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INFLUENCE OF PHYSICAL FACTORS ON LOGARITHMIC, QUIESCENT AND NON-QUIESCENT *S. CEREVISIAE* CELLS

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ABSTRACT

The influence of environmental conditions on eukaryotic cells, including Saccharomyces cerevisiae, has been the subject of numerous scientific studies. Among the most important physical factors affecting cellular growth are temperature, pH, UV, osmotic pressure, mechanical, gravitational force, and ultrasound. Furthermore, those physical parameters refer to the abiotic factors of an ecosystem. They affect living organisms and can have either stimulating or negative effects on cells. In this respect, quiescent (Q), non-quiescent (NQ), and logarithmic cells (Log) of S. cerevisiae were used as a model system to assess the effect of different physical stresses on cellular survival rate. Obtained results revealed that cells in the G_0 state are more resilient to various physical impacts from the environment. At the same time, logarithmic and NQ cells showed from 10 % to 90 % lower resistance depending on the physical factor. Moreover, the complex action of various abiotic factors on a living cell determines its biological path, namely entering different phases of the cell cycle, triggering proliferation, or formation of resting forms, which directs its adaptability to changed environmental conditions.

Keywords: physical factors, yeast, quiescence, non-quiescence, logarithmic growth.

INTRODUCTION

Yeasts are ubiquitous microorganisms that are part of the microbiota of the majority, if not all, natural habitats. Numerous different yeast species can be found in soil, freshwater, and marine environments, as well as in different associations with plants and animals [1, 2]. The metabolic activity, growth, and survival of yeasts are determined by the environmental factors present in these natural and artificial habitats. The life of yeasts is influenced by a wide range of abiotic and biotic variables, which create stress conditions that the cells must endure and adapt to in order to survive. Light, temperature, pH, free water, pressure, sound, radiation, gravitational force, chemical compounds, and others are among the most crucial abiotic environmental elements. Their intricate influence on a living cell shapes its biological course, determining when it enters one or other cellular phases of the life cycle: starts to proliferate or enters quiescence [3, 4]. Abiotic environmental factors also influence how well a cell can adapt to changing environmental conditions and, in some extreme cases, can result in irreparable harm to the cellular systems and the death of the organism [1 - 4]. The mechanisms by which yeast cells respond to environmental influence have been thoroughly investigated in logarithmically grown populations and compiled in a number of reviews [5 - 7]. The majority of wild yeast cells, however, spend part of their lives in quiescence a momentary non-proliferating condition, and little is known about how the response to environmental influence is altered based on the specific cellular state [8, 9]. Moreover, having a basic understanding of these is crucial for comprehending the ecology and biodiversity of yeasts as well as for managing environmental conditions to improve the exploitation of yeasts or to prevent or suppress their

detrimental and destructive activities. Therefore, populations of quiescent (Q), non-quiescent (NQ), and logarithmic cells of *S. cerevisiae* were used as a model to examine the deleterious effect of these exogenous stressors (temperature, pH, UV, osmotic pressure, mechanical, gravitational force, and ultrasound) with subsequent assessment of cell viability.

EXPERIMENTAL

Microorganisms and growth conditions

Yeast strain *Saccharomyces cerevisiae* BY4741 (*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*) was obtained from the EUROSCARF Frankfurt collection (Germany). Yeast cells were grown on a liquid YPD medium at 30°C on a reciprocal shaker for 168 hours. Samples were withdrawn at exponential (12 hours) and late stationary phase (168 hours). The biomass was harvested by centrifugation at 2 740 x g for 10 min at 4°C. The pellet was washed twice with distilled water and used for further analyses.

Isolation of quiescent (G_0/Q) and non-quiescent (NQ) cells

Isolation of Q and NQ *S. cerevisiae* BY4741 stationary phase yeast cells (168 h) was performed in Percoll density gradient according to the protocol described by Allen [10]. The density gradient was formed from Percol and 1.5 M NaCl in ratio 9:1 (v/v) and further centrifugation at 19 240 x g for 15 min at 20°C. Yeasts biomass in stationary phase was suspended to OD_{540} = 200 (2 x 10^9 cells/mL) in 0.1 M Tris-HCl buffer, pH 7.5. The obtained suspension was layered on to the formed gradient and subsequently centrifuged at 400 x g for 60 min at 20°C. The resulted two layers of cell fractions - the denser one composed of G_0 (Q) cells (lower fraction) and less dense fraction of NQ cells (upper fraction) were separately isolated. Both cell fractions were washed with 0.1 M Tris-HCl buffer solution with pH 7.5 and stored at 4°C.

