ISSN 0026-2617, Microbiology, 2006, Vol. 75, No. 2, pp. 154–160. © Pleiades Publishing, Inc., 2006.
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EXPERIMENTAL ARTICLES

The Viability Assessment of Ethanol-Producing Yeast by Computer-Aided Fluorescence Microscopy

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Received June 1, 2005; in final form, September 22, 2005

Abstract—A vital staining of the ethanol-producing yeast *Saccharomyces cerevisiae* with ethidium bromide and DAPI allows intact and damaged cells to be differentiated by fluorescence microscopy. A computer image analysis procedure is developed for the automatic determination of the relative number of damaged cells using ImageJ software (National Institute of Health, United States; http://rsb.info.nih.gov./ij/). A good correlation has been found between the viability rates determined by the plate count method and the relative numbers of intact cells assessed by the developed procedure in the dry-yeast preparations rehydrated under various conditions.

DOI: 10.1134/S0026261706020081

Key words: ethanol-producing yeast, *Saccharomyces cerevisiae*, fluorescence microscopy, viability, computer-aided image analysis.

The efficiency of production processes based on the fermenting ability of yeasts (such as ethanol production) is largely determined by the relative content of viable cells in the process culture (i.e., its viability). A microbiological assessment of this parameter from the reproduction ability of cells is cumbersome and time-consuming. This problem can be solved either through an improvement of existing cultural methods or through an employment of indirect methods of cell viability assessment [1–3].

The use of fluorescent dyes (fluorochromes, fluorescent probes) forms the basis of three types of methods for the differentiation of damaged (nonviable) and intact (viable) cells: flow cyto(fluoro)metry [4], fluorometry [5], and fluorescence microscopy [2]. The drawback of flow cyto(fluoro)metry is high cost of the required equipment and the use of fluorochromes, whose excitation is only possible in the visible spectral range. The fluorometry of cell suspensions often provides ambiguous results, since changes in the integral fluorescence of samples can reflect processes occurring both in the whole population and in subpopulations [6]. Conventional fluorescence microscopy is a cumbersome, time-consuming, and subjectivistic technique, which can be improved by obtaining digital images of cells and developing special computer programs in order to analyze these images [7].

This study was undertaken to develop a procedure for counting damaged cells by fluorescence microscopy, associated with a computer image analysis, and an evaluation of its applicability to the assessment of culture viability.

MATERIALS AND METHODS

Experiments were carried out with *Saccharomyces cerevisiae* cells present in dry commercial Fermiol preparation (DSM Food Specialties Beverage Ingredients, The Netherlands).

Cultivation and rehydration conditions. Cultivation was performed in a sterile wort (13°B) with pH ranging from 2.5 to 4.5. Dry yeast cells were rehydrated by placing 10 mg of Fermiol into 100-ml Erlenmeyer flasks containing 20 ml of sterile wort. The flasks were incubated at 30°C for 2–24 h. To obtain 100% viable cultures, the suspension of rehydrated cells was diluted twenty-fold with fresh wort (pH 4.5) and incubated for 12–24 h under the same conditions as indicated above.

Viability assay. The concentration of viable cells in a suspension was estimated by plating ten-fold serial dilutions of this suspension on 1.5% agar plates containing wort (7°B). The total concentration of yeast cells was determined under a Carl Zeiss light microscope (Germany) using a Goryaev counting chamber (magnification, 200×). A mother cell with a bud or an undetached daughter cell was considered as one cell. The viability of a cell culture was defined as the ratio of the concentration of viable cells to the total concentration of cells in this culture.

