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ORIGINAL PAPER

Differences in stationary-phase cells of a commercial *Saccharomyces cerevisiae* wine yeast grown in aerobic and microaerophilic batch cultures assessed by electric particle analysis, light diffraction and flow cytometry

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Abstract We applied electric particle analysis, light diffraction and flow cytometry to obtain information on the morphological changes during the stationary phase of *Saccharomyces cerevisiae*. The reported analyses of *S. cerevisiae* populations were obtained under two different conditions, aerobic and microaerophilic, at 27°C. The samples analysed were taken at between 20 and 50 h from the beginning of culture. To assist in the interpretation of the observed distributions a complexity index was used. The aerobically grown culture reached significantly greater cell density. Under these conditions, the cell density experienced a much lower reduction (3%) compared with the microaerophilic conditions (30%). Under aerobic conditions, the mean cell size determined by both electric particle analysis and light diffraction was lower and remained similar throughout the experiment. Under microaerophilic conditions, the mean cell size determined by electric particle analysis decreased slightly as the culture progressed through the stationary phase. Forward and side scatter distributions revealed two cell subpopulations under both growth conditions. However, in the aerobic growing culture the two subpopulations were more separated and hence easier to distinguish. The distributions obtained with the three experimental techniques were analysed using the complexity index. This analysis suggested that a complexity index is a good descriptor of the changes that take place in a yeast population in the stationary phase, and that it aids in the discussion and understanding of the implications of these distributions obtained by these experimental techniques.

Keywords Aerobic and microaerophilic growth · Yeast stationary phase · Cell size distributions · Flow cytometry

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Introduction

Saccharomyces cerevisiae is a eukaryotic organism capable of growing aerobically, in low oxygen concentration and in anaerobic conditions. Both aerobic and anaerobic growing conditions have importance at the industrial level for production of bioethanol, cell biomass and proteins, among other substances, for example squalene and β-glucan [9, 18]. Comprehension of respirofermentative growth is important to optimise the different biotechnological processes in which this yeast is involved.

The stationary phase of *S. cerevisiae* cells is being studied to extend our understanding of the eukaryotic cell lifecycle, and especially eukaryotic aging mechanisms [10, 17, 20]. Peculiarities of brewing industry processes cause

several stresses on yeast cells, even when they are in the stationary phase [11]. Taking into account the common practice of reusing yeast a number of times before disposing of it, which is typical of this industry, knowledge of the changes in biomass during the stationary phase is crucial to understand and model the process of maintaining and optimising subsequent fermentations [24]. Several reviews of the *S. cerevisiae* stationary phase have been published [12, 31, 32], given that the entry of the cells into a specialised nondividing resting state, accompanied by a decrease in overall growth rate and increased resistance to a variety of environmental stresses, is of great importance. It provides important insights into the control of two of the most fundamental aspects of eukaryotic cell biology: cell proliferation and long-term cell survival.

Two of the current techniques used to assess cell size distribution are electric particle analysis and light diffraction. Electric particle analysis is a valuable and advanced method that combines direct and rapid assays to determine numbers and cell size distributions. Montesinos et al. [21] used electric particle analysis to evaluate two electron microscopy methods, testing among other microorganisms, two yeast species: *S. cerevisiae* and *Candida albicans*. Erlebach et al. [7] used the Coulter® Multisizer II to study changes in small bacteria size distributions (mean cell size about 1 μm). In that study, Erlebach and co-workers concluded that the Coulter® Multisizer II provides a feasible method for acquiring information, not only about mean cell size, but also about cell size distributions. More recently, with the arrival of light diffraction methods, the forward diffraction of a laser beam by a particle has been used to determine its size. The angle of diffraction is inversely proportional to particle size, and the intensity of the diffracted beam at any angle is a measure of the number of particles with a specific cross-sectional area in the beam's path [8]. The absence of mathematical tools capable of satisfactory quantitative measurement of the differences among irregular and highly variable morphotypes is a limitation in this regard [15]. Consequently, sizing devices have to use the equivalent sphere concept, i.e. the sphere that will cause the measured response, which depends on the sizing principle used by the device. Because the relation between the volume and the diameter or cellular surface of a sphere is known, any of these three parameters can be used without any conceptual difference [13].

Flow cytometry, which measures individual parameters, can be used to identify subpopulations and to count microorganisms [6, 25, 29]. These uses include indirect assessment of size distribution in the population and study of its statistical characteristics [13–15, 30], development of protocols to distinguish between normal and arrested fermentations [4], validation of yeast population growth models [5, 27] as well as monitoring of microbial evolution during the transition from exponential to stationary phases [1]. In flow cytometry, the incident light scattered from one cell is collected at two different angles: a narrow forward angle (forward scatter) and approximately a right angle from the light beam (side scatter). Forward scatter is a complex parameter, as it varies not only with cell size, but also with cell shape and refractive index. Side scatter is also a complex parameter that is mainly dependent on the number of intracellular dielectric interfaces. This signal is thought to indicate variations in cell surface structure or internal structure, usually referred to in the literature as "cellular granularity" [3]. Therefore, flow cytometry, which combines direct and rapid assays to determine the number, cell size distribution and other biochemical information regarding individual cells, is particularly attractive for studying heterogeneous populations [15].

We have to confront batch cultures that exhibit high cell variability at the unicellular level, and we also encounter different cell aggregates generated during cell division and individual cell growth. We aim, then, to assess the complexity variations of the culture. A variety of definitions of complexity exist that commonly combine two aspects: how complicated a system is in terms of the number of different elementary components, and the hierarchical complexity in terms of the number of hierarchical levels in which these components are arranged [16]. Irrespective of the preferred definition of complexity, the concept contains an implicit, fundamental premise: whether functional or structural, the maintenance of components in specific relationships to each other involves hierarchy. So, by using a complexity measure to associate numerical values throughout the history of the system, one can study the dynamic behaviour of the system involved, and provide an idea of the organisation of the system.