Survival rate analysis

The effect of different physical stresses was assessed using *S. cerevisiae* cell suspensions with OD_{540} = 1.0. The percentage of surviving cells in all experiments was determined based on the colony-forming unit counts (CFU/ml). Untreated cells were used as controls on YPD solid media.

Temperature stress

To determine the effect of temperature extremes on yeast viability, cells were incubated for 20 minutes at different temperatures: -5°C, 4°C, 30°C, 50°C and 70°C.

pH stress

To apply alkaline and acid stress, cells were suspended in 0.2 M glycine adjusted to pH 2.0 with 1 M HCl, and in 0.1 M TRIS adjusted to pH 10.0 with 1 N NaOH followed by incubation for 30 and 60 min at room temperature.

Osmotic stress

The effect of mild osmotic stress was studied after cells were suspended in 0.4 M and 0.7 M sodium chloride solution, then incubated for 20 min at room temperature. Furthermore, the outcome from hyperosmotic and hypoosmotic stress was also analysed. The hyperosmotic stress was achieved through the treatment with 30 %, 50 % and 70 % sucrose and 40 %, 60 % and 87 % glycerol for 1, 2, 3, and 4 hours, respectively. The hypoosmotic shock was simulated after resuspending the yeast cells in sterile water and incubating them for 1, 2, 3, 4, 24, and 48 hours.

UV radiation

The effect of UV rays was determined after exposure of yeast cells for 1, 3, 5, 7, 10, 15, 20, 30, 40, and 60 min at UV irradiation intensity (wavelength 254 nm) of 200 mW cm^{-2}. To stop photoreactions, suspensions were kept in the dark for 24 h.

Gravitation stress

The application of the gravitational force (3 000 x g, 30 000 x g and 50 000 x g) on cells was carried out by centrifugation for 1, 2, 3, and 4 hours.

Mechanical stress

The effect of mechanical force on cellular survival rate was studied after using a Bullet Blender homogenizer, and the cells were treated with glass beads (425 - 600 μm) in a 1:2:1 ratio (biomass : 0.05 M K-Na phosphate buffer, pH 7.8 : beads) for different periods of time - 1, 2, 3, 4, 5, 6 minutes.

Ultrasound stress

A Sonifier® Cell Disruptor (Branson, USA), model

SLPe equipped with a 3,2 mm diameter tip was used to study the effect of ultrasound. The three cell populations were sonicated for defined periods of time (1, 3, 5, 10, and 15 minutes) at an amplitude of 40 % and a frequency of 40 kHz.

Data analysis

Used data represent the mean values with Standard error of the mean (± SEM) of three independent experiments. The statistical analysis was performed using MICROSOFT OFFICE 365 EXCEL 2020 software. Differences in means were analysed using Student's t-test with independent measures. Differences were considered statistically significant at the $p < 0.05$ level.

RESULTS AND DISCUSSION

Temperature stress

Temperature is one of the most important physical factors affecting the growth and development of microorganisms. With this respect, the effect of several supra- (- 5°C and 4°C) and super optimal (50°C and 70°C) temperatures have been analysed. As a control, growth at 30°C has been used. From the results obtained (Fig. 1), it was obvious that Q cells were most resistant at the tested temperatures between -5 and 50°C. They retain more than 50 % of their viability for 20 min at 50°C. Similar data have been obtained for the low temperatures tested - more than 60 % survival rate after treatment with sub-zero temperatures. In comparison, NQ cells were two times more susceptible to both low and high temperatures applied. Similarly, scientists reported that sensitivity to heat shock was related to the growth phase: cells in the stationary phase showed higher resistance to temperature imbalance compared to those growing in the exponential phase [5].