Preparation of specimens for microscopy. Cells were harvested by centrifugation, washed with distilled water, and resuspended in water. The suspension was

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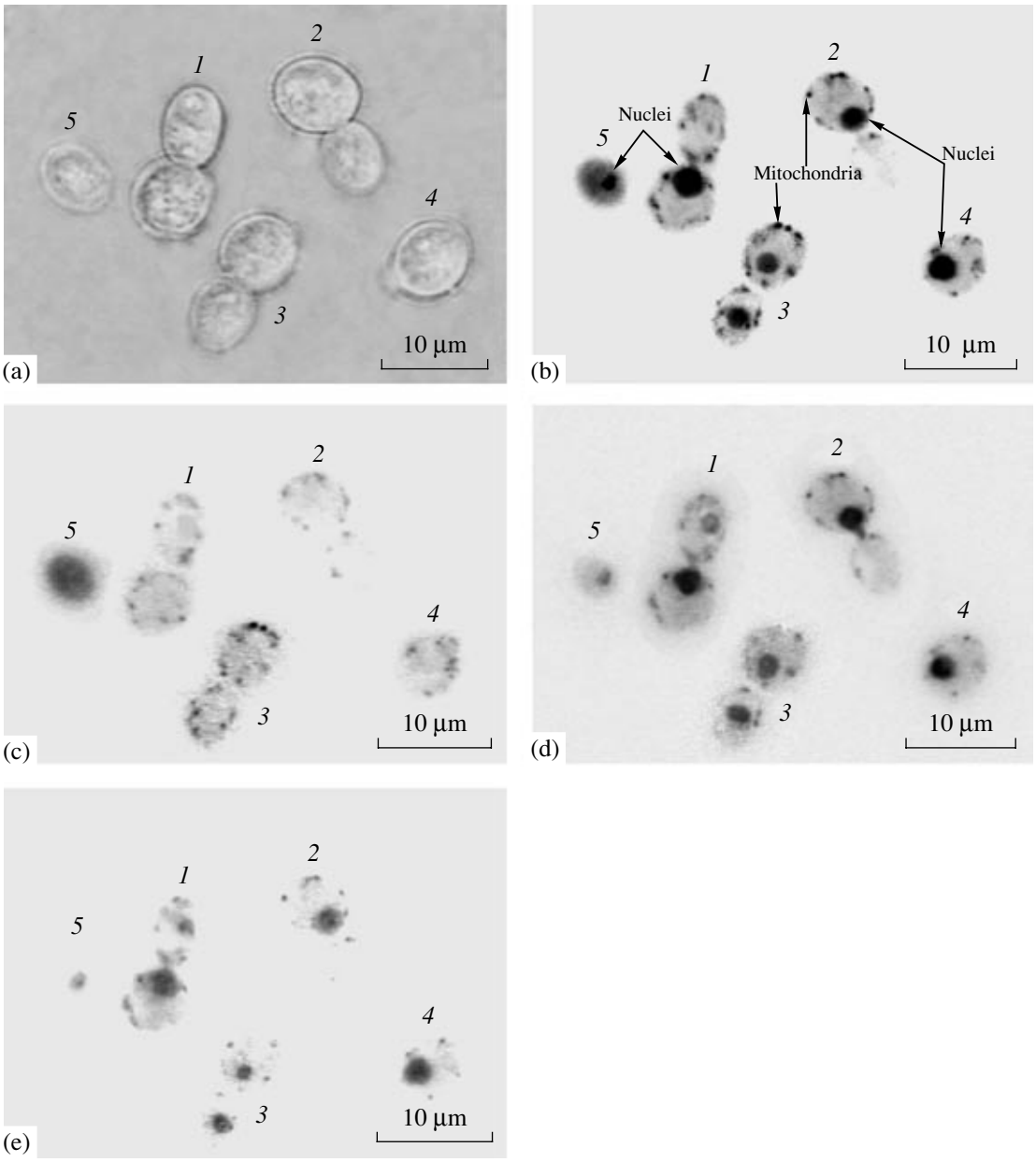

Fig. 1. *S. cerevisiae* cells from the Fermiol preparation stained with ethidium bromide and DAPI and observed by (a) light microscopy and (b) fluorescence microscopy. Panels c–e show, respectively, the red, green and blue components of the fluorescence images of yeast cells. After rehydration, the cells were incubated in wort (13°B) at pH 4.5 for 24 h. Magnification, ×900. *1–5,* see text.

