SYSTEMATIC ERROR AND COMPARISON OF FOUR METHODS FOR ASSESSING THE VIABILITY OF *SACCHAROMYCES CEREVISIAE* SUSPENSIONS

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Four methods for the determination of cell viability were compared : the plate count technique, the flow cytometer, and two microscopic numerations -one after methylene blue staining and the other one with epifluorescence. The experimental error of these techniques was for the first time estimated : 8% for both numerations under microscope and 13% for the plate count technique. The staining mechanisms were explained by comparing the numerations under microscope and the flow cytometer analysis.

INTRODUCTION

For many biotechnological investigations, it is of great importance to determine the viability of cellular suspensions. For yeast cultures, several methods are available to determine cell viability but the measurement of viability sets two problems. The first one has already been described by Jones (1987a and 1987b) : all methods give different values because they are based on varying criteria. The classical plate counts are based on cell replication (Bibek, 1979 ; Postgate, 1967). Some techniques are based on the ability of the plasma membrane to exclude the penetration of the dye like methylene blue (MB) (Bonara and Mares, 1982 ; Postgate, 1967). Literature presents also methods based on the ability of cell to breathe (King *et al.*, 1981), like the tetrazolium salts technique (Trevors, 1982 ; Trevors, 1983). The second problem has not been developed in literature but it is of great importance : the experimental error made when different methods are used.

In this paper, a comparison of four methods for assessing yeast cell viability was carried out. The experimental errors made when using standard plate counts and microscopic observations were evaluated. Another part of this work consists in comparing MB or Mg-ANS stainings to flow cytometry in order to understand the staining mechanisms which were merely hypothetical until today.

MATERIALS AND METHODS

Organism employed. The yeast *Saccharomyces cerevisiae* (UG5) was used for all experiments. The strain was maintained on agar slants at 4°C. The medium consists of the following composition : glucose, 50g ; yeast extract, 5g ; $MgSO_4$, 0.4g ; $(NH_4)_2SO_4$, 2g ; KH_2PO_4, 5g ; agar, 20g ; distilled water, to 1L.
The strain of yeast was grown in Erlenmeyer flasks in the same medium (without agar). Cultures were incubated at 30°C and pH was adjusted to 4.0 with H_3PO_4 at 85%.

Plate count technique. Sterile Petri dishes were filled with conservation medium at around 50°C. 100 mL of cellular suspension were spread over dried agar plate, and the colonies were counted after 3 days incubation at 30°C. An ideal count lay in the range of 30 to 300 colonies (Lee *et al.*, 1981).

MB staining. 100 mg of MB were diluted into one litre of 2% (w/v) sodium tri-citrate. This solution was stored in darkness at ambiant temperature. MB solution was added v/v to cellular suspension. After mixing, a staining time of 10 min at ambiant temperature was required before microscopic examination. Viable cells remained white, while dead cells were blue.

Mg-ANS staining. 0.3g Mg-ANS were dissolved in 100mL of Ringer solution (NaCl, 9 g ; KCl, 0.42g ; $CaCl_2$-$6H_2O$, 0.48g ; $NaHCO_3$, 0.2g ; glucose, 10g ; distilled water, to 1L). When stored in a dark bottle at 4°C, the stock solution can be kept for up to six months. Mg-ANS solution was added v/v to yeast sample. Epifluorescence examination was done after a staining time of 5 min. Dead cells fluoresced green, viable cells were not stained.

Classical microscopy and epifluorescent microscopy. For all MB stainings, the viability was obtained by enumerating the viable and the non viable yeasts with a hemacytometer (Thoma slide) observed under microscope. As the cells were distributed aleatory on the slide, they were always counted in the same manner on five large squares (the second from the right and from the top of the microscope field of view and those at the angles) for each slide. Work was carried out using a transmitted light microscope (Olympus system microscope model BHS). For Mg-ANS staining, a fluorescence system (Olympus : model BH2-RFC) and a deuterium illuminator were added. The fluor cluster used was that designed for use with Mg-ANS, so the exciting filter was at 490 nm and the barrier filter was at 515 nm. The viability was evaluated by first counting the fluorescing cells under epifluorescence, then the total number of cells was enumerated in the brightfield mode.

Cytofluorometry. Two fluorochromes were used to label viable and non viable cells. Fluorescein di-acetate (FDA) stock solution was prepared by diluting 2 mg of FDA per mL of acetone. For propidium-iodure (PI) stock solution, 80 mg of PI were diluted in 1 mL of water. These solutions were stored at 4°C. One mL of cellular suspension was mixed with 0.5 mL of FDA solution and stored for 30 min in darkness on ice, before 0.5 mL of PI solution were added. Ten min after, cell sample was examined by the flow cytometer, which was from Coulter and it was rented by INSERM at Purpan hospital in Toulouse. Flow cytometer used optical and electronic methods to measure cellular characteristics. Emissions from both of the fluorochromes were quantified separately by using a serie of beamsplitting dichroic mirrors and filters which select appropriate wavelengths. Cells stained by PI fluoresced red and were considered to be non viable ; cells stained by FDA fluoresced green and were considered to be viable.

