

COMPARATIVE STRUCTURAL ANALYSIS OF YEASTS
CANDIDA GUILLIERMONDII NP-4 CULTIVATED WITH AND
WITHOUT NITROGEN SOURCE

K. O. HOVNANYAN^{1*}, H. V. GASPARYAN^{1**}, S. V. MARUTYAN^{2***},
L. H. NAVASARDYAN^{2****}, A. H. TRCHOUNIAN^{2*****}

¹ *Scientific Technological Center of Organic and
Pharmaceutical Chemistry, NAS of RA*

² *Chair of Biochemistry, Microbiology and
Biotechnology YSU, Armenia*

Comparative study of yeast growth and morphology of cells in nitrogen containing medium and under nitrogen starvation conditions has not been done before. The aim of this study was to investigate the ultrastructural changes in yeasts *C. guilliermondii* NP-4 cultivated with and without nitrogen. The yeast biomass cultivated under nitrogen starvation conditions has been twice lower, than in case of growth in nitrogen containing medium. Ultrastructural analysis of cells, carried out with scanning electron microscope, showed morphological changes in cells. A negative correlation of biomass and cell size was observed.

Keywords: *Candida guilliermondii*, yeast growth, nitrogen starvation, scanning electron microscopy, cell morphology.

Introduction. The yeast cells, especially *Candida guilliermondii*, which taxonomy, virulence and pathogenicity have been investigated in recent years in details, are considered as uncommon yeasts, the incidence of which appears as low even among compromised hosts. Although these yeasts show a reduced innate virulence compared with *Candida albicans*, their role as an agent of serious pathologies (mostly fungaemia and deep-seated infections in cancer patients) has been emphasized throughout the literature [1].

The biochemical studies of protein requirement and flow in the yeast cultures have revealed deep qualitative and quantitative changes occurring in cells [2]. In protein flow nitrogen is required for yeasts cultivation however its requirement and consequences are also important to reveal structural features, viability and survival of the yeasts. In different protein fractions, moreover, effects of nitrogen on gene expression are still poorly studied [3, 4]. The molecular biological study of yeasts in the practical aspect is also highlighted by the role of temperature fluctuations in biotechnology and food industry, for example, in wine fermentation [5].

* E-mail: hovkarl@mail.ru

*** E-mail: marsed@ysu.am

***** E-mail: trchounian@ysu.am

** E-mail: hrachikgasparyan@mail.ru

**** E-mail: l.navasardyan@ysu.am

Yeasts are able to use a wide variety of compounds as a carbon and nitrogen source. Nitrogen is one of the main elements found in many macromolecules of living organisms, playing a central role in structure and function, and most organisms have elaborate control mechanisms to provide a constant supply of nitrogen. Thus, a substantial proportion of cellular activity is concerned with procuring and assimilating of nitrogen. It has been observed that ammonia, asparagine, glutamine and glutamate are preferentially used by yeasts. When these primary nitrogen sources are absent, or present in concentrations low enough to limit growth, other nitrogen sources such as nitrite, nitrate, amides, amino acids and peptides can be used. The utilization of secondary nitrogen sources requires the synthesis of specific-catabolic enzymes and permeases, the expression of which is highly regulated by a process known as nitrogen catabolic repression. In nature, carbon and/or nitrogen compounds occur in diverse and complex structural compositions, such as polysaccharides and proteins. Brewer's wort is an example of a typical complex environment where the yeast has to adapt its metabolism during the course of fermentation. Wort contains the sugars sucrose, fructose, glucose, maltose, maltotriose, dextrin material, as well as a complex mixture of amino acids, peptides, proteins, vitamins, ions, nucleic acids and other constituents. In order to select the best options out of the large diversity of available nitrogen and carbon sources, the yeast has developed molecular mechanisms of sensing and regulation, which include induction and repression of key systems [6].

Both the nitrogen composition and level had significant impacts on the yeast biomass accumulation, ethanol production, and free amino nitrogen and sugars consumption rates. Worts supplemented with wheat gluten hydrolysates increased 11.5% of the biomass, 5.9% of fermentability, and 0.6% of ethanol content and decreased 25.6% of residual sugar content during wort fermentation. Moreover, yeast cells assimilated peptides with various molecular weights (MW) differently during fermentation. Peptides with MW below 1 *kDa* decreased quickly, and the rate of assimilation was more than 50% at the end of fermentation, while those with MW above 10 *kDa* almost could not be assimilated by yeast. So, peptides with MW below 1 *kDa* were one of preferred nitrogen sources for yeasts [7].