In this paper, we report analyses of *S. cerevisiae* cell populations in the stationary phase of batch cultures under two different growth conditions, aerobic and microaerophilic, using three experimental techniques: electric particle analysis, light diffraction and flow cytometry. We specifically aim to: (1) describe the cell size during these two stationary phases of the yeast populations by using descriptive statistics and histograms obtained by electric particle analysis and light diffraction, (2) compare the size distributions obtained by electric particle size analysis with those obtained by light diffraction, (3) assess the complexity of the distributions obtained with the three experimental techniques using a complexity index [23] to assist in the interpretation of this data and (4) combine, integrate and compare the different sets of data generated by these experimental techniques with the aerobic and microaerophilic conditions of the two cultures, to gain better understanding of the population structure and its behaviour during the stationary phase.

Materials and methods

Strains, media and culture conditions

The yeast strain used was the commercial wine yeast *S. cerevisiae* strain DV10 from the Oenological Station of Champagne (Epernay, France). In the oenological context, this strain is designated as variety *bayanus*, which is commonly used in the production of alcoholic beverages because of its high flocculation capacity that facilitates cell separation. Furthermore, it is particularly fast fermenting in difficult conditions such as low pH and high alcoholic graduation [19, 28]. The strain is supplied as active dry yeast. The yeast was rehydrated in saline solution at 27°C for 30 min in accordance with the manufacturer's instructions. The rehydrated yeast was spread on Sabouraud dextrose agar, and a single characteristic colony was used as the initial inoculum.

Two experimental conditions were used: aerobic and microaerophilic growth conditions. Under aerobic conditions, the medium contained 10 g L^{-1} glucose, 5 g L^{-1} yeast extract and 3 g L^{-1} casein peptone. Under microaerophilic conditions, the aerobic medium was supplemented by adding 0.5 g L^{-1} sodium thioglycolate and 0.001 g L^{-1} resazurine. In both cases, components were dissolved in tap water, and pH was initially adjusted to 3.5 using orthophosphoric acid. Both media were autoclaved for 15 min at 121°C.

The inoculum used in the aerobic conditions was cultured using 250-mL cotton-plugged flasks with 100 mL medium, and then magnetically stirred. The inoculum used in microaerophilic conditions was cultured using 50-mL screw-cap tubes completely filled with medium. Both cases were incubated at 27°C for approximately 72 h to guarantee that the two cultures reached the stationary phase.

The cultures for the experimental data were cultivated in 1,000-mL Erlenmeyer flasks, with 500 mL of corresponding medium. The experimental cultures were inoculated with 2 and 3 mL of the same medium, respectively, for aerobic and microaerophilic conditions, and both were incubated at 27°C using a magnetic stirrer (150 rpm) for approximately 50 h. The initial oxygen concentrations in the media were 8.2 and 4.9 mg O_2 L^{-1} for aerobic and microaerophilic growth conditions, respectively. Samples were not sonicated before being processed.

Electric particle analysis

Counts and cell size distribution were determined by electric particle analysis using a Multisizer II electric particle analyser (Coulter Corporation, Miami, FL), with an aperture tube 30 μm in diameter and processing capacity of 500 μL cell suspension in 0.9% NaCl, previously filtered through a 0.2-μm membrane filter. The instrument provides three types of size measurement after the transformation of the electric pulses generated by the counter: diameter, volume and revolution surface. Data were analysed using AccuComp software version 1.15 (Coulter Corporation).

Light diffraction analysis

A light diffraction technique was also used to determine the cell size distribution. A Beckman Coulter LS13320 laser diffraction particle size analyser equipped with a universal liquid module was used. This method passes a laser through the sample and measures the intensity of the scattered light at different angles. Mie or Fraunhofer theory can be applied to the intensity data to generate the distribution of particle sizes. This instrument measures particle diameters from 0.04 to 2,000 μm. The instrument vessel was filled with distilled water, and auto-alignment and a background measurement were performed before each analysis. The files generated by the Beckman Coulter LS13320 were analysed using Beckman Coulter LS software version 5.01 with 1.332 as the fluid refractive index and (1.430, 0.01) as the sample refractive index (real and imaginary parts, respectively). Cell sizes were given as diameters.

Flow cytometry

Flow cytometry was carried out using a cytomics FC 500 MPL flow cytometer (Coulter Corporation). A standard 488-nm air-cooled argon-ion laser with power of 15 mW was used. The forward scatter and side scatter distributions were obtained for each measurement. The forward scatter detector in the Elite flow cytometer is a photodiode that collects light between 1° and 19° from the laser axis, and which is able to detect particles as small as 0.5 μm in diameter. The side scatter detector is situated at a 90° angle from the laser axis. Data were analysed using Summit® V3.1 software (Cytomation, Inc., Fort Collins, CO).

Statistical analysis

The shape, spread and location of the data sets obtained by the three instruments were analysed using the SAS statistical package (SAS Institute, Cary, NC; 1992). The SAS procedures provide data summarisation methods and statistical tests for all of these distributions. The assessment of the size distributions provided by the Multisizer II was performed using graphs and descriptive statistics. Knowing the distribution of a data set can reveal a great deal about the data itself, and can be critical in selecting appropriate future analyses and interpreting their results. Visual assessments using histograms

can give instant insight into data distribution. The size distribution metrics, obtained via descriptive statistics, illustrate the central tendency and spread of these data with numeric values, and add a layer of detail which can be used to make comparisons. The assessment of the two size distributions obtained by electric particle analysis and light diffraction in the stationary phase was performed with the chi-square test for specified proportions, taking the light diffraction distribution as a reference at each time point.

