Comparison of methods used for assessing the viability and vitality of yeast cells

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Abstract
Determination of cell viability is the most commonly used method for assessing the impact of various types of stressors in toxicity research and in industrial microbiology studies. Viability is defined as a percentage of live cells in a whole population. Although cell death is one of the consequences of toxicity, chemical or physical factors may exert their toxic effects through a number of cellular alterations that may compromise cell ability to divide without necessarily leading to cell death. This aspect represents the term ‘cell vitality’ defined as physiological capabilities of cells. It is important to note that cell viability and cell vitality represent two different aspects of cell functions, and both are required for the estimation of the physiological state of a cell after exposure to various types of stressors and chemical or physical factors. In this paper, we introduced a classification of available methods for estimating both viability and vitality in Saccharomyces cerevisiae yeast cells (wild-type and Δsod1 mutant) in which the effects of selected oxidants causing oxidative stress is evaluated. We present the advantages as well as disadvantages of the selected methods and assess their usefulness in different types of research.

Introduction
The yeast Saccharomyces cerevisiae is a very useful model organism for studies of cellular response to various types of stresses. Determination of cell viability is one of the most commonly used methods in an analysis of cyto- or genotoxicity under different kinds of chemical, physical, or environmental factors. The analysis of the viability parameter is also very important for industrial processes where microorganisms are used (Nikolova et al., 2000–2002). Generally, cell viability is defined as a percentage of live cells in a whole population. This parameter can be determined by different methods described in a number of research papers. For a better understanding of the cell viability problem, we introduced a classification of available methods according to their performance and types of the obtained results, which is shown in Fig. 1. (1) The first category includes methods based on the ability of yeast cells to grow on solid or liquid medium. One of the most commonly used methods in this category is the analysis of the number of colonies (CFU – colony-forming unit; Longo et al., 2012); there are also other methods such as spotting test, measurement of the growth inhibition zone, and culture on liquid medium. The undoubted advantages of these methods are ease of use and low costs, whereas the disadvantage is the long waiting time for the results. More importantly, even though the aforementioned tests enable estimation of the degree of growth inhibition, they fail to provide estimation of viable yeast cells or cells unable to reproduce. (2) The other category of viability measurement methods includes stain-based methods. In this case, both the colorimetric and the fluorescent dyes are used. The mechanism of action of these dyes depends on the properties of the cell membrane. The cell membrane separates the inside of the cell from the external environment; thus, cell membrane damage usually leads to cell death. The stains that are used to estimate cell viability may be divided into two subcategories. (1) Dyes that are dependent on the changes in the integrity and functionality of the cell membrane. Dyes that at physiological pH are anionic and do not penetrate into living cells (due to the negative charge of the cell membrane). They are blocked by the intact membrane of viable cells and penetrate into dead or damaged
CELL VIABILITY

Methods useful in assessing cell viability

Fig. 1. Methods used to determine viability and vitality of yeast cells.
cells only, staining the nucleus or cytoplasm. The presence of the dyes within the cells indicates damage of the cell membrane and cell death. Commonly used dyes from this group include DNA-binding fluorescent dyes such as propidium iodide (Lopezamoros et al., 1995; Fanjiang et al., 2004) or ethidium bromide (Aeschbacher et al., 1986), or colorimetric dyes such as trypan blue (McGahan et al., 1995) or erythrosine B (Bochner et al., 1989).

(2) Dyes that penetrate both into alive and dead cells. Living cells are able to pump out the dye (e.g. phloxine B; Minois et al., 2005) or reduce the dye (such as methylene blue; Painting & Kir sop, 1990; Bapat et al., 2006) and remain colorless, whereas dead cells are unable to do it and therefore are stained red (phloxine B) or blue (methylene blue). The above methods provide rapid and objective results. Moreover, they enable observation of a single yeast cell, making distinction between alive or dead cells and measuring the percentage of these two categories of cells in the whole population.

The methods for assessing cell viability presented above provide information only on alive and dead cells in the whole population. However, in many cases, toxic effects of chemical or physical factors do not lead directly to cell death. Such factors may cause a number of morphological, intracellular, or metabolic alterations that will result in the inability of a cell to divide, yet the cell itself may still be alive. This aspect represents cell vitality, defined as physiological capabilities of a cell.