supplemented with ethidium bromide (3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide) at a concentration of 50 μM and DAPI (4,6-diamidino-2-phenylindole, dilactate) at a concentration of 15 μM. Ethidium bromide and DAPI were purchased from Sigma (United States) and Serva (Germany), respectively. After 30 min of incubation at 20–23°C, 5-μl aliquots of this suspension were placed on specimen slides, covered with coverslips, and sealed with a nail lacquer on the periphery of the coverslips. All of these procedures were performed under dull illumination in order to prevent dye bleaching. The microscopic specimens thus prepared retained their principal characteristics for at least 12 h.

Fluorescence microscopy. Experiments were carried out on an ML-2 microscope (LOMO, Russia), equipped with a DSC-S85 digital camera (Sony, Japan), and two objectives, 40 × 0.95 and 90 × 1.30 (Carl Zeiss, Germany). The light source was a DRSh-250 mercury lamp. In the light transmission mode of operation, the microscope was furnished with four glass filters (SZS-20, ZhS-18, NS-8, and NS-3) in front of an object and one glass filter (ZhS-3) behind it. In the fluorescence mode of operation, a UFS6-3 filter was

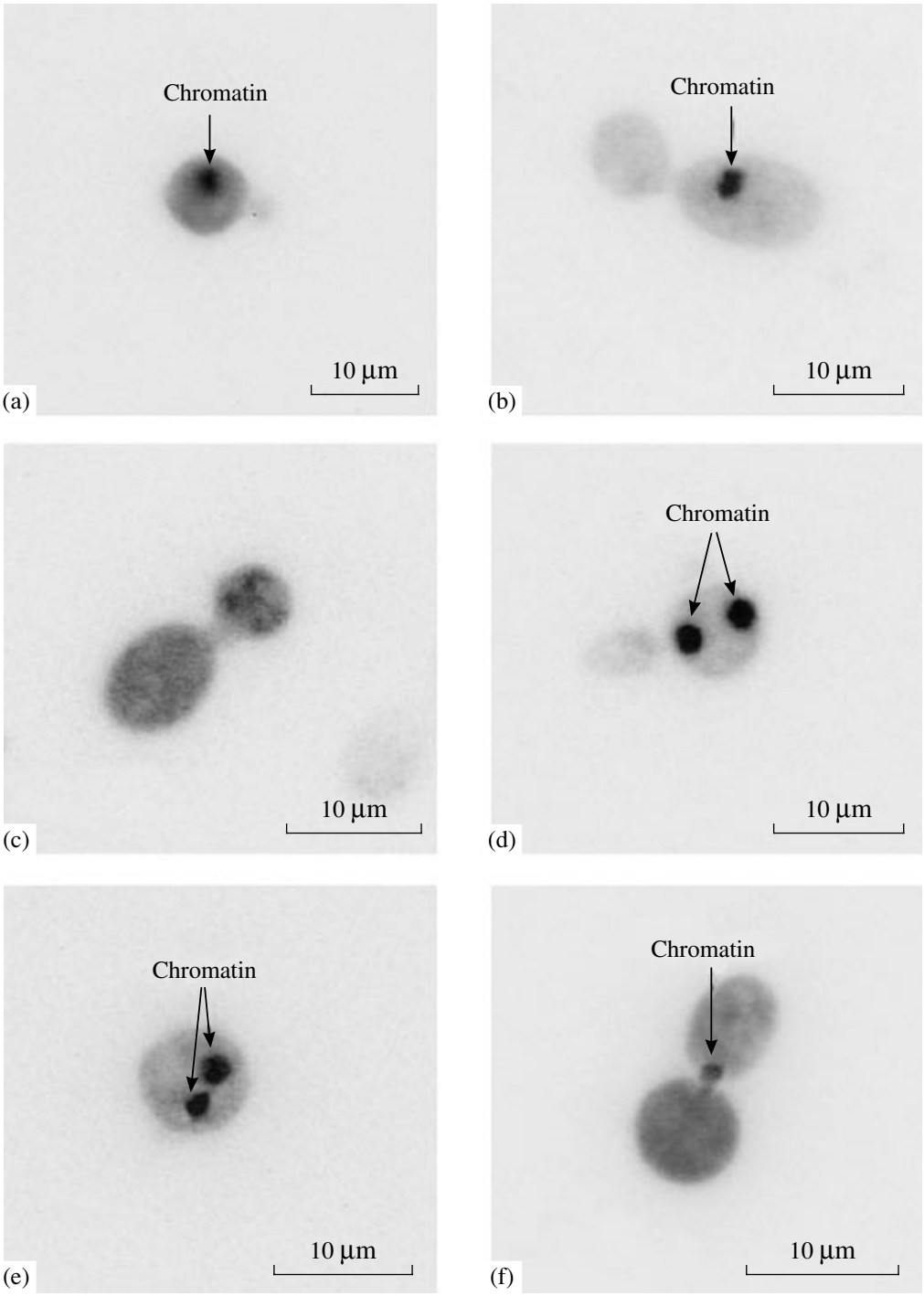

Fig. 2. The main morphotypes of damaged *S. cerevisiae* cells from a Fermiol preparation stained with ethidium bromide and DAPI and visualized by fluorescence microscopy at a magnification of 900×. After rehydration, the cells were incubated in wort (13°B) for 6 h.

installed in the excitation light path to induce fluorescence with the 366-nm mercury line. In most experiments, fluorescence was recorded at wavelengths above 400 nm (ZhS-3 filter). However, in some cases, it was recorded at wavelengths above 600 nm (KS-11 filter).