Complexity index

We used the complexity index initially created and applied to plants and animals by Piqueira et al. [23]. We calculated this complexity index from the electric particle analysis, light diffraction and flow cytometry data along the stationary stage of yeast growth to study the dynamic behaviour of its value, and to provide an idea about the organisation of the yeast system. In addition, these values help with the comparison of growth of the yeast in aerobic and microaerophilic culture conditions. Taking as their starting point the concept of informational entropy (see H_e below) and attributing no complexity to situations presenting zero or maximum informational entropy, Piqueira et al. [23] define a new index of complexity (C) as

$$C = \left(\tilde{1} - \frac{H_e}{H_e^{max}}\right)\left(\frac{H_e}{H_e^{max}}\right), \quad (1)$$

where H_e is the informational entropy associated with the system structure, and H_e^{max} is the maximum informational entropy, reached when all states have the same probability. H_e^{max} is designated by

$$H_e^{max} = \log_2 N, \quad (2)$$

and H_e is given by Eq. 3:

$$H_e = \sum_{i=1}^{N}\left[p_i \log\left(\frac{1}{p_i}\right)\right]. \quad (3)$$

For both equations, p_i is the probability of the ith state, and N is the total number of possible states or system length. In our application, N is determined by the number of instrument channels, and p_i is the frequency observed in the ith channel.

Results

Growth assessment using the cell density

Cell density evolution of batch cultures was obtained using the Multisizer II. After inoculation, the initial population densities in the Erlenmeyer flasks in both media were 2.6×10^5 and 1.8×10^5 cells mL^{-1} for aerobic and microaerophilic conditions, respectively. To represent the stationary phase reached by the cultures, four samples for each growth condition were selected (Fig. 1). Considering these points, the final mean cell density achieved by the two cultures was approximately 5.9×10^7 and 3.9×10^7 cells mL^{-1}, respectively, under aerobic and microaerophilic conditions. The difference between these two means proved be statistically significant as compared by the parametric pooled t-test (p-value = 0.023). Taking into account this significant difference, it would be interesting to compare the total cell volume of yeast reached in the two situations. To do this, we used the cell count from the Multisizer II instrument and size distributions obtained by both electric particle size analysis and the light diffraction technique. So, the total volume (V) for a time sample can be estimated using the following expression:

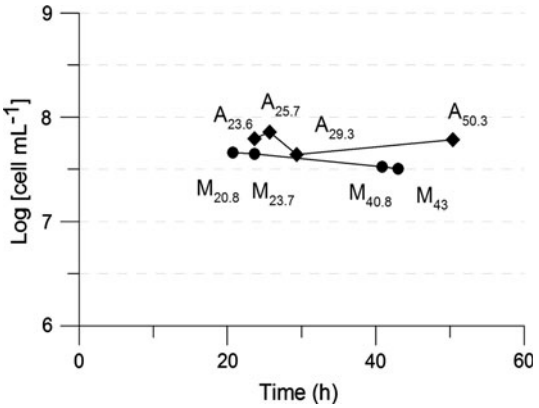

Fig. 1 Samples taken in the stationary phase, and cell density reached with an aerobic culture (A at 23.6, 25.7, 29.3 and 50.3 h, *diamonds*) and microaerophilic culture (M at 20.8, 23.7, 40.8 and 43.0 h, *circles*)

$$V = \sum_{i=1}^{N}(D \times f_i \times m_i), \quad (4)$$

where D is the total cell density obtained by the Multisizer II instrument, f_i is the relative frequency in the ith channel, m_i is the mark class of the ith channel and N is determined by the number of instrument channels. Using electric particle size analysis data, the mean volume obtained from the four samples was 3.5×10^9 and 3.1×10^9 μm^3 mL^{-1}, respectively, for aerobic and microaerophilic conditions. Using light diffraction data, the mean volume obtained was 2.8×10^{10} and 2.0×10^{10} μm^3 mL^{-1}, respectively, for aerobic and microaerophilic conditions. Taking into account that the binning of the data is not the same, the results are consistent and in agreement.

Cell size assessment and cell size distributions by electric particle analysis

Descriptive statistics for the data from the Multisizer II were obtained by SAS [26] and are presented in Table 1. In aerobic conditions the mean fluctuated between 4.46 and 4.67 μm. Cells evolving under microaerophilic conditions were bigger, with mean cell size of 4.93–5.23 μm, and they showed, moreover, a clear decreasing tendency during the stationary phase. The central tendency expressed by the median shows the same tendency as that expressed by the mean, and the values obtained are very similar. Taking into account the time samples $A_{23.6}$, $A_{25.7}$ and $A_{50.3}$ from aerobic culture, variability in cell diameter measured by the standard error was similar, evolving from 0.0050 to 0.0054 μm. However the standard error obtained for the time sample $A_{29.3}$ showed the highest value: 0.0072 μm. The tendency shown by the range (maximum minus minimum measured diameter) and the coefficient of variation were analogous to those described by the standard error. The range values varied from 17.98 to 33.30 μm, and the coefficient of variation varied from 19.75 to 23%. Variability can be assessed as the range of 50% of the central data via the interquartile range (Q3–Q1, Table 1), which was less sensitive, with values ranging from 1.04, 1.17, 1.15 to 1.17 μm. Under microaerophilic conditions, the standard errors (0.0064–0074 μm) were greater than those obtained for aerobic conditions and, moreover, there seemed to be a tendency toward an increase. The range values varied from 9.55 to 14.99 μm, smaller than those found in the aerobic culture. The coefficient of variation values varied from 18.02 to 19.33%, and interquartile ranges varied from 1.09, 1.08, 1.08 to 1.03 μm. It is worth mentioning that, in contrast to what was observed under aerobic conditions, these variability measures showed a decreasing tendency over time and lower values than those observed under aerobic conditions. There are fewer cells with the microaerophilic conditions, and they are larger than those found under aerobic conditions, but this is not sufficient to offset the final volume achieved. That is, the final volume achieved with aerobic conditions is greater than with the microaerophilic conditions.