Methods for determination of yeast cell vitality are based on studies of various aspects of the physiological state of cells. These methods fall into three categories: (1) determination of the cellular ATP content based on the luciferin reaction (Ansehn & Nilsson, 1984); (2) determination of the mitochondrial membrane potential based on staining with rhodamine 123 (Ludovic et al., 2001) or rhodamine B (Marchi & Cavalieri, 2008); and (3) determination of the activity of enzymes, for example, esterase [staining with fluorescein diacetate (FDA; Chrzanowski et al., 1984; Breeuwer et al., 1995), oxidoreductases (staining with tetrazolium salts MTT (Leviz & Diamond, 1985), XTT (Kuhn et al., 2003), WST-8 (Kuhn et al., 2003; Tsukatani et al., 2009)], or several different redox enzymes (staining with resazurin; O’Brien et al., 2000; Czekanska, 2011). The category should also include the yeast-specific dye FUN-1 (Millard et al., 1997; Essay & Marshall, 2009), a membrane-permeate nonfluorescent precursor converted by the activity of intracellular enzymes to a fluorescent product. The dye makes it possible to distinguish among alive (metabolically active), metabolically weakened, and dead cells. These methods require the use of advanced equipment; however, they give very objective and measurable results. They allow for detailed characterization of the metabolic state of the cells, providing a better basis for drawing conclusions about the mechanism of action of the factors under examination.

The aim of this study was to develop a clear classification of methods for analyzing both viability and vitality of yeast cells. A comparative analysis of the results obtained by different methods was performed based on the effect of selected oxidants that cause oxidative stress. Depending on the oxidant type and concentration, oxidative stress may not necessarily lead to cell death but may cause a variety of metabolic and physiological consequences (Kwolek-Mirek et al., 2009, 2011a, b; Kwolek-Mirek et al., 2012), thus making a good model for the comparison of methods assessing viability and vitality of cells. We used the Δsod1 mutant, which is hypersensitive to oxidative stress, and a wild-type strain as a control. We then estimated viability of yeast cells after incubation with well-known oxidants such as hydrogen peroxide, allyl alcohol, and menadione.

**Materials and methods**

**Chemicals**

Allyl alcohol, menadione, phloxine B, propidium iodide, FDA, and Cell Counting Kit-8 were from Sigma-Aldrich (Poznan, Poland). H$_2$O$_2$ and Methylene blue were from POCH (Gliwice, Poland). FUN-1, rhodamine B, rhodamine 123 stains were from Molecular Probes (Eugene, OR). BacTiter-Glo™ Microbial Cell Viability Assay was from Promega (Madison, WI). Components of culture media were from BD Difco (Becton Dickinson and Company, Spark, MD) except for glucose (POCH, Gliwice, Poland).

**Yeast strains and growth conditions**

The following yeast strains were used: wild-type SP4 MATα leu1 arg4 (Bilinski et al., 1978) and Δsod1 mutant, isogenic to SP4, MATα leu1 arg4 sod1::natMX (Koziol et al., 2005). Yeast was grown in a standard liquid YPD medium (1% yeast extract, 1% yeast bacto-peptone, 2% glucose) on a rotary shaker at 150 r.p.m. or on a solid YPD medium containing 2% agar, at a temperature of 28 °C.

**Cell viability and vitality assays**

To determine cell viability, yeast cells from the exponential phase culture were centrifuged, washed with sterile water, and suspended to the final density of 10$^8$ cells mL$^{-1}$ in 100 mM phosphate buffer with pH 7.0, containing 0.1% glucose and 1 mM EDTA with 10 mM...
H₂O₂, 0.4 mM allyl alcohol, and 0.105 mM menadione. After 1-h incubation, the cells were pelleted by centrifugation, then washed twice with sterile water, and suspended in water or in a buffer solution as appropriate.

To determine cell viability and vitality, the following methods were used:

1. **Spotting test**
   Cells were suspended in sterile water and diluted to give 10⁷, 10⁶, 10⁵, or 10⁴ cells mL⁻¹. Samples (5 μL) of each suspension were inoculated on solid YPD medium and incubated at 28 °C. Colony growth was inspected after 48 h.