Computer image analysis. The digital images of cells were processed by the aid of Adobe Photoshop, v. 8.0 (Adobe Systems Inc., United States) and ImageJ software, v. 1.32 (National Institute of Health, United States; http://rsb.info.nih.gov/ij). The images were split into red, green, and blue components (pseudospectral analysis), with the RGB split option of ImageJ. For this publication, the color images were transformed into inverted black-and-white images.

The data obtained were statistically processed using MS Office calculator software. The table and the figures show the representative data of the experiments, which were performed at least in triplicate.

RESULTS AND DISCUSSION

Detection of damaged yeast cells using vital staining with ethidium bromide and DAPI. Fluorescence microscopy of rehydrated yeast cells from the Fermiol preparation, stained with ethidium bromide and DAPI, revealed the presence of two types of cells. Some cells showed blue–green fluorescence tinted red on the cell periphery (Fig. 1b, cells 1–4), whereas other cells showed bright orange–red fluorescence distributed evenly over the cytoplasm (Fig. 1a, cell 5). In a cell culture with an almost 100% level of viability, more than 99% of cells showed a fluorescence of the first type, and less than 1% of cells showed fluorescence of the second type. After heating this culture in the cultivation medium at 60°C for 5 min or subjecting it to two freezing (–70°C) and thawing cycles, culture viability fell to zero, and all cells in the culture showed orange–red fluorescence. These data indicates that the first type of fluorescence is typical of intact cells and the second type is typical of damaged cells.

According to the available data [8], DAPI can interact with the mitochondrial and nuclear DNA of intact yeast cells, causing them to fluoresce in the blue–green region. Ethidium can interact with the mitochondrial DNA (but not nuclear) of intact yeast cells, causing their mitochondria to fluoresce in the orange–red region [9].

