Research article

Institute of Brewing & Distilling

Received: 9 March 2016 Revised: 15 November 2016 Accepted: 15 November 2016 Published online in Wiley Online Library: 16 March 2017

(wileyonlinelibrary.com) DOI 10.1002/jib.400

Improvement of the slide culture technique for the assessment of yeast viability

Frederik Cruyt,[1,2] Cátia A. Sousa,[1,3,4] Manuela D. Machado[1,4] and Eduardo V. Soares[1,4]*

This work aimed to improve the slide culture technique (SCT) for the assessment of yeast viability. Thus, all the steps of the SCT were standardized: a sample of 20 μL containing 1×10^5 cells/mL was placed in a ~ 20 × 20 mm YPD agar block and incubated for 16–24 h, at 25°C. It was proposed the use of calcofluor white (CFW) to facilitate the microscopic observation of yeast cells. The viability of cell populations in different physiological states (healthy, ethanol stressed and starved cells), assessed by SCT (without or with CFW), did not differ significantly ($p < 0.01$). In addition, the viability of healthy and ethanol stressed cells determined by the SCT and the standard plate count technique (PCT) did not differ significantly ($p < 0.01$). In conclusion, the improved SCT is a fast and reliable alternative to PCT for the evaluation of yeast viability in research and in the industry.

Keywords: ethanol stress; micro-colony count; *Saccharomyces cerevisiae*; starvation; viability

Introduction

Viability determination of a yeast population is a common procedure both at laboratory and industrial level. Beer, wine and bioethanol production methods share many similarities, chiefly the use of the yeast *Saccharomyces cerevisiae*. In these industries, at the end of fermentation, yeast cells are removed, washed, stored and re-used in many fermentation cycles *(1,2)*. The practice of serial harvesting and re-inoculations imposes on yeast cells several stresses, such as cold shock, starvation and ethanol stress, which have a detrimental impact on yeast viability *(3)*. Knowledge of cell viability determines the amount of yeast to be inoculated since the rate of fermentation is deeply influenced by the rate of cellular growth. Thus, the viability determination is of great importance in order to achieve consistent fermentations and produce high-quality alcoholic beverages (beer or wine) or high bio-ethanol yields *(4)*.

The yeast *S. cerevisiae* is one of the most useful and studied model organisms since is easy to grow, store and manipulate under laboratory conditions, does not present any ethical constrains, has the genome sequenced and an appreciable percentage of yeast genes are members of orthologous gene families associated with human diseases *(5,6)*. For these reasons, this yeast has been used in the study of cellular responses to different stresses. Cell viability is one of the most common determinations used to evaluate the toxic impact of different chemical or physical agents.

The differentiation between life and dead yeast cells is not straightforward, being problematic from both practical and conceptual stand points *(7)*. The most common processes to evaluate yeast viability include methods based on the measurement of: (a) the integrity of the cell membrane; (b) the metabolic activity; and (c) the ability of cells to reproduce *(8,9)*.

Cell viability determination by the plate count technique (PCT) constitutes the 'gold standard' method in quality control and research laboratories *(10)*. This method is based on the (in)ability, of yeast cells to divide successively and form a colony on the surface of an appropriate agar medium after a defined period of incubation (clonogenic assay). PCT gives direct information of the surviving and dead cells of a given population of wild-type, mutant strains or any other genetic yeast construct being used in the detection of cell death *(6)*. Nevertheless, with this technique, a limited number of cultures can be evaluated simultaneously, even when using an automated cell counter and/or an automated colony counter *(11)*. In addition, PCT requires large amounts of reagents and gives a slow answer as 2–4 days are needed to obtain colony-forming units with a size that can be clearly seen.

These problems (the amount of reagents required and the speed of the analysis) can be overcome using the slide counting technique (SCT). In this technique, a thin layer of an appropriate agar medium is poured on a haemocytometer or a microscope slide (reducing the amount of culture medium); subsequently, yeast cells are placed on the culture medium, covered with a sterilized cover-slip and incubated, usually at room temperature or 25°C, for 18 h *(10,12)*. After incubation, the slides are observed under a microscope. Viable cells produce micro-colonies, whereas dead cells are single. In SCT the results are obtained in up to 24 h.