However, correlation between yeasts growth and biomass formation and cell morphology in nitrogen containing medium and under nitrogen starvation conditions has been not known yet.

The purpose of this work was to find out the specific ultrastructural changes of yeasts *C. guilliermondii* incubated in mediums with and without nitrogen.

The results obtained showed significant differences in biomass yield and cell morphology and sizes under the mentioned conditions.

Materials and Methods.

Yeast Growth and Biomass Determination. Yeasts *C. guilliermondii* NP-4 (wild type lab culture) were incubated on plates with 2% wort-agar, then yeast biomass was obtained by their growth in the liquid medium (17.5 *mM* $\text{NH}_4\text{H}_2\text{PO}_4$, 3.8 *mM* $(\text{NH}_4)_2\text{HPO}_4$, 0.6 *mM* K_2SO_4 , 0.8 *mM* MgSO_4 , 100 *mM* glucose, pH 5.5) on a shaker (200–250 *rpm* medium). The yeasts were grown at 30°C under 4000 *Lux* of light during 24 *h*. The yeast biomass was isolated by centrifugation (3500 *g*, 10 *min*). The amount of biomass was determined by nephelometric method using the spectrophotometer GENESIS 10S UV-VIS (Thermo Fisher Scientific Inc., USA) at wavelength of 590 *nm*. For investigation of yeasts growth under nitrogen starvation

conditions liquid medium, which contains only 100 *mM* glucose and does not contain any sources of nitrogen ($\text{NH}_4\text{H}_2\text{PO}_4$, $(\text{NH}_4)_2\text{HPO}_4$) or other salts was used [8]. For comparison of obtained data, yeasts were incubated also in distilled water.

Electron Microscopy Study. Preparations for image analysis of yeasts by electron microscopic methods were done by fixing in 2.5% glutaraldehyde prepared in 0.1 *M* cacodylate buffer (pH 6.8–7.0). Then, after fixation by 1% osmium tetroxide prepared in 0.1 *M* cacodylate buffer (pH 6.8–7.0) [9] being coated by vacuum-evaporations the sample surfaces were covered with a thin layer of aluminum. Specimens treated by the mentioned technique were observed with [10, 11]. Scanning electron microscopy (SEM) imaging analysis was performed using the digital program system “Morphology-Tesla” in SEM Vega Tescan, Program AxioVision and of post hoc test.

Reagents and Data Processing. The reagents of analytical grade were used throughout; glucose was from Reanal (Hungary).

Each experiment was repeated at least five times; error bars were presented on figures. Standard errors such as standard deviation were calculated using appropriate function of Microsoft Excel 2013. The area of ellipsoid cells was calculated by equation $S=\pi ab$, where S is an area of ellipsoid cell, a is a length of cell, b is a diameter of cell. The changes between data were validated by calculation of Student’s validity criteria (P); the differences between experiments (starved and non-starved cells) were valid if $p<0.05$.

Results and Discussion.

Yeasts Growth in Nitrogen Containing Medium and under Nitrogen Starvation Conditions. For investigation of yeast growth in nitrogen containing medium and in conditions of nitrogen starvation, three different kinds of medium were used: nitrogen containing medium (see Materials and Methods), and two kinds of nitrogen starvation medium: the medium of 100 *mM* glucose (without of nitrogen sources), and distilled water (without nitrogen and carbon sources; for comparison). After 24 *h* growth in these mediums it has been shown that the yeast biomass yields were different (Fig. 1). In nitrogen containing medium, with about 20 *mM* of nitrogen, 100 *mM* carbon and 0.6–0.8 *mM* mineral salts, the biomass of yeasts was the highest – 2 *mg/mL*.

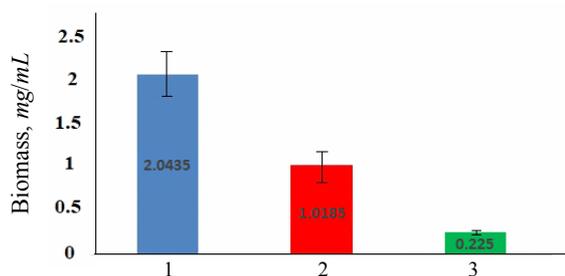


Fig. 1. The amounts of biomass of yeasts *C. guilliermondii* NP-4 in different growth mediums: 1 – nitrogen containing medium; 2 – medium containing only 100 *mM* glucose; 3 – distilled water (for comparison).