The cell size distributions obtained with the Multisizer II (first column in Figs. 2 and 3) were similar to unimodal triangular distributions. As Table 1 showed, distributions of aerobically growing yeast remain practically constant throughout the different determinations, whereas those from microaerophilic growing yeast cells are centred more to the right (greater mean size) but move to the left along the stationary phase. Cell size distributions obtained with the yeast growing under aerobic conditions are slightly positively skewed (Fig. 2).

Table 1 Descriptive statistics for each sample of the stationary phase obtained with the Multisizer II: diameter (μm) distributions

	M (SE)	Q1–Q2–Q3	R	CV
Aerobic				
$A_{23.6}$	4.46 (0.0050)	3.94–4.46–4.98	17.98	19.75
$A_{25.7}$	4.67 (0.0052)	4.10–4.65–5.27	23.82	20.96
$A_{29.3}$	4.60 (0.0072)	4.05–4.59–5.20	33.30	23.15
$A_{50.3}$	4.67 (0.0054)	4.10–4.65–5.27	21.81	20.19
Microaerophilic				
$M_{20.8}$	5.23 (0.0065)	4.72–5.27–5.81	14.99	19.00
$M_{23.7}$	5.19 (0.0064)	4.65–5.20–5.73	13.67	18.53
$M_{40.8}$	5.09 (0.0070)	4.59–5.05–5.65	13.89	18.02
$M_{43.0}$	4.93 (0.0074)	4.46–4.92–5.49	09.55	19.33

Aerobic samples (A) were taken at 23.6, 25.7, 29.3 and 50.3 h, whereas microaerophilic samples (M) were taken at 20.8, 23.7, 40.8 and 43.0 h

The numbers of yeast cells analysed are: $N_{A23.6} = 30{,}885$, $N_{A25.7} = 35{,}790$, $N_{A29.3} = 21{,}767$, $N_{A50.3} = 30{,}266$, $N_{M20.8} = 23{,}655$, $N_{M23.7} = 22{,}676$, $N_{M40.8} = 17{,}063$, $N_{M43.0} = 16{,}507$

M mean, *SE* standard error, *Q1* first quartile, *Q2* second quartile or median, *Q3* third quartile, *R* range, *CV* coefficient of variation (%)

Cell size assessment and cell size distributions by light diffraction technique

Descriptive statistics for the data from the light diffraction technique were also obtained by SAS [26] and are presented in Table 2. Under aerobic conditions the mean fluctuated between 4.50 and 4.66 μm, with the same behaviour as shown by particle size analysis data. The mean cell size under microaerophilic conditions was greater, with mean values from 4.98 to 5.00 μm. However, in contrast to what was shown by the particle size analysis, there was no reduction of mean cell size during the stationary phase. The central tendency as expressed by the median did not undergo any change, and was calculated to be 4.44 and 4.88 μm for aerobic and microaerophilic conditions, respectively. Variability as expressed by the standard error under aerobic conditions varied from 0.0597 to 0.0996 μm and showed a tendency toward a decrease during the stationary phase. In these growth conditions, the coefficient of variation (12.76–1.47%) and range (2.77–4.72 μm) followed approximately the same tendency as those followed by the standard error, but the interquartile range hardly varied. As seen in electric particle size analysis, the standard error under microaerophilic conditions (0.0806–0.0837 μm) was generally greater than that shown by aerobic culture. The coefficient of variation values (15.70–16.34%) followed the same tendency as followed by the standard error, but again range and interquartile range did not vary in any observation.