The computer pseudospectral analysis of the fluorescent cells showed that DAPI stained both the mitochondria and the nuclei of these cells (blue and green components), whereas ethidium was detected only in the mitochondria (red component) (Figs. 1c–1e). When intact yeast cells were stained with DAPI alone, the cell staining pattern was the same as in the case of combined staining with DAPI and ethidium bromide. In contrast, when intact cells were stained with ethidium bromide alone, the cells did not fluoresce. Taking into account the design of fluorescence microscopy in our experiments (see the Materials and Methods section) and the spectral properties of DAPI and ethidium [10], we could suggest that the fluorescence of ethidium in the mitochondria of cells stained with DAPI and ethidium bromide is due to energy transfer [11] from DAPI to ethidium, in the sites of their joint localization [11]. This suggestion received direct confirmation from the experiments on isolated DNA [12].

The fluorescence microscopy of damaged cells revealed the presence of several cell morphotypes (Fig. 2), which largely differed in the structure of nuclear chromatin (visualized with DAPI), when present. Mitochondria in the damaged cells were not visible. The cytoplasm of such cells showed diffuse orange–red fluorescence, which was observed irrespective of whether cells were stained with ethidium bromide alone or in combination with DAPI. In general, damaged cells accumulated much more ethidium than the intact cells did.

In order to understand the difference in the staining mechanisms of intact and damaged cells, one should consider the following. First, there is evidence that the cytoplasmic membrane of yeast cells has a transport system similar to the ATP-dependent system of multiple drug resistance in higher eukaryotes [13]. Such a system provides for the excretion of cationic organic molecules, including ethidium, from the cells that the molecules have penetrated by means of diffusion. This excretion reduces the level of ethidium in intact cells. Second, as shown in experiments with isolated mitochondria [14] and bacteria [15], ethidium cation is distributed in membrane systems according to the membrane potential. With this in mind, the staining of mitochondria with ethidium in intact cells can be conceived as an equilibrium result of the following processes: (a) the transport of this dye into and from cells through the cytoplasmic membrane, (b) the accumulation of part of the absorbed dye molecules in mitochondria, and (c) their binding by mitochondrial DNA. The energy status of cells plays an important part in the process of staining, since the ATP level influences the rate of ethidium excretion through the cytoplasmic membrane, while the membrane potential of mitochondria favors the accumulation of ethidium in these organelles.

The mechanism of intact yeast cell staining with DAPI is different and so far poorly understood. We only know that the permeation of DAPI to its binding molecules (mitochondrial and nuclear DNA) in cells is less dependent on their energy status than in the case of ethidium.

In this context, the uptake of ethidium by damaged cells can be due to the impairment of the barrier function of the cytoplasmic membrane and/or the inhibition of the energy metabolism of such cells. The substances that bind ethidium in damaged cells are unknown. It should be noted that only the amphiphilic ethidium cation has an affinity for hydrophobic cellular structures [16] and polyanions [17] and that the quantum yield of its fluorescence considerably increases in nonpolar media [18].

Evaluation of the viability of yeast populations with the aid of ImageJ software. Fluorescence microscopy of cells stained with ethidium bromide and DAPI allows one to count intact and damaged cells separately. This count can be carried out manually over the digital color images of microscopic fields, using the *Cell counter* plugin of the ImageJ program.

The red component of the fluorescence of ethidium in damaged cells is much more intense than that in intact cells (Figs. 1c, 3c), due to which fine tuning of the contrast in the red channel of the Brightness/Contrast option causes only damaged cells to become visible (Fig. 3d). The green component of DAPI fluorescence is almost the same in intact and damaged cells