2. **Colony-forming units**
   Cells were suspended in sterile water and diluted to a final concentration of 10⁷ cells mL⁻¹. A sample (100 μL) of the suspension was inoculated on solid YPD medium and incubated at 28 °C. CFUs were counted after 48 h.

3. **Growth in the presence of phloxine B**
   Samples (5 μL) of the cell suspension in 100 mM phosphate buffer with pH 7.0, containing 0.1% glucose and 1 mM EDTA, were dropped on YPD plates with solid medium with 10 mM Na-HEPES buffer with pH 7.2, containing 2% glucose. The metabolic activity of cells was estimated with 0.5 μM FUN-1 (100 mM stock solution, dissolved in DMSO). The metabolic activity of cells was expressed as a change in ratio of red (λ = 575 nm) to green (λ = 535 nm) fluorescence. The fluorescence of the cell suspension was measured after 15 min using the TECAN Infinite 200 microplate reader at λ_em = 500–650 nm. However, the fluorescence pictures were taken with a fluorescence microscope. Metabolically active and inactive cells were examined from at least 200 cells in one biological replicate. Metabolically active cells contain cylindrical, red-fluorescent structures in their vacuoles, while dead cells or cells with little or no metabolic activity exhibit extremely bright diffuse green cytoplasmic fluorescence and lack fluorescent intravacuolar inclusions.

4. **Staining with methylene blue**
   Cells were suspended in PBS, and a sample (100 μL) of the cell suspension was mixed with 100 μL methylene blue (0.1 mg mL⁻¹ stock solution, dissolved in a 2% dihydrate sodium citrate solution) and incubated for 5 min at room temperature. Viability was examined under microscope from at least 200 cells in one biological replicate. Viable cells were colorless, and dead cells were blue.

5. **Staining with PI and FDA**
   Cells were suspended in PBS and stained with both 5 μg mL⁻¹ PI (1 mg mL⁻¹ stock solution, dissolved in water) and 10 μg mL⁻¹ FDA (1 mg mL⁻¹ stock solution, dissolved in acetone) for 20 min in the dark at room temperature. FDA/PI fluorescence was examined from at least 400 cells in one biological replicate under a fluorescence microscope at λ_ex = 480 nm. Viable cells were green fluorescent, and dead cells were red fluorescent.

6. **Determination with Cell Counting Kit-8**
   The number of viable yeast cells in the proliferation was determined with Cell Counting Kit-8 based on monosodium salt WST-8 according to the manufacturer’s protocol (Sigma-Aldrich). Cells were suspended in 100 mM phosphate buffer with pH 7.0, containing 0.1% glucose and 1 mM EDTA. A sample (100 μL) of the cell suspension with density 10⁷ cells mL⁻¹ was used for assay purposes. WST-8 was reduced by cellular dehydrogenases to an orange formazan product that was soluble in buffer. The absorbance of the cell suspension was measured after 3 h using TECAN Infinite 200 microplate reader at λ = 450 nm. The amount of formazan produced was directly proportional to the number of living cells.

7. **Estimation with the FUN-1 stain**
   The metabolic activity of yeast cells was assessed with FUN-1 according to the manufacturer’s protocol (Molecular Probes). Cells were suspended in 10 mM Na-HEPES with pH 7.2, containing 2% glucose. The metabolic activity of cells was estimated with 0.5 μM FUN-1 (100 mM stock solution, dissolved in DMSO). The metabolic activity of cells was expressed as a change in ratio of red (λ = 575 nm) to green (λ = 535 nm) fluorescence. The fluorescence of the cell suspension was measured after 15 min using the TECAN Infinite 200 microplate reader at λ_em = 480 nm and λ_em = 500–650 nm. However, the fluorescence pictures were taken with a fluorescence microscope. Metabolically active and inactive cells were examined from at least 200 cells in one biological replicate. Metabolically active cells contain cylindrical, red-fluorescent structures in their vacuoles, while dead cells or cells with little or no metabolic activity exhibit extremely bright diffuse green cytoplasmic fluorescence and lack fluorescent intravacuolar inclusions.

