic features of these compartments [1–3, 5, 16, 17].

In view of the above, the goal of this work was to study the content of polyP fractions in *S. cerevisiae* with cultivation on different carbon sources (glucose and ethanol) and under different aeration modes.

MATERIALS AND METHODS

The object of research was the yeast *Saccharomyces cerevisiae* VKM Y-1173. Cells were grown on a shaker at 29°C in flasks with the Reader medium [7] in two different aeration modes: weak (rate of stirring 100 rpm; $V_{flask} : V_{medium} = 3.75 : 1$; with 2% glucose as a carbon source) and intensive (rate of stirring 200 rpm; $V_{flask} : V_{medium} = 15 : 1$; with 2% glucose or 1 vol % ethanol). At inoculation, the density was 0.018 OD units at $\lambda = 530$ nm and cuvette width of 3.07 mm.

The cells harvested in the logarithmic growth phase ($OD_{530} \sim 0.8$) were separated from the medium by centrifugation at 3000 *g*, washed twice with cold (4°C) distilled water, and frozen at –70°C.

PolyP fractions were obtained by the method of Langen and Liss [6] by sequential extraction from cells with acid, salt, and alkali solutions. As a result, the following polyP fractions were obtained: acid-soluble (polyP1), salt-soluble (polyP2), and two alkali-soluble fractions (polyP3, pH 9–10, and polyP4, pH 12). The content of polyP5 was assessed by orthophosphate formed after the treatment of residual biomass with 0.5 M $HClO_4$ at 90°C, two times for 20 min.

In order to determine polymer chain lengths by PAG electrophoresis, polyP from the obtained extracts was precipitated with saturated $Ba(NO_3)_2$ solution and then converted into a soluble NH_4^+ form using ion-exchange resin [7]. For the assay of polyP5 chain length, the residual biomass after removal of polyP1, polyP2, polyP3, and polyP4 fractions was suspended in distilled water at 0°C and neutralized with 0.1 M HCl, followed by addition of EDTA solution to the final concentration of 30 mM. The biomass was separated by centrifugation. PAG electrophoresis was performed as described [17]. The amount of preparation applied to the gel was 1.5–3.3 μg P defined as P_i.

The content of cytochromes in intact yeast cells was determined by differential absorption spectra registered in a double-beam spectrophotometer (Shimadzu, Japan). The coefficients of molar extinction of cytochromes for $c + c_1$, b, and $a + a_3$ were 24, 17.8, and 14 $mM^{-1}\ cm^{-1}$, respectively [18].

Phosphorus compounds of the acid-soluble fraction were determined as described [19]; P_i, glucose, and the weight of dry yeast biomass were determined by the known methods [6].

The presented experimental results were obtained from three statistically processed biological experiments [20].

RESULTS AND DISCUSSION

S. cerevisiae is known to metabolize glucose to ethanol both under anaerobic and aerobic conditions [21, 22].

The need of active aeration for complete fermentation of carbohydrate substrates and effective production of end products is well-known in the microbiological industry [23]. It has been established that the expression of more than 500 yeast genes varies depending on the presence of O_2 in the medium, including the genes responsible for the synthesis of hemoproteins, NAD, uracyl, some structural proteins of cells wall, etc. Molecular oxygen is needed for synthesis of the most important membrane components: sterols and unsaturated fatty acids [24]. Enhanced synthesis of lipids increases resistance of yeasts to ethanol and makes higher yield of alcohol possible [25]. The change of membrane structure due to the absence of oxygen may affect the activity of many, if not all, membrane-bound enzymes [26]. Moreover, the activity of pyruvate decarboxylase (the key participant of the last stage of glucose catabolism in alcohol fermentation) was found to increase significantly in the presence of oxygen [24].

Via catabolite repression, glucose as a carbon source inhibits the synthesis of functional components of the metabolic pathways not involved in glycolysis (Krebs cycle, respiratory chain and oxidative phosphorylation, glyoxylate shunt and gluconeogenesis) [27].

Thus, fermentation under aerobic conditions (Crabtree effect) results in suppression of respiratory metabolism by another energy-yielding process, glycolysis, while the process as a whole is often termed "aerobic fermentation" [28].