* Correspondence to: Eduardo V. Soares, Bioengineering Laboratory-CIETI, Chemical Engineering Department, ISEP-School of Engineering of Polytechnic Institute of Porto, Rua Dr António Bernardino de Almeida, 431, 4200-072 Porto, Portugal. E-mail: evs@isep.ipp.pt

[1] Bioengineering Laboratory-CIETI, Chemical Engineering Department, ISEP-School of Engineering of Polytechnic Institute of Porto, 4200-072, Porto, Portugal

[2] KU Leuven, Faculty of Engineering Technology, B-9000, Ghent, Belgium

[3] REQUIMTE/LAQV, Chemical Engineering Department, Faculty of Engineering, University of Porto, 4200-465, Porto, Portugal

[4] CEB-Centre of Biological Engineering, University of Minho, 4710-057, Braga, Portugal

Despite the advantages and the simplicity of SCT, this method has been used in a very limited way. Possible reasons for this low use may be the lack of a complete standardization of the method and the difficulty of focusing on the cells, particularly the dead cells, which can lead to the overestimation of the viability.

The present work aims to standardize all the steps of the SCT. In addition, the problem of the focusing on the dead cells will be addressed. For this purpose, calcofluor white (CFW), a widely available low-cost dye, will be used to stain yeast cell walls. The results obtained with the SCT in healthy and stressed cells will be compared with those obtained with the conventional PCT.

Material and methods

Yeast, media and growth conditions

In this work, the strain of *S. cerevisiae* BY4741 was used. The strain was purchased from the EUROSCARF collection (Frankfurt, Germany) and was routinely maintained at 4°C on YPD agar slants [10 g/L yeast extract (Difco-BD, Sparks, MD, USA), 20 g/L peptone (Difco-BD), 20 g/L glucose (Merck, Darmstadt, Germany) and 20 g/L agar (Merck)].

Yeast pre-cultures were prepared in 10 mL of YPD broth in 100 mL Erlenmeyer flasks. Cells were incubated at 25°C on an orbital shaker at 150 rpm for 8–10 h. Cultures in exponential growth phase were obtained by inoculating 40 mL YPD broth in 100 mL Erlenmeyer flasks with pre-cultures and then incubating overnight to an OD_{600} of ~1.0 under the same conditions as the pre-cultures. Cultures in stationary growth phase were prepared by inoculating 40 mL YPD broth in 100 mL Erlenmeyer flasks with pre-cultures, at an OD_{600} of ~0.050; then, cells were grown in the conditions described above for 48 h. After growth, cells were harvested by centrifugation (2000 ***g***, 5 min) and then washed twice and resuspended in deionized water.

Effect of CFW on yeast cell growth

Cells in exponential phase of growth (OD_{600} ~ 1.0) were harvested by centrifugation (2000 ***g***, 5 min) and suspended in YPD (double concentrated) at 2×10^6 cells/mL. A volume of 100 μL of yeast cell suspensions was combined with 100 μL of double the desired concentration of CFW (Sigma-Aldrich), in sterile flat-bottomed clear 96-well microplates (Orange Scientific). A stock solution of 5.0 mg/mL CFW was prepared in deionized water and filter sterilized. Subsequently, the stock solution was diluted, in sterile deionized water, in order to obtain the desired concentrations of CFW. Five replicates of each CFW concentration were carried out. A final volume of 200 μL per well and a final cell concentration of 1×10^6 cells/mL was used. Cells were incubated in the dark, at 25°C. After 24 h of incubation, the OD_{600} was measured on a PerkinElmer (Victor3) microplate reader, after appropriate dilution. The absorbance was corrected by subtracting the absorbance of culture medium with a given CFW concentration.

Exposure of yeast to stress conditions

Two stress conditions were used: starvation and ethanol. For this purpose, cells at a final concentration of 1×10^7cells/mL were placed in 10 mmol/L PBS buffer, pH 6.0, at 25°C, on an orbital shaker at 150 rpm for 48 h or were exposed to 20% (*v*/v) ethanol, in PBS buffer, for 120 min, in starvation or ethanol stress, respectively.

Slide culture technique

For preparing slide cultures, a defined volume of YPD agar (12.5 or 25 mL) was poured into a sterile 90 mm plastic Petri dishes and allowed to solidify. A block of ~20 × 20 mm YDP agar was cut and placed in a sterile microscopy slide. Cell suspensions (10–20 μL) containing 5×10^4, 1×10^5 or 2×10^5 cells/mL were poured over the YPD block. The starting cell concentration was determined by direct microscopic counting using a Neubauer chamber. Subsequently, cells were covered with a 24 × 24 mm sterile coverslip and placed in a wetting chamber, containing 100 μL of sterile water. All procedures were carried out in sterile conditions. The slides were incubated at 25°C and observed under a light microscope after the times indicated in the figures.

When appropriate, CFW was added to the medium. YPD was autoclaved and cooled to 50°C. Then, CFW was added (from a sterile water stock solution of 5.0 mg/mL) at a final concentration of 2.5 μg/mL. The medium was poured into Petri dishes as described above.