8. **Determination with BactTiter-Glo™ Microbial Cell Viability Assay**
   Determination of the number of viable yeast cells was based on quantitation of ATP; cells were assessed with BactTiter-Glo™ Microbial Cell Viability Assay according to the manufacturer’s protocol (Promega). Cells were suspended in a 100 mM phosphate buffer with pH 7.0, containing 0.1% glucose and 1 mM EDTA. A sample (100 μL) of cell suspension with density 10⁷ cells mL⁻¹ was used for determination purposes. Luminescence was recorded after 5 min using TECAN Infinite 200 microplate reader. The luminescent signal was proportional to the amount of ATP present, which was directly proportional to the number of cells.

9. **Estimation with rhodamine B and rhodamine 123**
   Mitochondria were stained with rhodamine B hexyl ester (red-fluorescent stain) and rhodamine 123 (green-fluorescent stain) which place themselves in mitochondria depending on the mitochondrial membrane potential. For rhodamine B staining, cells were suspended in a 10 mM HEPES buffer with pH 7.4, containing 5% glucose, and rhodamine B (10 μM stock solution, dissolved in DMSO) was added to a final concentration of 100 nM. For rhodamine 123 staining, cells were suspended in a 50 mM sodium citrate buffer with pH 5.0, containing 2% glucose,
and rhodamine 123 (25 mM stock solution, dissolved in DMSO) was added to a final concentration of 25 μM. After 15 min of incubation, mitochondrial membrane potential was visualized by fluorescence microscopy at λ<sub>ex</sub> = 555 nm and λ<sub>em</sub> = 579 nm for rhodamine B, and at λ<sub>ex</sub> = 505 nm and λ<sub>em</sub> = 534 nm for rhodamine 123.

**Microscopic observations**

All microscopic observations were carried out using the Olympus BX-51 epifluorescence microscope equipped with the DP-72 digital camera and CELL^D software. In each of the three biological replicates, at least 200 cells were examined.

The quantitative results are presented as mean ± SD from at least three independent experiments. They are presented as relative values (%) in each case compared to the control for the wild-type strain.

**Statistical analysis**

The statistical analysis was performed using SPSS 21.0. The statistical significance of the differences between the means of the two yeast strains compared was evaluated using the t-test for independent samples. The statistical significance of the differences between the means of the results for the CFU method compared to the results for other cell viability methods was estimated using one-way ANOVA and the Dunnett test when equal variances were assumed, or Dunnett T3 when equal variances were not found in a post hoc test. Homogeneity of variance was checked using Levene’s test. Values were considered significant at P < 0.05.