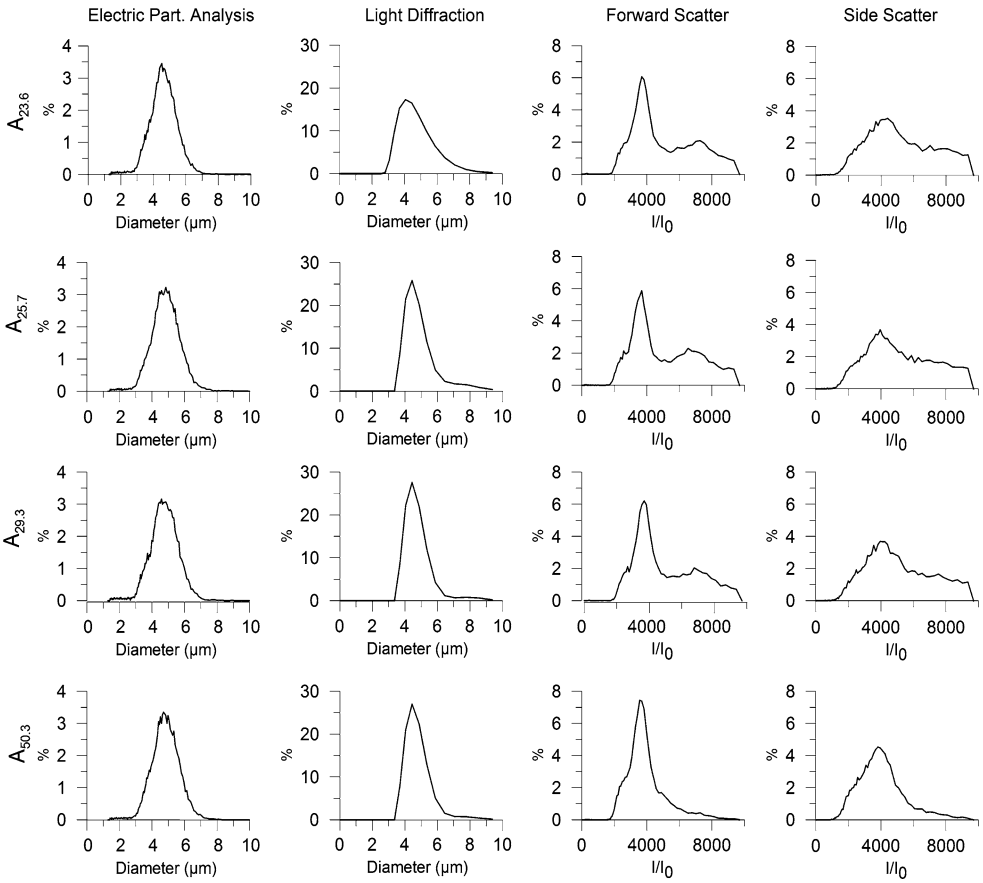

Fig. 2 Evolution of the distributions resulting from the electric particle size analysis, light diffraction technique and flow cytometry under aerobic conditions at different times of the stationary phase

Cell size distributions obtained by light diffraction (second column of Figs. 2 and 3) show a different shape from the electric particle analysis distributions; they are slightly positively skewed, with no left tail. Comparing histograms from aerobic and microaerophilic cultures, it can be seen that the aerobic ones are situated more to the left, with lower cell sizes. It is interesting to note that, with this method, almost all distributions taken during the course of the stationary phase of each experimental culture overlap each other. The only exception is time sample $A_{23.7}$ from the aerobic culture.

Comparison of size distributions obtained by electric particle analysis and light diffraction measurements

Although the light diffraction measurement is a very reliable technique for assessing cell size, it presents a serious drawback in the study of the entire evolution of a culture, since the volume of the sample as well as the cell density need to reach relatively high concentrations. These two requirements usually make it impractical to study the lag and initial exponential phases using this equipment. When comparing the stationary phase results from the two methods (Multisizer II and light diffraction), a redistribution of the primary distribution obtained by the Multisizer II into the size intervals provided by the light diffraction instrument—assuming that the values in each interval were uniformly distributed—was carried out. Comparing these new distributions from the Multisizer II with the light diffraction distributions (Fig. 4), three main points emerge: (1) the centres of the Multisizer II distributions are always situated further to the right, and therefore give higher mean sizes, than those obtained by light diffraction, (2) size distributions from the Multisizer II show a more symmetrical shape, similar to a Gaussian or normal distribution and (3) the distributions obtained with light diffraction show no left tail. Nevertheless, these sets of distributions, when compared, do not seem to display very strong discrepancies. To verify whether there are significant differences between the distributions, the chi-square test for specified proportions was used (taking the light diffraction distribution as the reference). The *p*-values obtained were lower than 0.0001 for all the time samples, so from a statistical point of view, the distributions are significantly different.

Forward and side scatter distributions

The number of yeast cells for each sample analysed with the flow cytometer was, in the case of aerobic conditions,

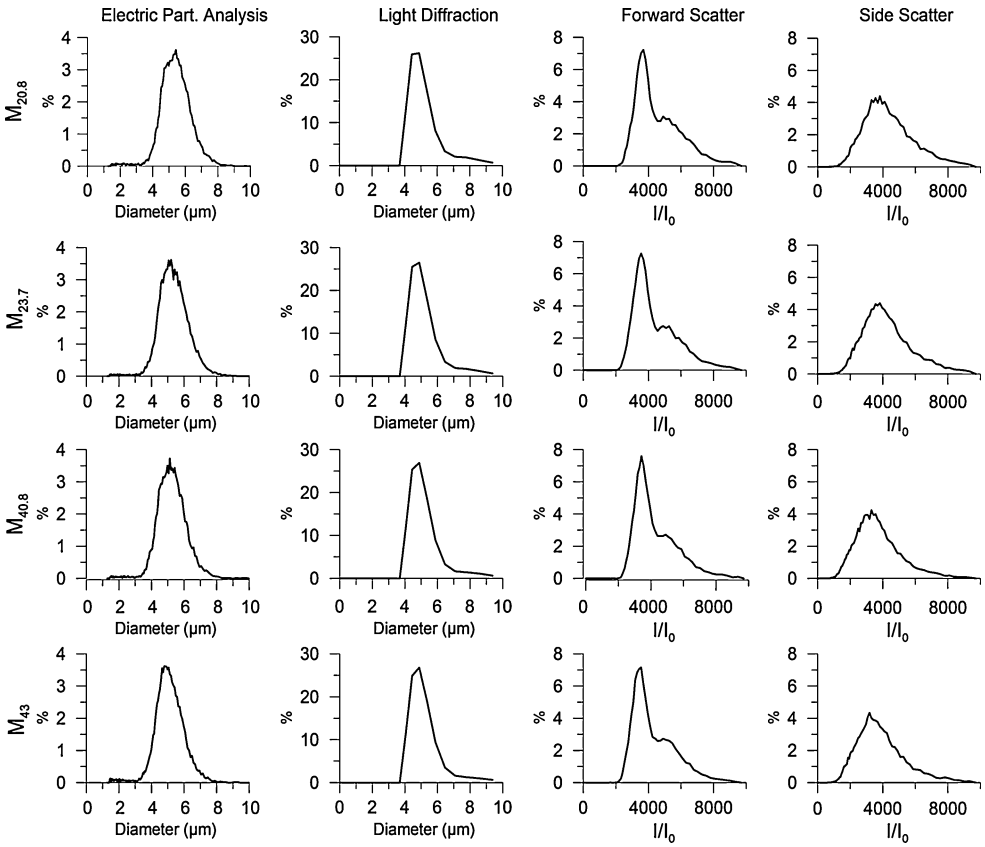

Fig. 3 Evolution of the distributions resulting from the electric particle size analysis, light diffraction technique and flow cytometry under microaerophilic conditions at different times of the stationary phase