Experimental error calculation. For the flow cytometer, experimental error was 2%, evaluated by the constructor. For colorimetric methods, experimental error was estimated by repeating the numeration n times (n hemacytometer were counted).
The arithmetic mean (X) and the standard deviation (σ_{n-1}) were calculated :

$$X = \frac{\Sigma X_i}{n} \quad \text{and} \quad \sigma_{n-1}(X) = \sqrt{\frac{(X_i - X)^2}{n-1}}$$

The experimental error was then :

$$\varepsilon_n(X) = \frac{\sigma_{n-1}(X) \times 100}{X}.$$

RESULTS

Plate count method. Three cellular suspensions about 24 hours old, were spread over 10 Petri dishes. For each solution, the experimental error is calculated (table 1).

Numeration under microscope. Twenty samples of *Saccharomyces cerevisiae* at different concentrations and about 24 hours old were stained with Mg-ANS and observed under epifluorescence. For each sample, 10 slides were counted ; ε_{10}(N), ε_{10}(Via) were calculated. For each sample, when the ten counts were taken in fours, seven ε_4(N) and seven ε_4(Via) could be calculated. The highest value of each serie of ε_4 is used to define the ε_4(N) and the ε_4(Via) of one sample. ε_{10}(N) and ε_4(N) are represented versus the total number of cells counted per slide on figure 1 ; ε_{10}(Via) and ε_4(Via) are represented versus the total number of cells counted per slide on figure 2.

Comparison of MB or Mg-ANS stainings to flow cytometry. Viability of ten cultures of different ages was determined by MB staining, by epifluorescence and by flow cytometry. Results are presented in figure 3. When stained with PI and FDA for flow cytometry analysis, some yeasts were red and green at one and the same time.

Experimental error of plate counts and microscopic numerations. With the plate method, yeast cell concentration is known with a 13.8% error when around 140 colonies were counted 10 times. The experimental error of this method is too high for routine experiments when reproducibility of this method is unsatisfactory when it is obviously impossible to realise10 plate counts but only 3 or 4.

Fig.1 and fig.2 show that the experimental errors decrease when the total number of cells counted per slide increases. This work gives estimations of the experimental error of microscopic numerations : for example, when around 400 cells were counted 4 times, the experimental error on viability is less than 1.8% and the experimental error on total cell concentration is less than 8%. So this work clearly shows, it is better to use microscopic numerations than plate counts to determine precise cell concentrations.

When fig.1 and fig.2 were compared, we also see that $\varepsilon 4$(Via) is always lower than $\varepsilon 4$(N) and this can be explained : systematic error on total cell count is due to differences in the volume confined in the hemacytometer from one time to another and is also due to direct mistake in numeration (some cells could be counted twice or forgotten). On the other hand, systematic error in viability depends only on the latter cause. It is then not surprising to find that reproducibility of viability measurement is better than reproducibility of total cell number measurement.

Comparison of MB and Mg-ANS stainings to flow cytometry. Before comparing any methods, it is essential to understand their principles and why some cells are considered to be viable or not. In this part, we show the mechanisms of MB and Mg-ANS stainings which were controversial or hypothetical until today. The principle of MB staining is under debate. MB is said to enter all cells and is reduced by a deshydrogenase in living cells, which becomes white (Postgate, 1967). Another explanation for MB staining is found in literature : MB is supposed to enter only cells in which selective permeability of the plasma membrane is severely compromised (Bonara and Mares, 1982). The mechanism of Mg-ANS stain is described hypothetically (King, 1981). The fluorochrome passes into the cells by selective permeability, and stains the proteins of the plasma membrane and cytoplasm of damaged cells. To clarify the staining mechanisms of MB and Mg-ANS, they were compared to the mechanism of the double staining of flow cytometry. FDA enters every cell by simple diffusion and is hydrolysed by the cytoplasmic esterases of living cells and the resulting compound is green fluorescent (Ch Iver *et al.*, 1978). PI fluoresces red in cells when its penetration is not prevented by the intact plasma membrane (Vol, 1985).

With the double staining, three sorts of cells are differentiated : Green cells are assumed to be viable (they possess the esterase activity and intact plasma membrane). Red cells are assumed to be dead (they do not possess the esterase activity and their plasma membraneis altered). Red and green cells are called moribund cells (they have lost their membrane integrity but possess the esterase activity).