In case of nitrogen starvation medium, which contains only 100 *mM* glucose and does not contain any sources of nitrogen or mineral salts, there was the 2-fold decrease in the amount of accumulated biomass, and it was only 1 *mg/mL*. For comparison of yeast growth data, we have incubated yeasts in distilled water, which contains neither nitrogen, nor carbon or mineral salts

sources. Yeast biomass amount in this case was very small: only 0.225 *mg/mL*. We suggest that in distilled water the yeast growth was limited by internal resources of yeast cells and was interrupted when the cell resources of nitrogen and carbon were

exhausted. Since then the growth of yeast has stopped, so it has resulted in about 10 times less biomass than in nitrogen-containing medium and about 5 times less than in nitrogen starvation conditions.

Ultrastructural Analysis of Yeast Grown in Nitrogen Containing and Nitrogen Starvation Mediums. SEM of *C. guilliermondii* NP-4 cells cultivated in nitrogen containing medium has shown typical ultrastructural images for yeast cells, the clarification of the intercellular contacts zones was cell wall and fastening to the inorganic plate substrate. The yeast cells both are rounded and ellipsoidal in shape, the length is $4.21 \pm 0.3 \mu\text{m}$ and the diameter is $2.29 \pm 0.1 \mu\text{m}$ (Fig. 2). These data are in good correlation with our previous results [10, 11].

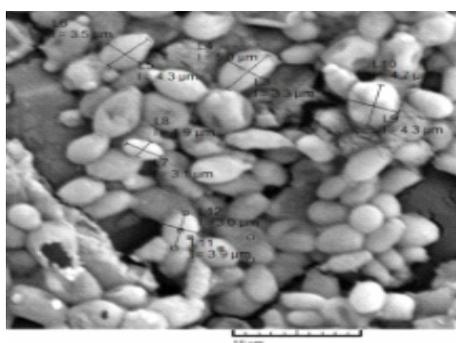


Fig. 2. SEM image of cells *C. guilliermondii* NP-4 cultivated in nitrogen containing medium. Scale bar was $10 \mu\text{m}$ (for details, see the text).

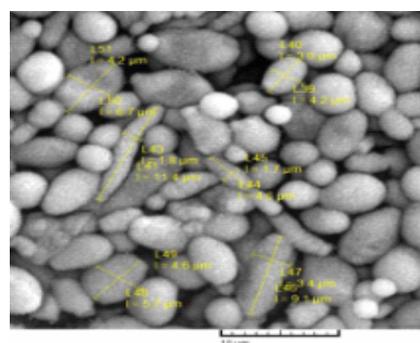


Fig. 3. SEM image of *C. guilliermondii* NP-4 cultivated under nitrogen starvation conditions. The growth medium contains only 100 mM glucose solution in distilled water.

The *C. guilliermondii* compliant SEM examination when yeasts were grown in 100 mM glucose (nitrogen starvation) medium has shown the presence of cells with the diameter of $3.02 \pm 0.1 \mu\text{m}$ and length of $7.99 \pm 0.30 \mu\text{m}$ (Fig. 3).

In distilled water medium (without any sources of nitrogen and carbon) the yeasts have been presented as giant vacuole-containing deformed cells with diameter of $4.37 \pm 0.2 \mu\text{m}$ and length of $6.75 \pm 0.3 \mu\text{m}$ (Fig. 4).

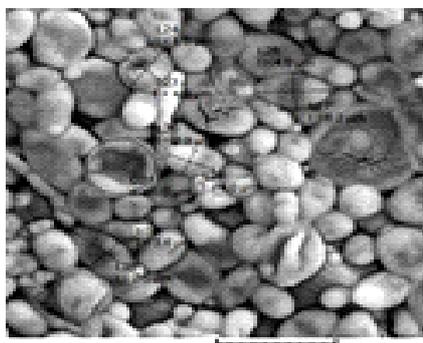


Fig. 4. SEM image of *C. guilliermondii* NP-4 yeasts incubated in distilled water (nitrogen and carbon starvation medium).

In many studies scientists found differences in nitrogen-related curves (e.g. curves of biomass versus metabolizing nitrogen) which indicated that the fermentation rate and biomass yield functions are distinct.