The cells that gave rise to a micro-colony (four cells or more) were deemed as viable. Single (unbudded); double or triple cells were considered as non-viable. A cell with a bud ('doublets') is considered a single cell until cytokinesis occurs. As a new cell cycle never begin before completion of a previous cycle *(13)*, four cells or more ('quartets') are observed when the 'original' mother cell originates a daughter cell and both cells (mother and daughter) give rise to a cell (bud). A similar criteria was proposed by Lodolo and Cantrell *(14)*.

The numbers of micro-colonies and of non-reproducing cells were counted on several view fields. The viability was determined as a percentage of micro-colonies of the total count. In each experiment and for each condition the counting was performed in duplicate (at least 200 events were scored: micro-colonies plus cells), in two YPD blocks. An overview of the main steps of the technique is presented in Fig. 1.

Plate culture technique

Cell suspensions were serially diluted with sterile deionized water and plated on YPD agar (two replicates of the convenient dilutions). The colonies were counted after 2–3 days of incubation at 25°C. No further colonies appeared after that incubation time. The percentage viability was calculated using the number of colony-forming units (CFU)/mL at zero time as reference (100%).

Microscopy

Cells were observed by phase-contrast or by epifluorescence microscopy using an epifluorescence microscope (Leica Microsystems, Wetzlar GmbH, Germany) equipped with an HBO 100 mercury lamp and the filter set A from Leica. The images were acquired with a Leica DC 300 F camera (Leica Microsystems, Heerbrugg, Switzerland) and processed using Leica IM 50- Image manager software.

Reproducibility of the results and statistical analysis

All experiments were repeated, independently, at least three times in duplicate ($n \geq 6$). The data reported are the mean values ± standard deviation (SD), presented with 95% confidence. The means values were subjected to one-way ANOVA followed by Tukey–Kramer multiple comparison method.

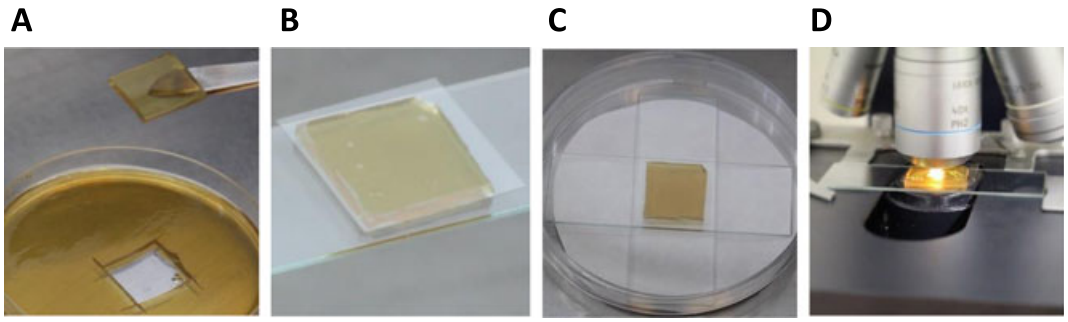

Figure 1. Overview of the main steps of the yeast slide culture technique. (A) A YPD agar block was cut using a sterile dissecting knife. (B) The agar block was placed in a microscopy slide; then, yeast cell suspension was poured and covered with a coverslip. (C) The slide culture was placed in a wetting chamber. (D) Observation of the slide culture under a light microscope. [Colour figure can be viewed at wileyonlinelibrary.com]

Results and discussion

Optimization of the slide culture technique

In the original slide culture technique, the yeast cell suspension was mixed with an equal volume of melted gelatine medium which was deposited on the surface of a haemocytometer *(15,16)*. More recently, different authors replaced the haemocytometer with a microscope slide. A small volume of molten YPD agar was poured over a slide to form a thin layer of the culture medium. Then, the cell suspension was pipetted onto the surface and a cover slip was placed over the sample *(17,18)*. This procedure originates small irregularities of medium surface, which makes the focus difficult and consequently the counting of single cells and micro-colonies. In order to overcome this difficulty, a defined volume of YPD was poured on Petri dishes and after solidification 20 × 20 mm medium blocks were cut (Fig. 1a). With this procedure, a flat surface culture medium was obtained.

The thickness of the medium blocks is an important issue. The medium should not be very thin for ensuring nutrient availability and reduced dehydration, which is particularly important for long incubation times (16–24 h). On the other hand, the medium should not be very thick to allow focusing with 100× objective. Preliminary experiments, using different volumes of YPD, considered that the use of 12.5 mL YPD, in 90 mm Petri dishes, was the optimal condition. For preventing the dehydration of culture medium, the microscope slide with the medium block was placed in a wetting chamber (Fig. 1C).