Table 2 Descriptive statistics for each sample of the stationary phase obtained with LS13320: diameter (μm) distributions

	M (SE)	Q1–Q2–Q3	R	CV
Aerobic				
$A_{23.6}$	4.50 (0.0996)	3.69–4.44–4.88	4.72	21.47
$A_{25.7}$	4.66 (0.0771)	4.05–4.44–4.88	4.09	15.95
$A_{29.3}$	4.58 (0.0597)	4.05–4.44–4.88	2.77	12.76
$A_{50.3}$	4.61 (0.0608)	4.05–4.44–4.88	2.77	12.85
Microaerophilic				
$M_{20.8}$	4.99 (0.0837)	4.44–4.88–5.35	4.49	16.34
$M_{23.7}$	4.98 (0.0810)	4.44–4.88–5.35	4.49	15.76
$M_{40.8}$	4.98 (0.0810)	4.44–4.88–5.35	4.49	15.76
$M_{43.0}$	5.00 (0.0806)	4.44–4.88–5.35	4.49	15.70

Aerobic samples (A) were taken at 23.6, 25.7, 29.3 and 50.3 h, whereas microaerophilic samples (M) were taken at 20.8, 23.7, 40.8 and 43.0 h

This device does not provide absolute values for the number of yeast cells; rather, it provides the distributions of relative frequencies

M mean, *SE* standard error, *Q1* first quartile, *Q2* second quartile or median, *Q3* third quartile, *R* range, *CV* coefficient of variation (%)

as follows: $N_{A23.6} = 20{,}681$, $N_{A25.7} = 20{,}450$, $N_{A29.3} = 20{,}355$ and $N_{A50.3} = 20{,}049$. In the microaerophilic case the numbers were $N_{M20.8} = 20{,}053$, $N_{M23.7} = 20{,}049$, $N_{M40.8} = 20{,}103$ and $N_{M43.0} = 20{,}099$. All the samples had more than 20,000 yeast cells. The distributions of forward and side scatter showed great heterogeneity in the internal structure of the population and cell size in both growth conditions (third and fourth columns of Figs. 2 and 3). However there were differences in this heterogeneity depending on the considered growth condition. Forward scatter distributions showed a similarly basic shape under the two growth conditions, with common features. They are non-symmetrical, unimodal and centred in a range of relative intensities between 3,500 and 3,700, with a long and noteworthy right tail. However, cells grown under microaerophilic conditions showed a subset with relative frequencies between 4,500 and 6,000; under aerobic conditions there was a higher proportion of cells with relative intensities between 6,000 and 10,000. It is interesting to note that almost all histograms obtained for each growth condition exhibit little variation. This general rule does not apply to the last determination taken under aerobic conditions ($A_{50.3}$). However this is due to the fact that the cell subpopulation with relative intensities between 6,000 and 10,000 disappeared. This causes a variation in the shape of the histogram. Similarly, side scatter distributions in both experiments are essentially the same: a triangular distribution, slightly positively skewed and centred at relative intensities between 4,000 and