Fig.3 shows that moribund cells are counted with the viable cells, when stained with MB or Mg-ANS. This experiment show then the hypothesis of Postgate (1967) is wrong or incomplete : either the dye enters only damaged cells and is reduced by an enzyme in moribund cells or the dye enters all cells and is reduced in viable and moribund cells.

For Mg-ANS staining, the hypothesis of dye exclusion by the membrane is wrong because moribund cells which have lost their integrity remain unstained.

Fig.3 shows a strong correlation between MB and Mg-ANS stainings.

Table 1 : Plate counts for three cellular suspensions of *Saccharomyces cerevisiae* : experimental error.

Plate	1	2	3	4	5	6	7	8	9	10	ε_{10}
Sample A	132	120	162	152	130	128	123	171	120	124	13,6%
Sample B	162	160	158	120	134	127	170	173	120	153	13,8%
Sample C	102	143	149	138	137	150	108	146	111	120	14.1%

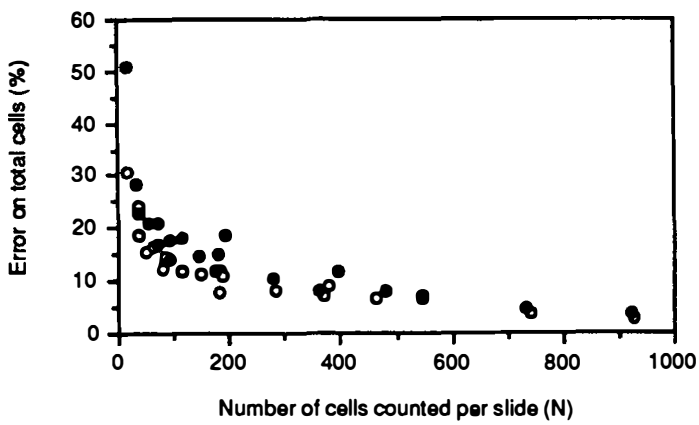

Figure 1 : Microscopic numeration of *Saccharomyces cerevisiae* suspensions after Mg-ANS staining : error on total cells with 10 slides counted (o) and with 4 slides counted (•), versus the total number of cells counted per slide.

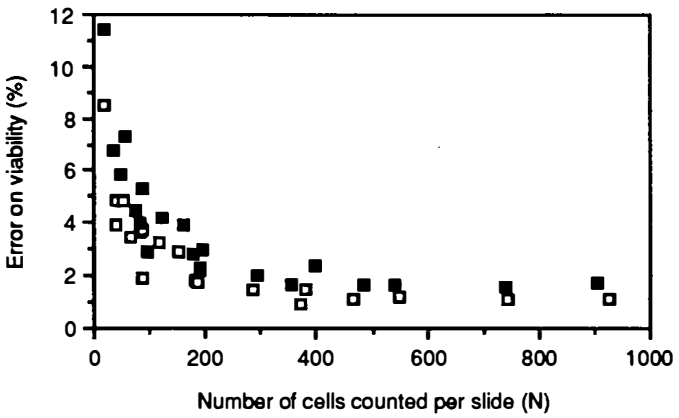

Figure 2 : Microscopic numeration of *Saccharomyces cerevisiae* suspensions after Mg-ANS staining : error on viability with 10 slides counted (□) and with 4 slides counted (■) ,versus the total number of cells counted per slide.

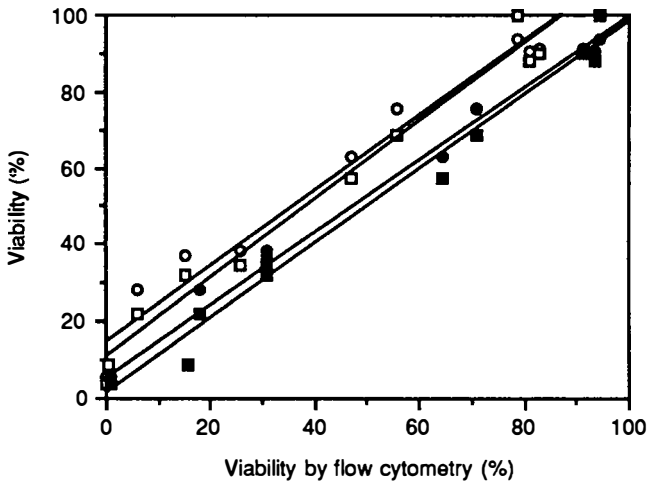

Figure 3 : % viability of 10 *Saccharomyces cerevisiae* suspensions measured by the MB staining method and by epifluorescence versus % viability found by flow cytometry. (• and ○ = Mg-ANS. ■ and □ = BM. • and ■ = moribund cells are counted as viable cells. ○ and □ = moribund cells are counted as dead cells.)

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