Based on the above, the following models of *S. cerevisiae* cultivation were chosen for studying the content of different polyP fractions:

(i) Growth on glucose under hypoxia (aeration intensity determined by the standard sulfite method was <0.1 mmol O_2/l min),

(ii) Growth on glucose under active aeration, i.e., aerobic fermentation (aeration intensity: 0.56 mmol O_2/l min), and

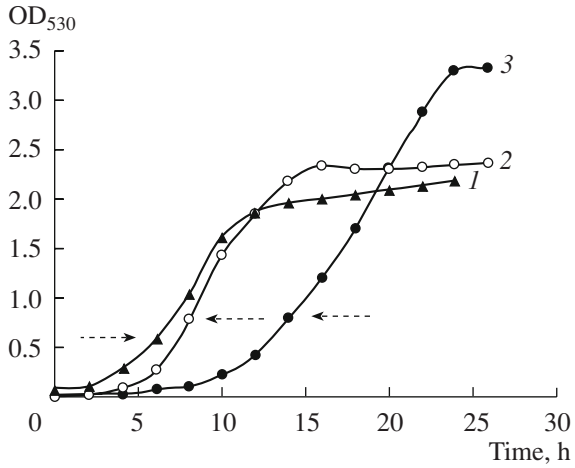

Fig. 1. Growth of *S. cerevisiae* on the Reader medium with glucose under hypoxia (*1*) and intensive aeration (*2*) and with ethanol under intensive aeration (*3*). Broken arrows indicate the points of biomass sampling for analysis.

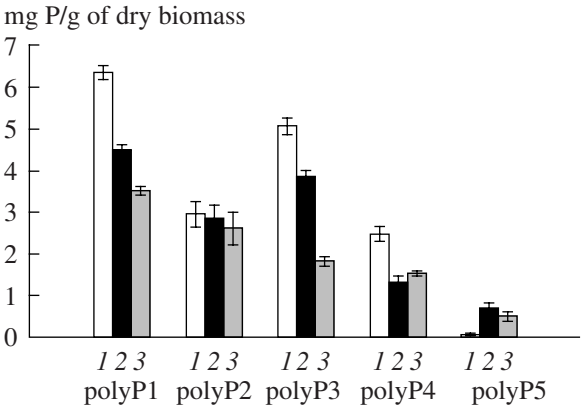

Fig. 2. Content of polyP fractions in *S. cerevisiae* cells of the logarithmic growth phase grown on the Reader medium with glucose under hypoxia (*1*) and intensive aeration (*2*) and with ethanol under intensive aeration (*3*).

(iii) Growth on ethanol under active aeration.

Figure 1 shows that both the duration of the logarithmic growth phase and biomass yield were higher under aerobic fermentation at a high growth rate ($\mu = 0.52\ h^{-1}$) than under hypoxia. On ethanol, after a long lag period the growth rate ($\mu = 0.35\ h^{-1}$) was nearly the same as on glucose under hypoxia ($\mu = 0.38\ h^{-1}$), but the logarithmic phase duration and biomass yield were maximal, which has been mentioned by other authors as well [29]. Samples to be analyzed were taken in the beginning of the logarithmic phase, after the yeast had utilized not more than 30–35% of glucose and 40% of ethanol (of their initial concentrations in the medium). At the same time, P_i concentration in the medium remained at a high level (8.6 mM).

As can be seen from Table 1, the amount of cytochromes was at a low "residual" level both under hypoxia and at aeration on glucose, [27, 30]. This amount significantly increased only at cultivation on a respiratory substrate. Hence, in the presence of glucose in the medium (independent of aeration), glycolysis was probably the main pathway of substrate degradation and energy formation in the given yeast, while growth on ethanol involves respiration and oxidative phosphorylation.

Figure 2 shows the content of different polyP fractions in *S. cerevisiae* cells. As can be seen from the figure, the maximal amount of polyP is accumulated for the growth on glucose under hypoxia (16.88 ± 1.0 mg P per g of dry biomass). The increase of oxygen content in the medium resulted in a decrease of total polyP by nearly 20% (13.40 ± 0.8 mg P/g of dry biomass). The cells growing on ethanol (9.81×0.7 mg P/g of dry biomass) had the minimal polyP content. Low polyP level on ethanol as compared with glucose was also found in *Kluyveromyces marxianus* [13]. Figure 2 shows that under the given experimental conditions the content of acid-soluble polyP1 was maximal (~33–37%), followed by alkali-soluble polyP3 (~20–30%), salt-soluble polyP2 (~18–26%), alkali-soluble polyP4 (~10–15%), and, finally, polyP5 (~0.3–5%). Such a pattern of polyP distribution by fractions in the beginning of the logarithmic growth phase has been already reported for this yeast [6] and, consequently, is typical of this growth phase and does not depend on the energy-yielding system predominant in cell metabolism. Further, Fig. 2 shows that the amounts of different polyP fractions decreased unevenly. At aerobic fermentation, as compared with hypoxia conditions, the content of polyP4 decreased most pronouncedly (~45%), followed by polyP1 (~30%) and polyP3 (~25%). The decrease of the content of these polyP fractions in ethanol-grown cells was less dramatic. On this background, two fractions (polyP2 and polyP5) stand out particularly. Salt-soluble polyP2 proved to be the least prone to variations independent of cultivation conditions. Such stability of this fraction probably results from the fact that its metabolism in fungi is closely associated with the synthesis of nucleic acids, which is active in this growth phase [1, 15].