**Results and discussion**

Hydrogen peroxide, allyl alcohol, and menadione are well-known compounds that are toxic to *S. cerevisiae*. They cause oxidative stress in the cells mainly through decreasing the level of reduced glutathione and increasing the level of reactive oxygen species. These compounds cause a number of negative changes in yeast cells, which result in reduction of their viability (Fortuniak & Chojnowski, 1997; Madeo *et al.*, 1999; Castro *et al.*, 2008; Marchi & Cavalieri, 2008; Kwolek-Mirek *et al.*, 2009). In our studies, we used two yeast strains, namely a Δsod1 mutant (MS2) lacking superoxide dismutase 1, which is hypersensitive to oxidative stress, and a wild-type strain (SP4) as a control. To determine cell viability after exposure to oxidants, we used a number of methods to compare their efficiency and the results obtained. Commonly used methods include analysis of the number of CFUs and the spotting test to enable the observation of only those cells that are able to reproduce. The results obtained by these two methods are not the same. In the case of the spotting test, 1-h incubation of cells with 10 mM H<sub>2</sub>O<sub>2</sub>, 0.4 mM allyl alcohol, and 0.105 mM menadione causes c. 50% inhibition of growth (which means growth of only two of the four spots) of the Δsod1 mutant compared to the control (cells incubated in buffer without oxidants). We did not observe changes of growth in the case of the wild-type strain (Fig. 2). A comparison of these results with those obtained by the CFU method shows a significant difference. In the case of the CFU method, we noted a complete growth reduction of the Δsod1 mutant and cell viability of c. 37, 14 and 99%, respectively, in the wild-type strain, in both cases compared to the control for the wild-type strain. For all tested oxidants, the differences between the wild-type strain and the Δsod1 mutant are statistically significant. The differences observed in the results obtained by these two methods may be explained in two ways; firstly, by decreasing the toxicity effect of oxidants in the case of the spotting test. Yeast cells in the spots, depending on their number (the spots initially contained 50 000, 5000, 500, and 50 cells, respectively), are placed not only next to one other but also on one another. It may reduce direct contact with oxidants and facilitate growth of some cells which are protected by other cells. Secondly, the observed growth (marked as ‘+’+) was marked in the test even if it consisted in growth of few cells out of all the cells placed on the spot. Moreover, the spotting test method is not a quantitative method like the CFU method, but a qualitative-semiquantitative method; therefore, it is recommended as a screening method to select a range of oxidants for further studies.
concentrations of the test compounds. Considering that the two presented methods fail to determine whether the cells unable to reproduce are dead, we propose an alternative method, namely time-lapse photomicroscopy of individual yeast cells in the presence of the vital dye phloxine B (Fig. 4a and b). Phloxine B is a dye absorbed by cells; metabolically active cells are able to pump it out and remain colorless, whereas dead cells are stained red (Minois et al., 2005). Using this dye allows the cells to be identified and distinguished into those alive and able to reproduce, those alive but unable to reproduce, and those that are dead. Cells unable to reproduce are often mistakenly regarded as dead (Mirisola et al., 2014). In a previous paper, we showed acrylamide action as an example of a stress factor that makes cells unable to reproduce rather than causing their death (Kwolek-Mirek et al., 2011b). That effect depends on exposure to specific circumstances and may be permanent or reversible (data not shown). In this study in the case of the wild-type strain, dividing cells were observed only for the control cells and for

Fig. 3. Cell viability determined by the number of colonies (CFU method). WT and Δsod1 cells were incubated with H₂O₂, allyl alcohol, and menadione for 1 h, and in each case, 1 x 10⁶ of cells were inoculated onto YPD plates. The growth was inspected after 48 h. The results are presented as relative values (%) compared to the control for the wild-type strain. Bars indicate SD; n = 3; letters a, b, and c indicate the differences between WT vs Δsod1 cells at P < 0.05, P < 0.01, and P < 0.001, respectively.

Fig. 4. Cell viability estimated by time-lapse photomicroscopy in the presence of vital dye phloxine B. WT (a) and Δsod1 (b) cells were incubated with H₂O₂, allyl alcohol, and menadione for 1 h and dropped onto YPD plates with 10 µg mL⁻¹ phloxine B. Images were captured after 0, 8, and 24 h. The dead cells are red due to accumulation of the dye inside the cell (for better contrast, the pictures are presented as a grayscale; the dead cells are black).
those incubated with menadione, while dividing, nondi-
ving, and dead cells were observed for the cells incu-
bated with H$_2$O$_2$ and allyl alcohol (Fig. 4a). These
differences are visible only after 8 h of culture, whereas
they are not visible after 24 h. Cells able to reproduce
and their offsprings produce a colony, thereby completely
masking the presence of the nondividing and dead cells
(Fig. 4a). In contrast, cells unable to divide and dead cells
in the case of the $\Delta$sod1 mutant can be observed immedi-
ately after incubation with oxidants and after 8 and 24 h
of culture. A high number of dead cells was noted in the
case of incubation of the $\Delta$sod1 mutant cells with allyl
alcohol, while a lower number was noted in the case of
incubation with H$_2$O$_2$ and menadione. The difference in
the growth of control cells and cells incubated with oxi-
dants is clearly visible after 8 and 24 h of culture (Fig. 4b).
This method, even though not a quantitative one, has an unquestionable advantage compared to the
CFU method as it is simple and fast (it takes few hours
depending on the reproductive cycle duration), allowing
the dead cells to be distinguished and counted, and also
enabling differentiation between the alive cells that are
able and unable to reproduce.