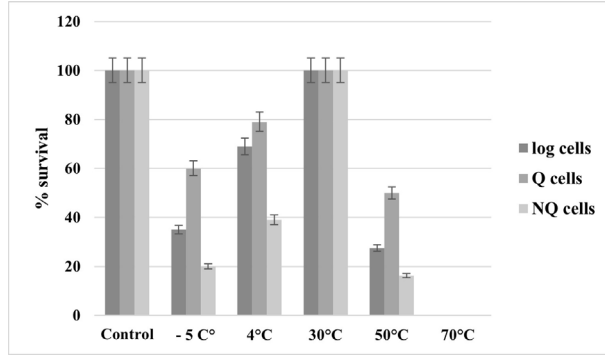

Fig. 1. Influence of temperature on Log, Q and NQ cell populations of *S. cerevisiae*.

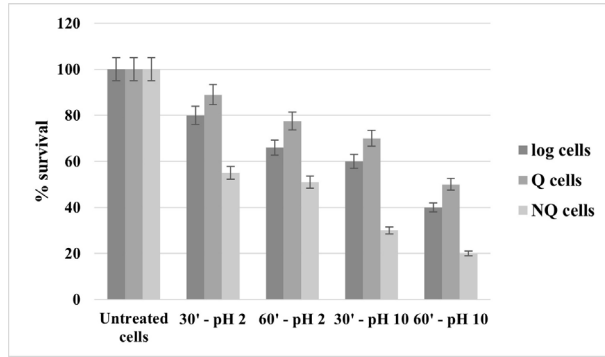

Fig. 2. Influence of pH on Log, Q and NQ cell populations of *S. cerevisiae*.

pH stress

Another important physicochemical factor greatly affecting cellular growth is the exogenous pH. Investigations of cell growth at different extreme values of pH (Fig. 2) showed that the highest percentage of survival was again observed for cells in the quiescence state, which is most likely due to their better resistance to adverse conditions. The observed higher survival percentage at acidic pH is consistent with literature data clearly describing that most strains of *S. cerevisiae* grow at pH between 2.5 and 8.5 but are inherently acidophilic microorganisms and grow better in acidic environments [11]. In cells derived from the exponential phase, lower survival was observed, which is assumed to be related to the higher metabolic rate and the corresponding higher susceptibility of their enzymes to the altered pH values [12]. Apoptotic NQ cells were with the lowest survival, most likely due to their nature – they retain viability but rapidly lose their ability to replicate, with ~ 50 % of viable cells unable to form daughter progeny [13].

Osmotic stress

After the application of hypoosmotic stress (Fig. 3), a nearly 50 % drop in the viability of NQ cells was observed in just four hours. Compared to them, the other two cellular populations showed 100 % survival even after 48 hours of incubation in distilled water. Studies involving *S. cerevisiae* have shown that a significant loss of viability following hypoosmotic shock occurs in cells that are defective in osmolyte export [14]. These

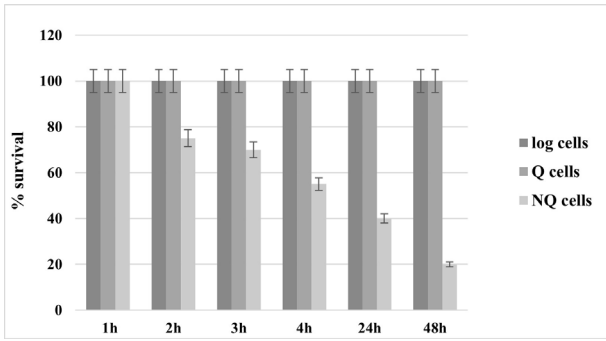

Fig. 3. Influence of hypoosmotic stress on Log, Q and NQ cell populations of *S. cerevisiae*.

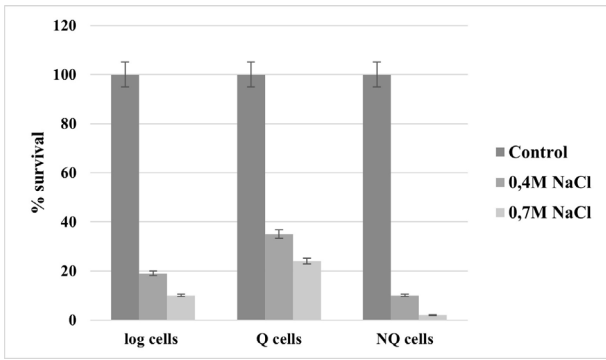

Fig. 4. Influence of hyperosmotic stress with 0.4 M and 0.7 M NaCl on Log, Q and NQ cell populations of *S. cerevisiae*.