Table 1. The content of cytochromes in *S. cerevisiae* cells under different cultivation conditions, mmol/g of dry biomass

Cultivation conditions	Cytochromes		
	$c + c_1$	b	$a + a_3$
Glucose, hypoxia	0.076	0.069	0.039
Glucose, intensive aeration	0.079	0.067	0.042
Ethanol, intensive aeration	0.167	0.097	0.104

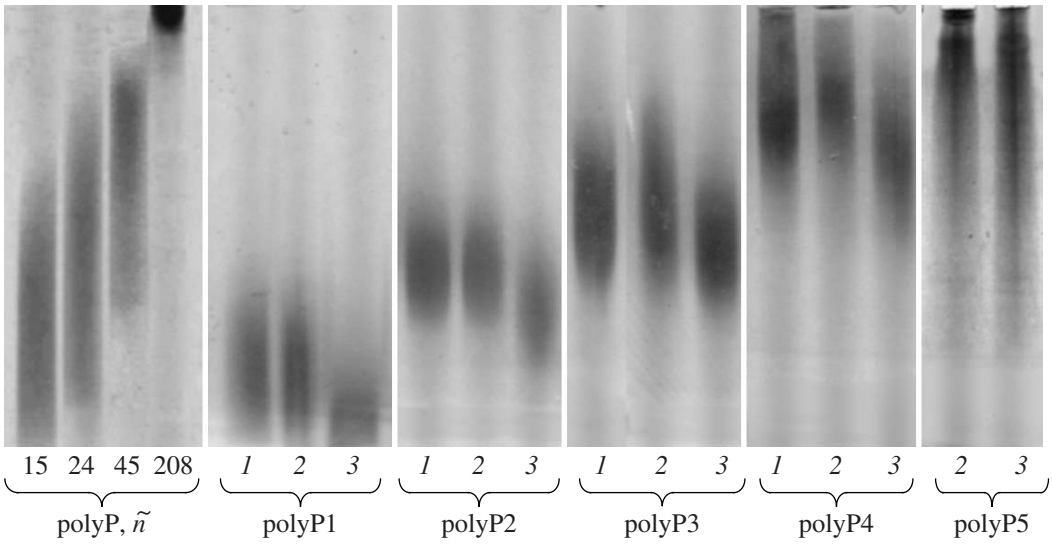

Fig. 3. Electrophoregram of polyP fractions isolated from *S. cerevisiae* grown on the Reader medium with glucose under hypoxia (*1*) and intensive aeration (*2*) and with ethanol under intensive aeration (*3*). $\tilde{n}$ is the number of orthophosphoric acid residues in standard polyP preparations.

PolyP5, unlike all other fractions, did not decrease and even drastically increased (~10- to 15-fold) for cultivation on ethanol and aerobic fermentation as compared with growth under hypoxia. This fact suggests that the synthesis and consumption of this polyP fraction lies apart from the main metabolic pathway of these polymers and is probably executed otherwise.

As Fig. 3 shows, the drop in the amount of the first four polyP fractions is accompanied by a decrease of the average chain length, especially in ethanol-grown yeasts. This fact may be evidence of more active polyP utilization under these growth conditions.

It is known that free nucleotides (primarily adenylic) are effectors of many metabolic pathways; the higher the ratio of mononucleotides to their high-energy analogues, the more active the consumption of energy for biochemical processes [31].

Table 2 shows that the lowest level of di- and triphosphates was revealed in the cells growing on the respiratory substrate and with aerobic fermentation. The low level of ATP for growth on ethanol, as compared with growth on glucose, has also been revealed in *K. marxianus* [13].

These findings support the opinion [6] that polyPs are involved in the general flow of energy during active growth processes as additional energy sources.

Under hypoxic conditions of growth on glucose, polyP5 in *S. cerevisiae* is usually represented by such a small amount that determination of its structure becomes problematic [6, 7]. Higher polyP5 content at the growth on ethanol and under aerobic fermentation made it possible to assess its structure by PAG electrophoresis (Fig. 3). As can be seen from Fig. 3, polyP5 is a high-molecular compound with an average chain length of more than 45 orthophosphoric acid resides. Further study will contribute to elucidation of the metabolic peculiarities of this polyP.

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Table 2. Phosphorus compounds of the acid-soluble fraction, mg P/g of dry biomass

Conditions	P_i	P of polyP	P of di- and trinucleotides	P of mononucleotides
Glucose, hypoxia	3.14	6.34	0.97	0.86
Glucose, intensive aeration	2.84	4.58	0.16	1.14
Ethanol, intensive aeration	2.32	3.30	0.05	0.96

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