An important step in SCT is the quantification of cell concentration in an unknown sample. This can be carried out by direct microscopic counting, using a haemocytometer (counting chamber), or using electronic devices such as an electronic particles counter or flow or image cytometers *(1,19,20)*. The volume of the sample to be placed on the culture medium and the respective cell concentration conditioned the total number of cells deposited on the surface of the medium. It is possible to find in the literature the use of samples with different volumes and cell concentrations. For instance, Palermo et al. *(17)* used a sample of 5 μL containing 6×10^6 cells/mL and Jenkins et al. *(18)* used a sample of 10 μL containing 1×10^6 cells/mL. In the present work, samples of 10 or 20 μL containing different cell concentrations (5×10^4, 1×10^5 and 2×10^5 cells/mL) were tested. A sample of 20 μL containing 1×10^5 cells/mL was selected. These conditions allowed a better distribution of the cell suspension on the medium agar block. This cell density is high enough to facilitate micro-colony counting without their overlapping, after a long incubation time (16–24 h). With a sample of 20 μL containing 2×10^5 cells/mL, an overlapping of the micro-colonies after 20 h of incubation was observed.

The minimal time required to determine, accurately, the viability was also studied. Lodolo and Cantrell *(14)*, proposed a culture time of 4 h as ideal to discriminate different viabilities. The European Brewing Convention and the American Society of Brewing Chemists (ASBC) recommend an incubation time of 12–16 h *(10,21)*. Palermo et al. *(17)* determined the cell viability after 24 h. In the present work, healthy cells (in exponential phase of growth) were placed in YPD blocks and determined the percentage of micro-colonies at different times. As can be seen in Fig. 2(A), the percentage of viable cells determined after 6 h of incubation was significantly lower ($p < 0.01$) than that obtained after 16 h of incubation. For healthy cells (in exponential phase of growth), the viability did not increase after 16 h and was not significantly different ($p < 0.01$) between 16 and 24 h of incubation (Fig. 2A). Similar results were observed with cells in stationary phase of growth (data not shown). Together, these results confirm the need for a minimum incubation time of 16 h to assess yeast viability in an accurate way. According to ASBC *(10)*, the incubation should not be longer than 18 h; a higher incubation time could result in the underestimation of the percentage of viable cells. Owing to the big size, the micro-colonies (Fig. 2D) can become confluent. However, as stated previously, with a sample of 20 μL containing 1×10^5 cells/mL, no overlapping of micro-colonies was observed until 24 h. An incubation period higher than 16–18 h may be useful, particularly, in the case of stressed cells, which may require long periods of time to recover and start the division process.

Use of CFW

In the conventional PCT, the determination of viability depends of the direct counting of the colony-forming surviving cells and the estimation of the number of total cells. The slide culture technique has the advantage of simultaneously determining both reproducing (alive) and non-reproducing cells, reducing sampling errors *(11,16)*. However, the accuracy of the technique is dependent on the counting of all events (micro-colonies and single cells). Although it is easy to focus a micro-colony, the focus of a single cell (not viable), even in a flat surface, can be more problematic. To overcome this difficulty, the simultaneous culture and staining of yeast cells (using CFW), was carried out. CFW is usually known as an optical brightener since it is used as an additive in domestic washing detergents to improve the 'whiteness' of clothes *(22)*. This dye has a big affinity for the chitin of the yeast cell wall *(23,24)*.