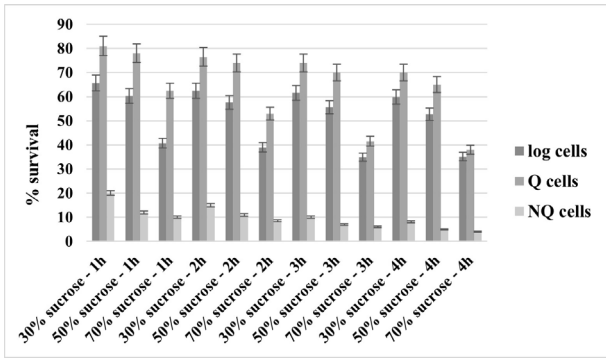

Fig. 5. Influence of hyperosmotic stress with sucrose on Log, Q and NQ cell populations of *S. cerevisiae*.

mutations generally affect the protein kinase C (PKC) signalling pathway, leading to weakened cell wall strength [15]. However, the effective functioning of the same signalling pathway and the formation of a tick cell wall is an important prerequisite for entering into the G_0 state. This makes the Q cells more resistant to the action of a number of lytic enzymes as well as to hypoosmotic stress conditions [16].

A commonly used osmolyte in hyperosmotic stress experiments is sodium chloride. Moreover, the intracellular glycerol concentration was found to increase in parallel with the external NaCl concentration [17]. In general, these elevated concentrations in the intracellular glycerol may result from upregulated glycerol synthesis, increased retention by cytoplasmic membranes, or decreased dissimilation or uptake of glycerol from the environment. Glycerol is synthesized during glycolysis by the reduction of dihydroxyacetone phosphate to glycerol 3 -phosphate by glycerol 3-phosphate dehydrogenase (GPD) [18 - 20]. Under osmotic stress, glycerol levels increase due to the enhanced action of GPD. Following the application of 0.4 M and 0.7 M NaCl, a trend towards the highest percent survival in Q cells was observed (Fig. 4), which correlates with claims that yeast accumulates trehalose (to survive adverse conditions) when starved for carbon, nitrogen, sulphur or phosphorus [21].

Different concentrations of sucrose (30 %, 50 %, 70 %) were also used to determine how the hyperosmotic stress affected the survival of proliferating, Q and NQ cells. During these investigations all the three cellular populations of *S. cerevisiae* BY4741 partially retained their viability after 4 hours of treatment. The sharpest drop in cell survival was reported 1 h after the beginning of the experiment, with about a 35 % decrease in cellular survival for the Log cells incubated in 30 % sucrose solution and about 60 % dying for those in 70 % sucrose solution (Fig. 5). Under the same conditions, 80 % of the apoptotic NQ cells lost their viability after treatment with 30 % sucrose, while in a 70 % sucrose solution, nearly 90 % of the cells died. However, Q cells maintained their viability within 81 % and 62.5 % in 30 % and 70 % sucrose solution, respectively. During the next 3 h, the trend of decreasing viability of the three types of cellular populations persisted, with survival decreasing smoothly. Generally, sucrose had a more favourable effect on all three cell populations, and higher concentrations were found to slow down the rate of fermentation [22].

Further, the role of osmotic stress on cellular survival was analysed by using different concentrations of glycerol (40 %, 60 %, 87 %). The results again showed (Fig. 6) a higher resistance to applied stress in Q cells compared to the NQ and exponential ones. However,

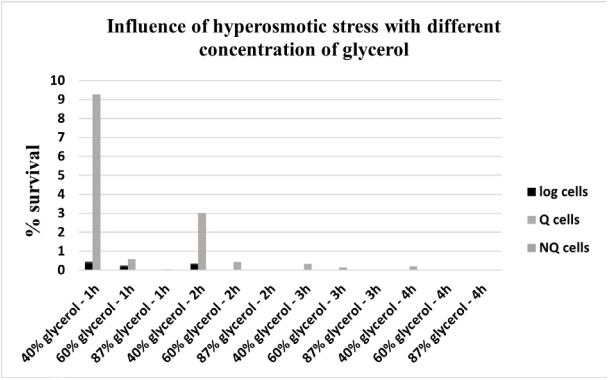

Fig. 6. Influence of hyperosmotic stress with glycerol on Log, Q and NQ cell populations of *S. cerevisiae*.