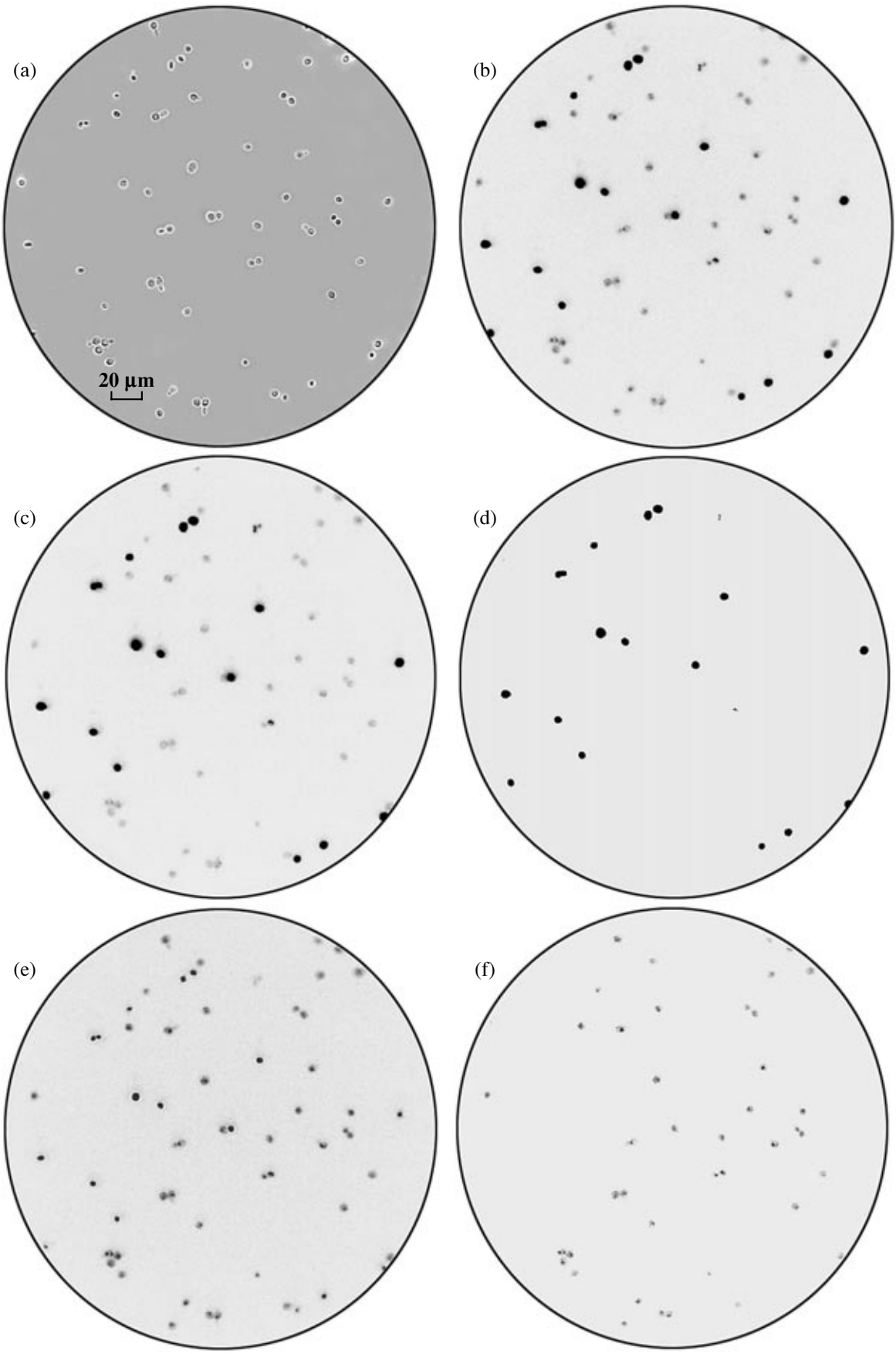
(a)
20 µm
(b)
(c)
(d)
(e)
(f)

Fig. 3. *S. cerevisiae* cells from the Fermiol preparation stained with ethidium bromide and DAPI and visualized by (a) light and (b) fluorescence microscopy. Panels c, e, and f show, respectively, the red, green, and blue components of the fluorescence images of yeast cells. Panel d shows the red component of the fluorescence image of yeast cells after contrast adjustment. Following rehydration, the cells were incubated in wort (13°B) at pH 4.5 for 24 h. Magnification, 400×.