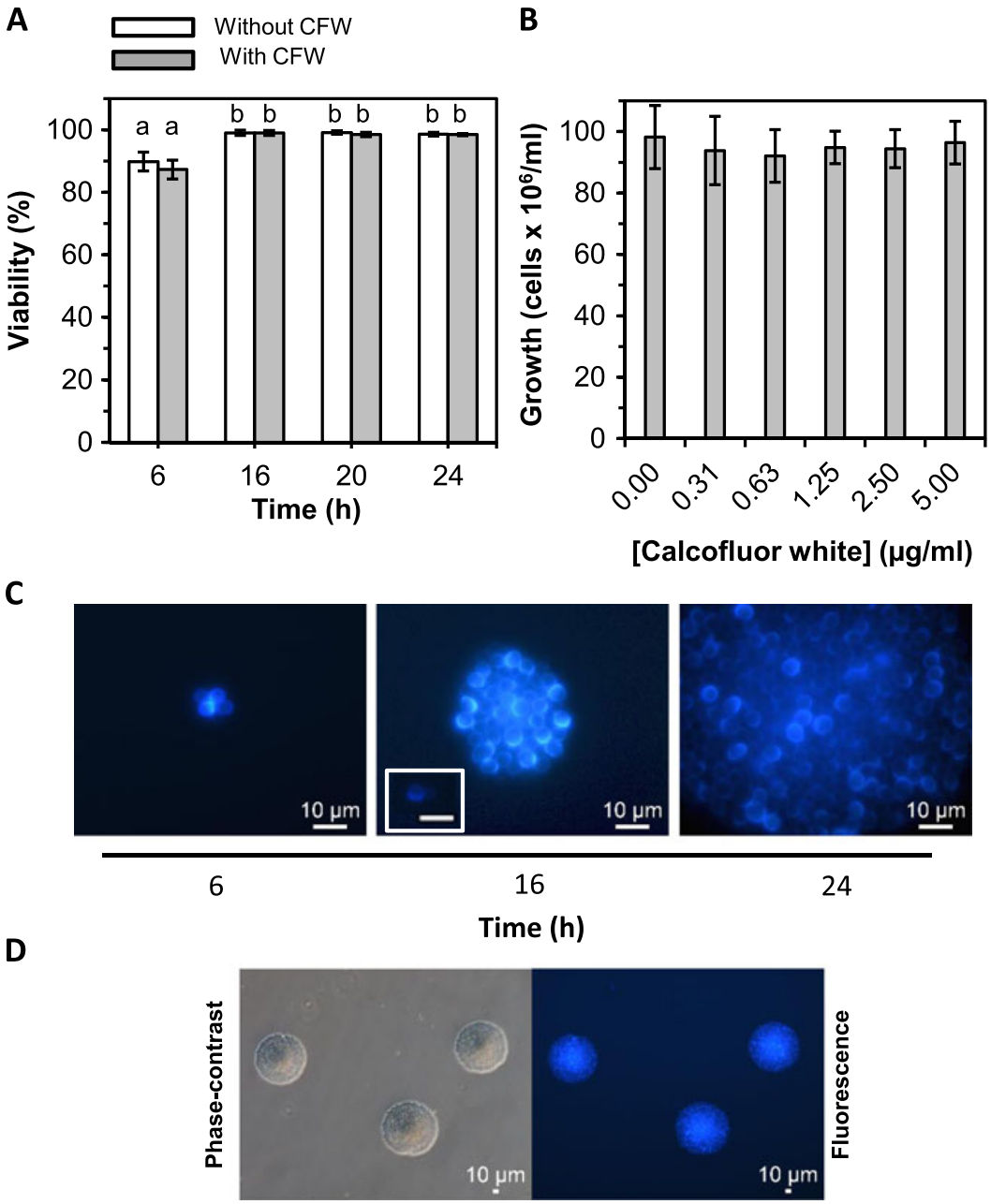

Figure 2. Effect of calcofluor white (CFW) on the growth of *Saccharomyces cerevisiae* BY4741. (A) Determination of viability by slide culture technique in the absence (without CFW) or in the presence of 2.5 µg/mL CFW (with CFW). (B) Yeast cells were incubated in YPD broth containing different concentrations of CFW. After 24 h of incubation the cell concentration was determined. (A) The means with different letters were significantly different ($p < 0.01$). (B) The means were not significantly different ($p < 0.01$). (C) Micrographs illustrative of the micro-colonies observed at different times by epifluorescence microscopy with a 100× N-Plan objective in YPD agar containing 2.5 µg/mL CFW. Insert at 16 h: dead cell. (D) Micrographs of the micro-colonies observed at 24 h with a 10× N-Plan objective. [Colour figure can be viewed at wileyonlinelibrary.com]

Once fixed to the chitin, the CFW presents a strong blue fluorescence when exposed to UV light, which facilitates the focusing of micro-colonies and single cells (Fig. 2C and D).

It is known that CFW, at 0.5–1 mg/mL, exhibits an antifungal activity provoking an abnormally thick septa between mother and daughter yeast cells *(25)*. However, no growth inhibition occurred when cells were exposed to 10 µg/mL *(26)*. In order to exploit the staining ability of CFW, yeast cells were exposed to different CFW concentrations (0.3–5.0 µg/mL). In the concentration range tested, the growth of the yeast *S. cerevisiae,* in the presence or absence of CFW, was not significantly different ($p < 0.01$; Fig. 2B). CFW concentrations <2.5 µg/mL resulted in a partial staining of the yeast cell wall. With 5 µg/mL some abnormal cell structures were observed. These results suggest that 2.5 µg/mL CFW is a suitable concentration to stain yeast cells. The incubation of yeast cells in YPD containing 2.5 µg/mL CFW did not inhibit cell growth, the cell wall was well stained and no morphological modifications of yeast cells were observed. The viability of healthy cells (in exponential or stationary phase of growth), assessed by SCT, in the absence or the presence of 2.5 µg/mL CFW was not significantly different ($p < 0.01$; Fig. 2A; data not shown for cells in stationary phase of growth). These results strongly suggest that CFW at 2.5 µg/mL can be used in SCT since it allows the staining of the yeast cell wall without apparent toxic effects. These results