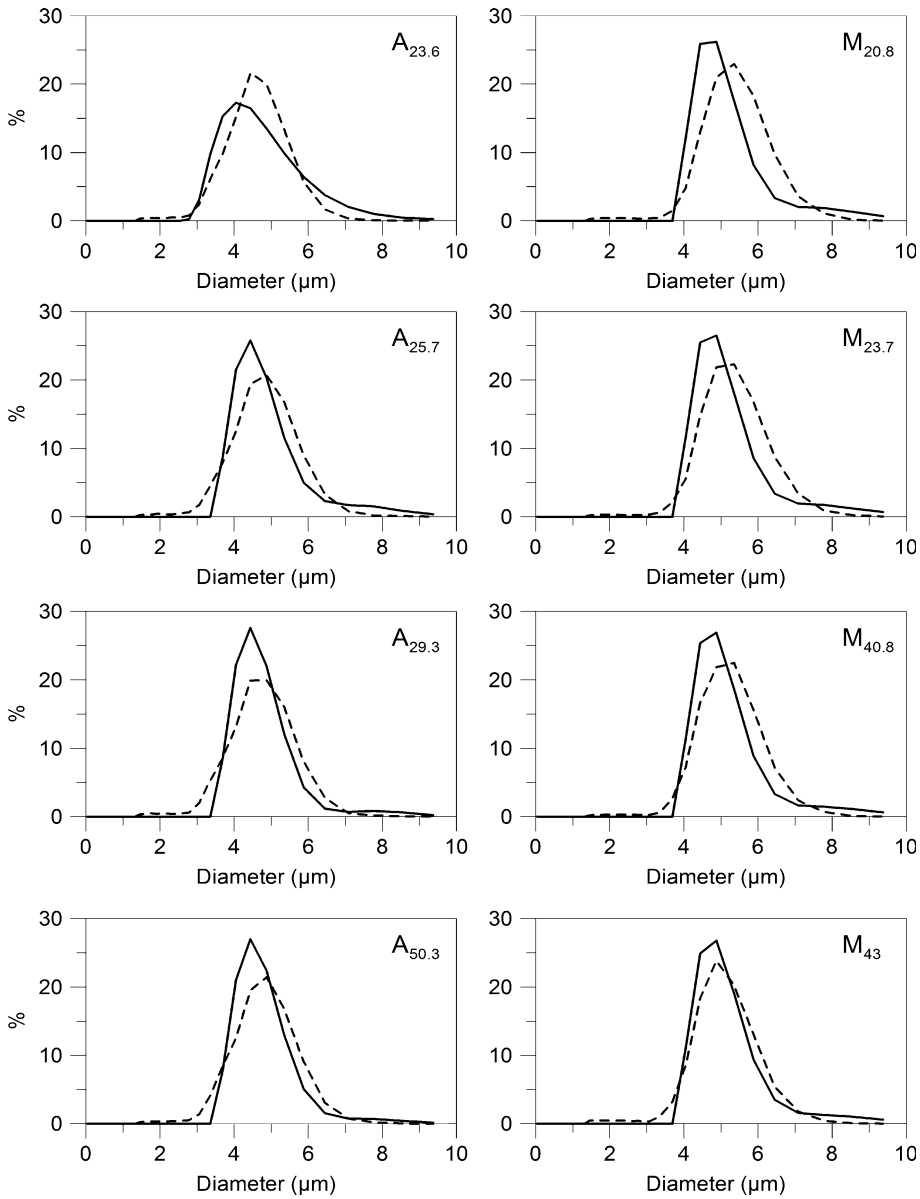

Fig. 4 Comparison of size distributions obtained by electric particle analysis and light diffraction techniques under the two growth conditions, aerobic (*left*) and microaerophilic (*right*). The size intervals for the data obtained with the Multisizer II (*dashed line*) were adapted to those of the light diffraction instrument (*solid line*)

4,200. However, in this measurement one can also observe a subpopulation of yeast cells with relative intensities between 6,000 and 10,000 under aerobic, but not microaerophilic, conditions. As before, almost all histograms obtained for each growth condition exhibit little variation except for the last aerobically taken sample ($A_{50.4}$). As reflected by forward scatter, for this time sample the subpopulation with relative intensities between 6,000 and 10,000 disappears. As a result, relative intensities lower than 6,000 are magnified at this time. Although under microaerophilic conditions there is no important change in the shape of the histograms, it should be noted that the histograms of time samples $M_{40.8}$ and $M_{43.0}$ are displaced slightly to the left.

Complexity analysis

It seems clear to us that the diversity and variability of the data obtained demand a complexity analysis if we are to progress further and better understand the information provided by the statistical values. Complexity values can be used to identify and compare evolution during the stationary stage of yeast population in these two different environments, aerobic and microaerophilic. Consequently, the population variability may also be studied by simple complexity analysis. Applying Eq. 1 to the data from the electric particle analysis, light diffraction and flow cytometry, we obtain the results depicted in Figs. 5 and 6 for aerobic and microaerophilic conditions, respectively.

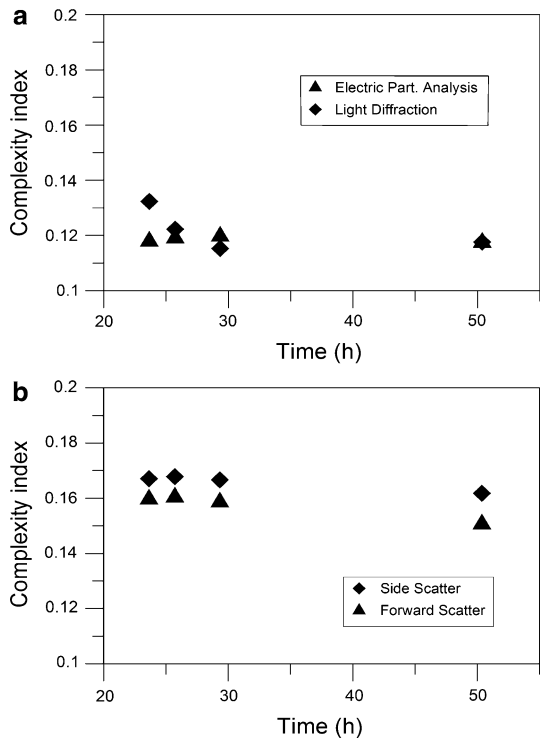

Fig. 5 Index of complexity calculated using data obtained from the stationary phase under aerobic conditions: **a** electric particle size analysis and light diffraction data; **b** forward scatter and side scatter data

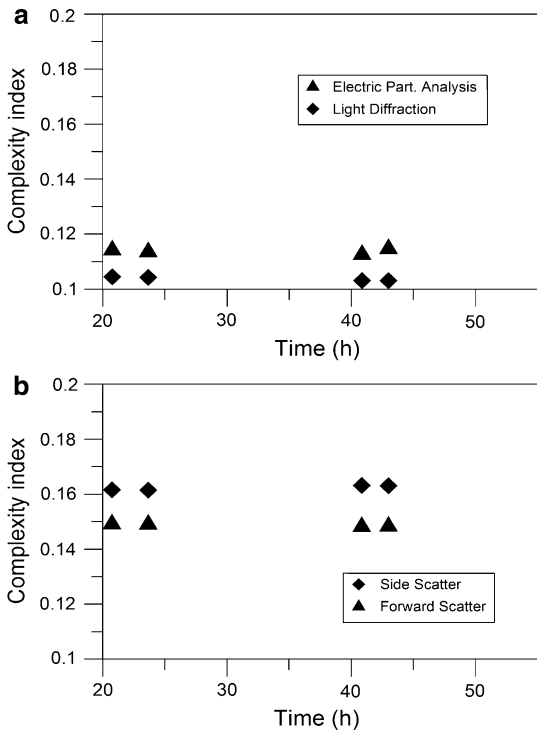

Fig. 6 Index of complexity calculated using data obtained from the stationary phase under microaerophilic conditions: **a** electric particle size analysis and light diffraction data; **b** forward scatter and side scatter data