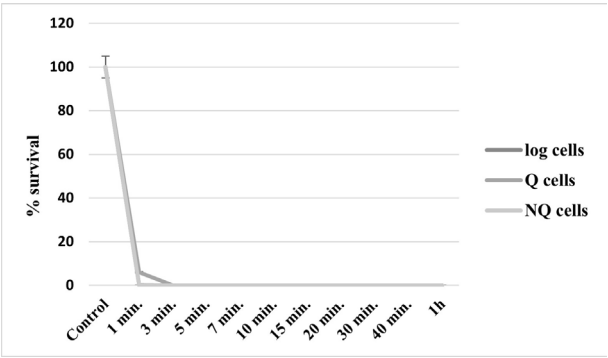

Fig. 7. Influence of UV rays on Log, Q and NQ cell populations of *S. cerevisiae*.

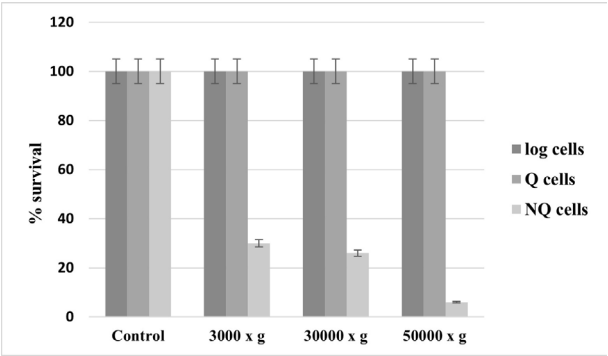

Fig. 8. Influence of gravitational force on Log, Q and NQ cell populations of *S. cerevisiae*.

viability drops sharply after treatment of the cells with 40 % glycerol, and in the first hour only 9.3 % survival was observed, even for the Q cells. As the time for exposure to glycerol increases, the tendency to lose viability persists. Q cells retained minimal survival rate up to 4 h at concentrations of 40 % and 60 % glycerol. This probably is due to the fact that at high glycerol concentrations in the environment, a significant role in maintaining cell viability is played by the HOG1 regulatory pathway, by which glycerol is synthesized *de novo* in the cell, but when cell-toxic levels are reached, yeast possess an exporter - Fps1p, which leads to the release of glycerol into the environment [23].

UV, gravitational, mechanical and ultrasonic stress

Direct exposure of yeast cells to UVC_{254} rays exerts a powerful fungicidal effect on them. Even after the first minute of exposure, almost 100 % growth inhibition was observed in all three cell populations, except for cells in a quiescence state (6 % survival) (Fig. 7). A 10 - fold lower viability was observed in cells in the exponential phase, which is most likely due to the fact that they are actively proliferating and consequently more susceptible to UV radiation. Thus, the possibility for the occurrence of DNA mutations is greater due to the higher rate of biosynthetic processes.

Similarly, to the results obtained in this study, other authors also reported a complete loss of viability after UVC_{254} treatment of exponential phase cells of *S. cerevisiae* after 9 minutes of irradiation [23]. In sporulating diploid strains of the genus *Saccharomyces*, sporulation is delayed or does not occur due to the presence of multiple lesions (pyrimidine dimers) in premeiotic DNA after UV irradiation [24].

Next, *S. cerevisiae* was used as a model organism to study the response of eukaryotic cells to hypergravity (Fig. 8). After subjecting the cells to 3 000 x g, no inhibition of survival rate was detected in both cell populations (Q and Log), even after four hours of centrifugation. In apoptotic (NQ) cells, viability was more than 3 - fold lower compared to the other two *S. cerevisiae* cell types, as well as compared to controls. Increasing the gravitational force by applying 30 000 x g. and 50,000 x g resulted in an almost complete loss of viability in NQ cells, 26 % and 6 %, respectively. The remaining two cell populations again maintained their survival (100 %) unchanged. The presented data correlates with those available in the literature, which revealed that yeast growth was completely suppressed only after the application of 74 558 x g [25].