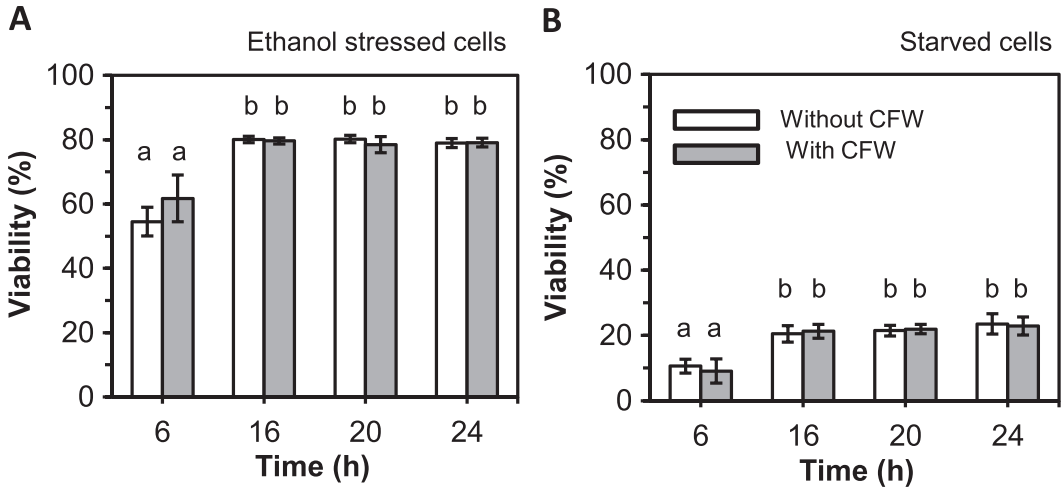

Figure 3. Influence of the incubation time on the percentage of viable cells of *S. cerevisiae* BY4741 exposed to stress conditions. (A) Yeast cells were exposed to 20% (*v*/v) ethanol. (B) Yeast cells were incubated in water for 48 h (starved cells). The percentage of viable cells was determined by slide culture technique in the absence (without CFW) or in the presence of 2.5 μg/mL CFW (with CFW). The means with different letters were significantly different ($p < 0.01$).

prompted us to evaluate the impact of 2.5 μg/mL CFW in the viability assessment of stressed cells.

Ethanol stress and starvation are two common stresses imposed on yeast strains used in the production of alcoholic beverages (wine and beer) as well as in the bio-ethanol production *(27,28)*. Thus, yeast cells were starved or submitted to ethanol stress. After treatment, cell viability was assessed by SCT in the absence or the presence of 2.5 μg/mL CFW. As was observed with healthy cells, an incubation period for 6 h leads to an underestimation of the yeast viability (Fig. 3). After 16 h of incubation, the viability of stressed populations was not significantly different ($p < 0.01$) from that observed after 24 h, with or without CFW (Fig. 3). These results suggests that: (a) a minimum of 16 h is required to assess yeast viability by SCT; (b) the presence of 2.5 μg/mL CFW facilitates the counting of isolated cells (dead cells) and micro-colonies (live cells); and (c) it did not affect the viability determination. The staining of *S. cerevisiae* with CFW can be useful in the semi-automatization of the technique. This can be done with automated microscope image acquisition combined with image processing methods as was described for microscopy cell counting *(29,30)*.

Possible weaknesses of SCT can be identified, such as the susceptibility to contamination. However, the performance of the main manipulations, such as the preparation of slides with an agar block on a clean bench and the incubation in a sterile Petri dish, reduces the possibility of contamination; in addition, during the microscopic observation, the samples are protected with a sterile coverslip, which also minimizes the possibility of contamination. The use of a fluorescence microscope can also be a possible disadvantage, particularly in small breweries; however, the advent of LED technology certainly will decrease the price of this equipment.