Comparison of the viability rates determined by the plate count method with the relative numbers of intact cells assessed by the developed microscopic method in the same samples of the Fermiol preparation rehydrated under various conditions

Viability	Sample A rehydrated for						Sample B rehydrated for			
	2 h		4 h		24 h		4 h		24 h	
	pH 4.5	pH 2.5	pH 4.5	pH 2.5	pH 4.5	pH 2.5	pH 4.5	pH 2.5	pH 4.5	pH 2.5
K_{cul}, %	63 ± 16	48 ± 18	55 ± 12	40 ± 15	110 ± 20	70 ± 10	6 ± 1	5 ± 2	48 ± 20	3 ± 1
K_{fl}, %	54 ± 8	30 ± 5	40 ± 10	25 ± 10	99 ± 0.5	58 ± 18	2 ± 0.8	2 ± 0.5	40 ± 18	1 ± 0.5

Note: After a container with the Fermiol preparation had been opened, sample A was stored at 4°C for no longer than 2 weeks, whereas sample B was stored aerobically at 14°C for 2 months before use. K_{cul} is the viability rate determined by cultural methods (plate counts). K_{fl} is the relative number of intact cells assessed by the developed microscopic method (see Fig. 3 and text).

(Figs. 1d, 3e). The blue component of DAPI fluorescence is faint, if at all, in virtually all damaged cells (Figs. 1e, 3f), which is probably due to an energy transfer from DAPI to ethidium (see above). Thus, the resolution of the initial color image of fluorescent cells into red, green, and blue components makes it possible to visualize and automatically count damaged cells via the red channel and both damaged and intact cells via the green channel, in one microscope field with the aid of the Analyze Particles option.

Alternatively, if a black-and-white CCD camera is used, the relative number of damaged cells can automatically be determined by separately recording cell images in the total fluorescence mode, with the ZhS-3 filter in the recording channel and in the red fluorescence mode with the KS-11 filter in the recording channel.

In our experiments, the automatic and manual counts of damaged cells agreed to an accuracy of 12%. It should be noted that the results of automatic count considerably depend on the quality of the original cell images, which depends on specimen properties (such as the relative number of aggregates in the specimen), the stability and evenness of the illumination of the microscopic field, and the degree of the chromatic aberration of the objectives.

A comparison of the viability rates of cultures, determined by the plate count method, and the relative numbers of intact cells, determined in the same cultures by the developed procedure, showed a good correlation between these parameters (see table), thereby indicating a possibility of using this procedure for the assessment of the viability of rehydrated Fermiol preparations. It should be noted, however, that fluorescence microscopy underestimates the viability of yeast populations by 10–15%, compared to cultural methods (table). This disagreement can probably be explained by the fact that some damaged cells may recover during the subsequent cultivation of rehydrated cells.

The main advantage of the proposed method is that it allows rapid assessment of cell viability. To illustrate, an analysis of the viability of one rehydrated Fermiol preparation, containing no less than 300 cells, takes no more than 2 h. The aforementioned underestimation of the viability of yeast populations, compared to cultural methods, can be taken into account during calculations.

The developed method can probably be used for an analysis of other microbial objects; these may require other fluorochromes with a fluorescence spectra that differs sufficiently to be resolved by either conventional filter sets or by a RGB-split of ImageJ.

ACKNOWLEDGMENTS

We are grateful to V.A. Yashin and K.S. Laurinavichyus for technical help and fruitful discussions, as well as to N.N. Martynenko for supplying the Fermiol preparation and for partial financial support of this work.

REFERENCES

1. Postgate, J.R., Viability Measurements and the Survival of Microbes under Minimum Stress, *Adv. Microb. Physiol.*, Rose, A.H. and Wilkinson, J.F., Eds., London: Academic, 1967, pp. 1–24.
2. Jones, R.P., Measures of Yeast Death and Deactivation and Their Meaning, *Process Biochem.*, 1987, vol. 22, no. 4, pp. 118–128.
3. Puchkov, E.O., Methods for Determining the Amount and Viability of Microorganisms, *Biotekhnol.*, 1988, vol. 4, no. 1, pp. 132–142.
4. Breeuwer, P., Drocourt, J.L., Rombouts, F.M., and Abee, T., Energy-Dependent, Carrier-Mediated Extrusion of Carboxyfluorescein from *Saccharomyces cerevisiae* Allows Rapid Assessment of Cell Viability by Flow

Cytometry, *Appl. Environ. Microbiol.*, 1994, vol. 60, no. 5, pp. 1467–1472.

