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Differences in stationary phase cells of *Saccharomyces cerevisiae* var. *bayanus* grown in aerobic and hypoxic bath cultures assessed by electric particle analysis, light diffraction and flow cytometry

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*Saccharomyces cerevisiae* is a yeast of widely recognized biotechnological interest and is also used as a model to understand the cell cycle progression of eukaryotic cells. In an asynchronously growing *S. cerevisiae* population, individual cells differ in their position (or phase) within the cell division cycle, their genealogical age and their size. All these variables drive the cell size distribution. Because of the tight coupling between cell growth and division, the study of cell size distributions of yeast populations at steady state, or under perturbed conditions, can reveal a wealth of information on the cell cycle regulatory mechanisms and adaptation to the environment.

From different principles, electric particle analysis and light diffraction are two current techniques that permit the obtaining of cell size distributions. In the field of microbiology, electric particle analysis is more usual, whereas light diffraction is used in most abiotic particles. Another current technique used to measure individual parameters, to identify subpopulations and to count microorganisms is flow cytometry. By this technique, the incident light scattered from one cell is collected in two different angles, in a narrow forward angle (Forward Scatter, FS) and in a proximally right angle (Side Scatter, SS) from the light beam. FS is a complex parameter as this function varies not only with cell size but also with cell shape, refractive index and number of intracellular dielectric interfaces. SS is also an intricate parameter. This signal is thought to indicate variations in cell surface or internal structure, usually referred to as “cellular granularity.” In this contribution, we report cell population analyses of *S. cerevisiae* in stationary phase grown in aerobic and hypoxic bath cultures by three experimental techniques: electric particle analysis, laser diffraction and flow cytometry.

The medium used in aerobic conditions contains: 10 g l\(^{-1}\) glucose, 5 g l\(^{-1}\) yeast extract and 3 g l\(^{-1}\) casein peptone, and pH was initially adjusted to 3.5 with orthophosphoric acid. To ensure hypoxic condition the medium was supplemented with 0.5 g l\(^{-1}\) sodium thioglycollate and 0.001 g l\(^{-1}\) resazurine. Both media were autoclaved for 15 min at 121\(^\circ\)C. The inocula, prepared and cultured in the same medium growing in aerobic and hypoxic conditions, respectively, were inoculated in 1000 ml flasks with 500 ml of the fresh medium and incubated at 27\(^\circ\)C, using magnetic shaking (150 r.p.m) for approximately 60 hours. Along this time, and after the steady state was confirmed, samples were removed to be analysed using an electronic particle analyser, a laser diffraction particle size analyzer and a flow cytometer.

Multiziter data shows that the cells under hypoxic conditions are greater (means from 4.93 to 5.23 µm) with size distributions moving to the left along the stationary phase, whereas cells grown aerobically are smaller (means from 4.46 to 4.67 µm) and with more stable size distributions. Although cell size distributions obtained by light diffraction show a different shape (slightly positively skewed with no left tail), the use of laser diffraction confirms that the cell sizes in aerobic conditions are smaller (means from 4.50 to 4.66 µm) than cells grown in hypoxic medium (means from 4.98 to 5.00 µm). However, with this method we observe that the stationary phase is not stable in aerobically grown cells, and there is a greater variability in cell size distributions from the two-hundred sample to sixty-hour sample. Basic shape of FS distributions shows a similarly basic shape in both growth conditions, centered in a range of relative intensities between 3500-3700 with a long right tail. In addition, cells grown in hypoxic conditions show a subset with relative frequencies between 4500 and 6000, whereas in aerobic conditions there is a higher proportion of cells with relative intensities between 6000 and 10000. This is consistent with the existence of two subpopulations, not observed in previous techniques. The two subpopulations show more differences in aerobic conditions than in hypoxia. Moreover, the subpopulations in aerobic conditions join at the advanced stationary phase. Similarly, SS distributions in both experiments are essentially the same: a triangular distribution slightly positively skewed and centered at relative intensities between 4000-4200. However, in this measure one can also observe a subpopulation of yeast cells with relative intensities between 6000 and 10000 in aerobic conditions but not in hypoxic conditions. Therefore, it is shown that in the stationary phase of culture in hypoxic conditions the population is more homogeneous than under the aerobic cultivation.

Dissecting Gene Expression in Micro-Colonies of *Aspergillus niger*

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The filamentous fungus *Aspergillus niger* forms centimeter scale macro-colonies on solid media, whereas (sub)millimeter micro-colonies are formed within a shaken culture. These colonies secrete large amounts of proteins to degrade polymers in the medium into compounds that can be taken up to serve as nutrients. Previously, it has been shown that macro-colonies of *A. niger* that had been grown on solid medium are heterogeneous with respect to gene expression and protein secretion. Here, we assessed whether heterogeneity can also be found between and within micro-colonies of a liquid shaken culture of *A. niger*.

Micro-colonies of *A. niger* strains expressing GFP from the promoter of the glucose-1,6-lactate and the furanolic acid esterase gene *faeA* were sorted on basis of their diameter and fluorescence of the reporter protein using the Complex Object Parametric Analyzer and Sorter (COPAS). Fluorescence intensity of the reporter correlated with the diameter of the micro-colonies. Both the distribution of fluorescence intensity and the micro-colony diameter were not normally distributed in the culture. 25% of the culture consisted out of smaller micro-colonies while 75% consisted out of larger micro-colonies. 27% and 73% of the micro-colonies of the strain expressing GFP from the *faeA* promoter were lowly and highly fluorescent, respectively. This implies that heterogeneity in this strain depends on the volume of the micro-colony only. In contrast, the lowly fluorescent micro-colonies of the strain expressing GFP from the *gladA* promoter comprised about 79% of the culture. This indicates that heterogeneity in *gladA* expression is not only determined by volume of the micro-colony but also by an unknown other factor.

Assess heterogeneity within a 750-800 µm wide micro-colony, central and peripheral parts of the mycelium were isolated by laser microdissection and pressure catapulting (LMPC). QPCR showed that *glaA* and the *faeA* promoter comprised about 79% of the culture. This indicates that heterogeneity in *glaA* expression is not only determined by volume of the micro-colony but also by an unknown other factor.

Therefore, it is shown that in the stationary phase of culture in hypoxic conditions the population is more homogeneous than under the aerobic cultivation.

**Keywords** Yeast populations; Cell size distribution; Electric particle; Light diffraction; Flow cytometry analysis; Stationary phase