Validation of the results obtained by the slide culture technique

To validate the results obtained by SCT with or without CFW, the viability of healthy and stressed cells was determined by PCT after 72 h of incubation at 25°C. The viability of healthy and ethanol stressed cells assessed by PCT and SCT, with or without CFW, was not significantly different ($p < 0.01$). However, in the case of starved cells the viability determined by SCT was underestimated

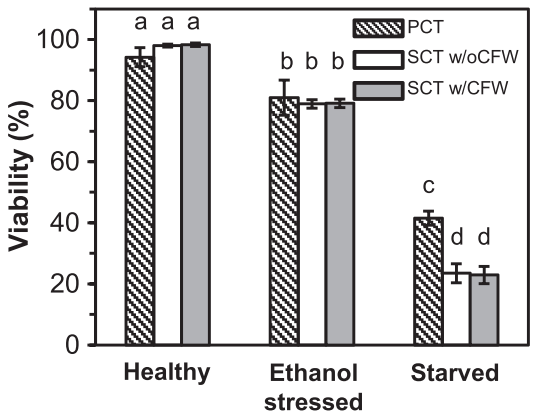

Figure 4. Comparison of the results obtained by slide culture technique (SCT) with plate count technique (PCT). The viability of cells in exponential growth-phase (healthy) or ethanol-stressed or starved cells (as described in Fig. 3) was evaluated by PCT or by SCT without (SCT w/o CFW) or with 2.5 μg/mL CFW (SCT w/CFW). The means with different letters were significantly different ($p < 0.01$).

compared with the viability determined by PCT (Fig. 4). Probably, some of the starved cells require a higher time of incubation (more than 24 h – the time used in SCT) to start the division process, owing to low internal glycogen reserves, or necessitate a long period of time to repair cell damage *(8)*.

Conclusions

The present work standardizes and improves the SCT. Using a thin YPD agar block of 20 × 20 mm, a yeast sample of 20 μL containing 1×10^5 cells/mL, allowed viability results to be obtained after 16–24 h, with healthy and ethanol stressed cells that did not differ significantly from those observed by PCT after 72 h of growth. The inclusion of 2.5 μg/mL CFW in YPD medium benefited the counting of individual cells and micro-colonies, without influencing the viability results. The SCT with CFW is a fast (results obtained in 16–24 h), accurate method (giving comparable results to those obtained with PCT) with reduced cost (comparatively with PCT, reduces the volume of culture medium and the costs associated

with disposable Petri dishes). This technique seems a reliable alternative to PCT for the evaluation of yeast viability. The staining of yeast cells with CFW facilitates the semi-automatization of the process (by using an automated microscope image acquisition combined with image processing methods, such as time-lapse photomicroscope) and allows an increased number of samples to be processed, which might be useful in high-throughput evaluation of yeast viability. For these reasons, the SCT here improved can be of great value in the assessment of viability, both at fundamental and at industrial level, or in the checking of new methods for yeast viability determination.

Acknowledgements

This study was supported by the Portuguese Foundation for Science and Technology under the scope of the strategic founding of UID/BIO/04469/2013 unit and COMPETE 2020 (POCI-01-0145-FEDER-006684) and the Project RECI/BBB-EBI/0179/2012 (FCOMP-01-0124-FEDER-027462). Manuela D. Machado and Cátia A. Sousa gratefully acknowledge the post-doctoral (SFRH/BPD/72816/2010) and the doctoral grants (SFRH/BD/101452/2014), from FCT, respectively.

Conflict of interest

The authors declare no conflict of interest.