5. Oh, K.B. and Matsuoka, H., Rapid Viability Assessment of Yeast Cells Using Vital Staining with 2-NBDG, a Fluorescent Derivative of Glucose, *Int. J. Food Microbiol.*, 2002, vol. 76, nos. 1-2, pp. 47–53.
6. Puchkov, E.O. and Melkozernov, A.N., Fluorimetric Assessment of *Pseudomonas fluorescens* Viability after Freeze-Thawing Using Ethidium Bromide, *Lett. Appl. Microbiol.*, 1995, vol. 21, pp. 368–372.
7. Brehm-Stecher, B.F. and Johnson, E., Single-Cell Microbiology: Tools, Technologies, and Applications, *Microbiol. Mol. Biol. Rev.*, 2004, vol. 68, no. 3, pp. 538–559.
8. Williamson, D.H. and Fennell, D.J., Visualization of Yeast Mitochondrial DNA with the Fluorescent Stain DAPI, *Methods Enzymol.*, 1979, vol. 56, pp. 728–733.
9. Borodina, V.M. and Meisel', M.N., Binding of Ethidium Bromide to the Nucleic Acids of Viable Microorganisms, *Dokl. Akad. Nauk SSSR*, 1974, vol. 216, no. 5, pp. 1168–1170.
10. Haugland, R.P., *Handbook of Fluorescent Probes and Research Chemicals*, Oregon: Molecular Probes, 2004 (http://www.probes.com/handbook).
11. Lakowicz, J.R., in *Principles of fluorescence spectroscopy*, Lakowicz, J.R., Ed., New York: Plenum, 1983, pp. 211–213.
12. Boger, D.L., Fink, B.E., Brunette, S.R., Tse, W.C., and Hedrick, M.P., A Simple, High-Resolution Method for Establishing DNA Binding Affinity and Sequence Selectivity, *J. Am. Chem. Soc.*, 2001, vol. 123, no. 25, pp. 5878–5891.
13. Ehrenhofer-Murray, A.E., Seitz, M.U., and Sengstag, C., The SGE1 Protein of *Saccharomyces cerevisiae* Is a Membrane-Associated Multidrug Transporter, *Yeast,* 1998, vol. 14, no. 1, pp. 49–65.
14. Rottenberg, H., Membrane Potential and Surface Potential in Mitochondria: Uptake and Binding of Lipophilic Cations, *J. Membr. Biol.*, 1984, vol. 81, no. 2, pp. 127–138.
15. Puchkov, E.O. and Melkozernov, A.N., Electrodiffusion of Ethidium Cation into *Micrococcus luteus* Cells, *Biochim. Biophys. Acta*, 1994, vol. 1192, no. 1, pp. 112–116.
16. Gitler, C., Rubalcava, B., and Caswell, A., Fluorescence Changes of Ethidium Bromide on Binding to Erythrocyte and Mitochondrial Membranes, *Biochim. Biophys. Acta*, 1969, vol. 193, no. 2, pp. 479–481.
17. Kido, N., Ohta, M., and Kato, N., Detection of Lipopolysaccharide by Ethidium Bromide Staining after Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis, *J. Bacteriol.,* 1990, vol. 172, no. 2, pp. 1145–1147.
18. LePecq, J.-B. and Paoletti, C., A Fluorescent Complex between Ethidium Bromide and Nucleic Acids, *J. Mol. Biol.*, 1967, vol. 27, no. 1, pp. 87–106.