Yeast cellular robustness to mechanical stress was tested after subjecting the Log, Q and NQ cells of *S. cerevisiae* BY4741 strain to mechanical disintegration with glass beads. Survival rate (%) was compared to those

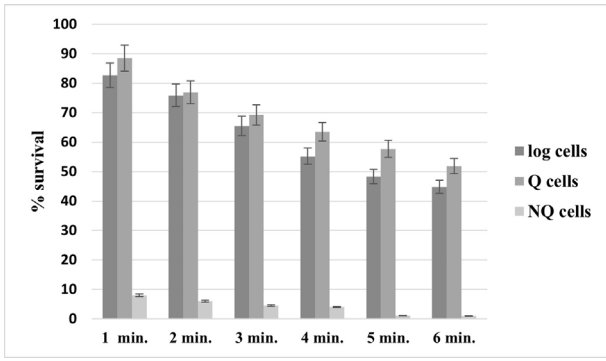

Fig. 9. Influence of mechanical force on Log, Q and NQ cell populations of *S. cerevisiae*.

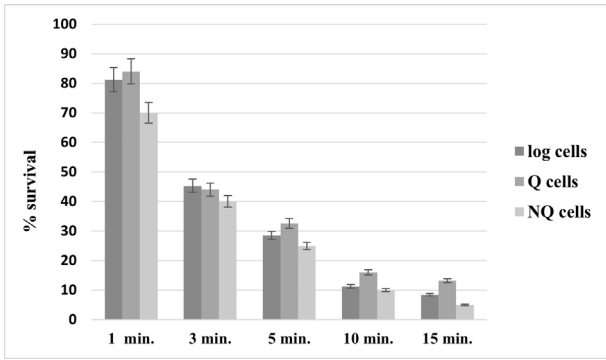

Fig. 10. Influence of ultrasound on Log, Q and NQ cell populations of *S. cerevisiae*.

of the non-disintegrated controls. A large percentage of disrupted cells from the three cell populations were found even after 1 min of disintegration - 17.3 %, 11.5 % and 92 % for Log, Q and NQ cells, respectively (Fig. 9). With each subsequent minute, the percentage of disintegrated cells increases. At the end of the study (6 min), the percentage of surviving cells was as follows: 44.8 %, 51.9 and 1 % for log, Q and NQ cells, respectively. Established higher resistance to mechanical stress in logarithmically grown and quiescent yeast cells is possibly related to the functioning of the *S. cerevisiae* specific mechanic sensors, which are essentially protein molecules [26]. They are mainly located in the cell wall, but their C-terminus binds to the cell membrane and transmits signals in the presence of mechanical stress to other signalling molecules located inside the cell. This leads to the activation of cellular regulatory pathways responsible for cell wall integrity and cell viability preservation (CWI pathway) [27].

Furthermore, the demonstrated higher resistance of Q cells after exposure to mechanical force is probably due to both their thicker cell wall and the observed smaller cellular sizes [9, 28]. According to Solecki, the number of hits on the cell surface depends largely on its dimensions, which in turn determines the extent to which the wall has broken open [29].

The resistance of the three *S. cerevisiae* cellular populations to ultrasound was tested after their sonification for 15 minutes and subsequent reporting of their survival rate as per cent compared to the control of non-sonicated cells (Fig. 10). During the first minute of ultrasound application, the trend for the highest survival was observed for Q cells, followed by Log and NQ, respectively (84 %, 82.5 % and 70 %). These results are in agreement with the other investigations performed, with the sole difference that, in our case, the applied ultrasound of 40 kHz, most likely has a stronger fungicidal effect compared to those applied by other authors [30, 31].

CONCLUSIONS

The growth rate or apoptosis of a given microorganism is influenced by various physical environmental factors. In nature, where many species coexist, fluctuations in environmental conditions cause dramatic changes in microbial ecosystems due to the different growth rates of innate microorganisms. In this regard, the results of this study present insights as to how the yeast cells being in the different stages of the cell cycle, respond to adverse physical stressors. Moreover, obtained data shed further light on the cell biology of yeast quiescence, showing that entrance in G_0 makes yeasts more resistant to rapid changes in the habitats, thus assuring their higher survival rate in adverse environments. Even more, studying the influence of abiotic factors on cellular survival has practical importance giving vital information on the robustness and applicability of different yeast strains for industrial fermentations.

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