References

1. Boulton, C., and Quain, D. (2001) *Brewing Yeast and Fermentation*, Blackwell Science, Oxford.
2. da Silva, T. L., Roseiro, J. C., and Reis, A. (2012) Applications and perspectives of multi-parameter flow cytometry to microbial biofuels production processes, *Trends Biotechnol. 30*, 225–232.
3. Gibson, B. R., Lawrence, S. J., Leclaire, J. P. R., Powell, C. D., and Smart, K. A. (2007) Yeast responses to stresses associated with industrial brewery handling, *FEMS Microbiol. Rev. 31*, 535–569.
4. Belini, V. L., Wiedemann, P., and Suhr, H. (2013) In situ microscopy: A perspective for industrial bioethanol production monitoring, *J. Microbiol. Meth. 93*, 224–232.
5. Karathia, H., Vilaprinyo, E., Sorribas, A., and Alves, R. (2011) *Saccharomyces cerevisiae* as a model organism: A comparative study, *PLoS One 6*, 1–10.
6. Wloch-Salamon, D. M., and Bem, A. E. (2013) Types of cell death and methods of their detection in yeast *Saccharomyces cerevisiae*, *J. Appl. Microbiol. 114*, 287–298.
7. Davey, H. M. (2011) Life, death, and in-between: Meanings and methods in microbiology, *Appl. Environ. Microb. 77*, 5571–5576.
8. Heggart, H., Margaritis, A., Stewart, R. J., Pikington, H., Sobczak, J., and Russell, I. (2000) Measurement of brewing yeast viability and vitality: A review of methods, *Tech. Q. Master Brew. Assoc. Am. 37*, 409–430.
9. Munoz, A. J., Wanichthanarak, K., Meza, E., and Petranovic, D. (2012) Systems biology of yeast cell death, *FEMS Yeast Res. 12*, 249–265.
10. American Society of Brewing Chemists (1984) Report of the subcommittee on microbiology, *J. Am. Soc. Brew. Chem. 42*, 132–134.
11. Mirisola, M. G., Braun, R. J., and Petranovic, D. (2014) Approaches to study yeast cell aging and death, *FEMS Yeast Res. 14*, 109–118.
12. Howard, G. A. (1971) Recommended methods of analysis, *J. Inst. Brew. 77*, 181–226.
13. Pringle, J. R., and Mor, J. R. (1975) Methods for monitoring the growth of yeast cultures and for dealing with the clumping problem, in *Methods in Cell Biology*, (Prescott, D. M. Ed.), *XI*, pp. 131–168, Academic Press, New York.
14. Lodolo, E. J., and Cantrell, I. C. (2007) Yeast vitality – A holistic approach toward an integrated solution to predict yeast performance, *J. Am. Soc. Brew. Chem. 65*, 202–207.
15. Pierce, J. S. (1970) Measurement of yeast viability, *J. Inst. Brew. 76*, 442–443.
16. Gilliland, R. B. (1959) Determination of yeast viability, *J. Inst. Brew. 65*, 424–429.
17. Palermo, V., Falcone, C., Calvani, M., and Mazzoni, C. (2010) Acetyl-L-carnitine protects yeast cells from apoptosis and aging and inhibits mitochondrial fission, *Aging Cell 9*, 570–579.
18. Jenkins, D. M., Powell, C. D., Fischborn, T., and Smart, K. A. (2011) Rehydration of active dry brewing yeast and its effect on cell viability, *J. Inst. Brew. 117*, 377–382.
19. Chan, L. L., Lyettefi, E. J., Pirani, A., Smith, T., Qiu, J., and Lin, B. (2011) Direct concentration and viability measurement of yeast in corn mash using a novel imaging cytometry method, *J. Ind. Microbiol. Biot. 38*, 1109–1115.
20. Madigan, M. T., Martinko, J. M., Bender, K. S., Buckley, D. H., and Stahl, D. A. (2012) *Brock Biology of Microorganisms*, Pearson Education, San Francisco, CA.
21. European, Brewing, and Convention (1962) Estimation of yeast viability, *J. Inst. Brew. 68*, 14–20.
22. Davey, H. M., and Kell, D. B. (1997) Fluorescent brighteners: Novel stains for the flow cytometric analysis of microorganisms, *Cytometry 28*, 311–315.
23. Costa-de-Oliveira, S., Silva, A. P., Miranda, I. M., Salvador, A., Azevedo, M. M., Munro, C. A., Rodrigues, A. G., and Pina-Vaz, C. (2013) Determination of chitin content in fungal cell wall: An alternative flow cytometric method, *Cytometry A 83A*, 324–328.
24. Pringle, J. R. (1991) Staining of bud scars and other cell-wall chitin with calcofluor, *Meth. Enzymol. 194*, 732–735.
25. Roncero, C., Valdivieso, M. H., Ribas, J. C., and Dúran, A. (1988) Isolation and characterization of *Saccharomyces cerevisiae*: Mutants resistant to calcofluor white, *J. Bacteriol. 170*, 1950–1954.
26. Boorsma, A., de Nobel, H., ter Riet, B., Bargmann, B., Brul, S., Hellingwerf, K. J., and Klis, F. M. (2004) Characterization of the transcriptional response to cell wall stress in *Saccharomyces cerevisiae*, *Yeast 21*, 413–427.
27. Ben Chaabane, F., Aldiguier, A. S., Alfenore, S., Cameleyre, X., Blanc, P., Bideaux, C., Guillouet, S. E., Roux, G., and Molina-Jouve, C. (2006) Very high ethanol productivity in an innovative continuous two-stage bioreactor with cell recycle, *Bioprocess Biosyst. Eng. 29*, 49–57.
28. Lodolo, E. J., Kock, J. L. F., Axcell, B. C., and Brooks, M. (2008) The yeast *Saccharomyces cerevisiae* – The main character in beer brewing, *FEMS Yeast Res. 8*, 1018–1036.
29. Geissmann, Q. (2013) OpenCFU, a new free and open-source software to count cell colonies and other circular objects, *PLoS One 8*, 1–10.
30. Piccinini, F., Tesei, A., Paganelli, G., Zoli, W., and Bevilacqua, A. (2014) Improving reliability of live/dead cell counting through automated image mosaicing, *Comput. Meth. Prog. Biol. 117*, 448–463.