Nitrogen and carbon are the main nutrients in nature, and this implies that the mutual interaction of these nutrients plays an important role in the metabolism of living organisms. It has been shown that the structural complexity of the nitrogen source, in correlation with sugar concentrations, greatly affects the fermentation performance of both baking and brewing yeast strains [6]. Quantitatively, nitrogen is the second most abundant nutrient, which is essential for yeast metabolism and growth. Consequently, a lack of nitrogen triggers sluggish fermentations.

In other studies, the researchers associated nitrogen deficiency with a high sugar transporter turnover rate, which resulted in a loss of sugar uptake capacity in the cells. The cellular membrane is the primary region for controlling sugar uptake. It is not possible to distinguish clearly between the effect of nitrogen on the fermentation rate and the effect of nitrogen on the biomass yield as the two effects are interdependent. It has been proposed that the rate of fermentation is indeed a two-component function comprising an intracellular component (a property of the cell metabolism) and a cellular component (which is dependent on the mass of cells actively fermenting) [5]. When the nitrogen source in the medium becomes depleted, protein and nucleic acid synthesis ceases but excess carbon continues to be metabolized to lipid. Under such conditions, non-oleaginous yeasts do not accumulate lipid. This states that intra-mitochondrial citrate is accumulated primarily due to the decrease in intracellular AMP concentration, which leads to a decline in activity of the AMP-dependent NAD^+ : isocitrate dehydrogenase in mitochondria. Citrate is then transported across the mitochondrial membrane in exchange for L-malate and is cleaved in the cytosol by ATP: citrate lyase to yield acetyl-CoA (and oxaloacetate), from which fatty acids are synthesized [12].

Results obtained by us show that during incubation of yeasts without any nitrogen source in incubation medium the morphological changes take place in yeast cells, in particular, occur some changes in the size of cells (see Table): the length of cells increases about 1.9 and the diameter increases 1.3 times. So the yeast cells are “swelled” and, possibly, form giant cells. In yeast cell population in this case there are also filamentous forms of cells. We have observed similar changes in the morphology of X-irradiated yeast cells [10]. On this base we can conclude that such morphological changes are the total reaction response of the yeasts to different external stress factors.

In case of incubation of yeasts without nitrogen and carbon sources (in distilled water), the cell size increase is more expressed: the length of cells increases by 1.6, and the diameter increases 1.9 times as compared to the yeasts grown in normal medium. In comparison with nitrogen-starved yeasts, the cell length during incubation of yeasts in distilled water is decreased 1.1 times, and the diameter is increased 1.4 times, so the yeast cells become more rounded. The results of calculations have shown that during starvation the area of ellipsoid yeast cells is increased: for yeasts incubated in normal conditions it is equal to $30.3 \mu\text{m}^2$, for nitrogen starved yeasts it is equal to $75.8 \mu\text{m}^2$ and for yeasts incubated in distilled water it is equal to $92.25 \mu\text{m}^2$.

Cell sizes of yeasts C. guilliermondii NP-4 incubated in different growth mediums ($p < 0.05$)

Incubation medium	Cell length, μm	Cell diameter, μm	Cell area, μm^2
Nitrogen containing medium	4.21 ± 0.3	2.29 ± 0.1	30.3 ± 0.7
100 mM glucose	7.99 ± 0.2	3.02 ± 0.1	75.8 ± 0.9
Distilled water	6.75 ± 0.3	4.37 ± 0.2	92.25 ± 0.91

Summarizing the results obtained by us, we can conclude that the increase in cell size during starvation is not accompanied by the increase of yeast biomass, i.e., the magnification of the cells probably breaks their division, as the metabolic processes in yeasts are being violated, and the negative correlation between the yeast cell size and accumulated biomass is observed.

It has been shown that by restoration of normal nutrition of nitrogen starved yeasts the metabolic activity of cells, including the protein synthesis, gradually is recovering [13]. From this point of view, it is interesting to study the morphological changes in yeast cells after restoration of normal nutrition.

Conclusion. Yeasts *C. guilliermondii* have formed significantly different (2-fold at least) biomass during the growth in nitrogen containing and nitrogen starvation conditions. SEM study of yeasts cultivated in different growth mediums visualized cellular changes in culture populations in the forms of longitudinal ones and vacuolization breaking the cell division process. The electron microscopy analysis results identified the changed cell morphology, inherent in the effect of stress factors. The results can be used in biotechnology to obtain greater biomass for feeding yeast, as well as in medicine, especially in relation to the choice of various dietary restrictions and the dangers to human beings in these diets.

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