Complexity index values calculated from electric particle size analysis data in aerobic conditions, with values from 0.117 to 0.120 (Fig. 5), are situated slightly above values obtained under microaerophilic conditions, 0.113–0.115 (Fig. 6). Similarly, complexity index values calculated from light diffraction data under aerobic conditions (0.115–0.132) are also situated above the values obtained under microaerophilic conditions (0.103–0.105). Excepting the complexity index value taken at time sample $A_{23.7}$ (0.132), the behaviour of the complexity index calculated from electric particle analysis and light diffraction data in the stationary phase of the two growth conditions is similar. In addition, under microaerophilic growth conditions, the difference between the complexity index based on electric particle analysis and light diffraction data is greater than under aerobic conditions. Complexity index values calculated from forward scatter data under aerobic conditions (0.151–0.160) show greater variability than under microaerophilic conditions (0.148–0.149). As with side scatter data, the complexity index values obtained under aerobic conditions (0.162–0.168) are also more variable than under microaerophilic conditions (0.162–0.163). Complexity index values calculated from forward and side scatter data obtained under aerobic conditions are higher than those obtained under microaerophilic conditions, except for the last time sample obtained under aerobic conditions ($A_{50.3}$), for which the two are the same.

Discussion

Taking initial sample points $A_{23.6}$ (6.2×10^7 cell mL^{-1}) and $M_{20.8}$ (4.6×10^7 cell mL^{-1}) and final samples points $A_{50.4}$ (6.0×10^7 cell mL^{-1}) and $M_{43.0}$ (3.2×10^7 cell mL^{-1}) as a reference, during the stationary phase there was a greater reduction in total cell count under microaerophilic (30%) than aerobic (3%) conditions. These results agree with MacLean et al. [17], who explain that cells initially cultured on a respiratory carbon source survived better in the stationary phase than cells pre-grown on fermentative media. In aerobic cell growth it is essential to counteract the oxidative damage induced in cells through gene expression to maximise resistance to oxidative stress, and these cells are probably provided with the most efficient repair systems.

The sizes of cells growing in microaerophilia were greater than under aerobic conditions. The mean cell size differences were about 0.21 or 0.51 μm, as measured by light diffraction and electric particle analysis, respectively. In addition, the cell size remained constant throughout the period. However, under microaerophilic conditions, and only by electric particle analysis, the size decreased

slightly as the stationary phase progressed. Nonetheless, this decrease was not confirmed by light diffraction. Taking into account the electric particle analysis distributions, we observe a symmetrical shape. Nevertheless, considering the flow cytometry distributions, both forward and side scatter reveal heterogeneous cell populations, especially under aerobic conditions. Distribution shapes could be explained by the existence of heterogeneous populations known as quiescent and non-quiescent cells [2]. Under aerobic conditions, the oxygen concentration decreases along the log phase despite the magnetic stirring, and the sample reaches the stationary phase with lower oxygen concentration [18, 22]. These environmental changes, in addition to nutrient depletion and pH changes, affect the physiology of yeast that changes from respirofermentative to fermentative [33]. In contrast, under microaerophilic conditions, the oxygen concentration changes are minimal during the experimental and stationary phases due to reducing agent added to the medium. Sodium thioglycolate reduces the redox potential of the culture medium, and resazurin acts as an indicator of oxidation–reduction. In microaerophilia, forward scatter results showed that there are two subpopulations, but much closer together and less distinct than those observed in aerobic growth. However, the results of side scatter showed a unimodal curve, typical of a single population of cells. The results could be explained by higher cell mortality in microaerophilia resulting from mortality of non-quiescent cells.

An important feature of these different distributions is the tail, that is, the data far from the central part of the distribution characterised by the 25% and 75% percentiles, the first and third quartiles. This part of the data was almost constant, but data below and above these values are the most sensitive to changes with time during the stationary phase, and between the two environmental conditions of growth. These data represent the yeast cells that are non-typical, smaller and simpler, or larger and not so plain in their cellular structure. The different experimental devices collect this kind of information in different ways.

As stated above, although significant according to the statistical analysis, comparison of the distributions obtained by electric particle size analysis and light diffraction does not show strong discrepancies. They may at times be equivalent, although not always, and this depends on the goal in using the two techniques. Both techniques make it possible to obtain a size distribution directly, and the processing time with the two techniques is approximately the same, but electric particle size analysis provides cell count information as well. However, as noted above, neither of these two techniques permits us to distinguish stationary phase subpopulations, while flow cytometry does. In evaluating the cell size, however, the latter technique reveals a serious drawback: it does not directly provide cell size. To overcome this limitation, it would be very interesting to be able to assume that these two measurements were related by a monotone increasing function that would enable their mathematical relationships to be studied. Julià et al. [13] showed that forward light scatter data cannot be linearly transformed into bacterial size values by an accurate universal function. However, second-order relationships seem to be the simplest satisfactory relationships between cell diameter and forward light scatter in eubacteria at a specific stage of growth of the culture. As far as we know, there is no such study for yeast cultures at any stage of growth. Such a study would help to overcome the above-mentioned drawback and would facilitate device applicability in the wine industry.

Although the size distributions obtained with electric particle size analysis and light diffraction were different, the behaviour of the corresponding complexity indexes was similar, especially in microaerophilic conditions. This strongly suggests that, although different sizing principles were used, the two size distributions were recorded in the same way as the changes that take place inside the yeast populations. The same is true when considering the forward and side scatter data. Although the distribution shapes were different, the changes detected by the complexity index were analogous. This last finding reinforces the well-known idea that these two parameters are related. Finally, it is interesting to note that the values for the complexity index obtained from flow cytometry data of sample $A_{50.3}$ (i.e. when the shapes of the distributions are very close under the two growth conditions) practically overlapped with the values obtained under microaerophilic conditions. This suggests that the shape of distributions clearly influences the value of the complexity index or, in turn, the complexity index assembles the shapes of the distributions in an accurate way. All these results suggest that this complexity index is a good descriptor of the changes that take place in a yeast population in the stationary phase, and that it aids in the analysis and understanding of the implications of these distributions obtained by these three experimental techniques.

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