Compared to the cell viability measurement methods
based on the cell growth, the methods based on colori-
metric or fluorescent dyes are much faster and give more
measurable results. A commonly used method is labeling
cells with dyes such as methylene blue, or PI and FDA.
The use of these dyes allows for analysis of individual
yeast cells. Methylene blue penetrates into every cell. Liv-
ing cells enzymatically reduce the dye to a colorless prod-
uct and become unstained, whereas dead cells are stained
blue (Painting & Kirsop, 1990; Bapat et al., 2006). We
observed cell viability of c. 90%, 60%, and 94% in the
case of the wild-type strain, and cell viability of c. 85%,
56%, and 91% in the case of the $\Delta$sod1 mutant after
incubation of cells with H$_2$O$_2$, allyl alcohol, and menad-
one, respectively; in both cases, the results were compared
to the control for the wild-type strain (Fig. 5). For all
tested oxidants, the differences between the wild-type
strain and the $\Delta$sod1 mutant are statistically significant.
These results strongly differ from those obtained using the
CFU method (Fig. 3), which are, respectively, 53%,
46%, and 5% in the case of the wild-type strain and 85%,
56%, and 91% in the case of the $\Delta$sod1 mutant. The dif-
ferences observed are statistically significant for all tested
oxidants and for both tested strains. The number of cells
considered dead in the case of the CFU method is signifi-
cantly higher than the number obtained by labeling cells
with dyes. This is because in the CFU method the cells
that are unable to reproduce are usually recognized as
dead. However, our results show that after exposure to
oxidants, there are many cells that are unable to repro-
duce but they are still alive (Fig. 4a and b). It seems
therefore that methods based on staining with dyes pro-
vide more objective results on the cells death rate. In this
study, we also applied double fluorescent staining of the
cells with PI and FDA (Fig. 6). Propidium iodide (PI)
penetrates only into dead cells, while FDA passively
crosses through cell membrane and is subsequently
hydrolyzed by intracellular esterases to fluorescein. This
double marking enables us to observe dead (PI-positive,
red-fluorescent) as well as live (FDA-positive, green-fluo-
rescent) cells (Nikolova et al., 2000–2002; Zheng et al.,
2007). Interestingly, we received almost the same results
of cell viability when staining with PI and FDA (Fig. 6),
and with methylene blue (Fig. 5). Both of these methods
give good results, and they may be used alternatively;

![Cell viability estimated with methylene blue dye.](https://academic.oup.com/femsyr/article-abstract/14/7/1068/531480)
however, in the case of staining with methylene blue, we should bear in mind that a longer time of exposure to the dye may give false-positive results due to its toxicity.

For all the oxidants tested, the differences between the wild-type strain and the $\Delta sod1$ mutant are statistically significant. Another method to determine cell viability (or rather vitality) is the Cell Counting Kit-8 method (Fig. 7). This assay is based on reduction of the sodium WST-8 salt by cellular dehydrogenase to water-soluble orange formazan. Because the sodium WST-8 salt is non-toxic for yeast cells, measurements can be taken both after short and long duration of the experiment (even 12–24 h of culture). After 3 h of cultivation, we observed cell viability of c. 66%, 57%, and 153% in the case of the wild-type strain, and cell viability of c. 53%, 47%, and 102% in the case of the $\Delta sod1$ mutant after incubating the cells with H$_2$O$_2$, allyl alcohol, and menadione, respectively; in both cases, the results were compared to the control for the wild-type strain (Fig. 7). The differences between the wild-type strain and the $\Delta sod1$ mutant were statistically significant only for the control sample and the menadione-treated sample. High viability of cells incubated with menadione may be caused by the presence of a small amount of ethanol (menadione solvent), which can impact cellular dehydrogenase activity and thus change the metabolism of the sodium WST-8 salt. Differences in viability of cells between the wild-type strain and the $\Delta sod1$ mutant may be caused by different times of generation (respectively c. 90 and 120 min, data not shown), because we obtained a different number of cells for each strain at the same time. Cell viability results obtained through this method (Fig. 7) are lower than those obtained with dyes (Figs 5 and 6), but higher than the results obtained from the CFU method (Fig. 3). The use of Cell Counting Kit-8 enables the determination of the number of viable yeast cells in suspension; however, when the experiment lasts longer, the obtained results are strongly dependent on the number of cells able to reproduce and on their time of generation. Statistical analysis indicates a statistically significant difference between the cell viability determined by the CFU method (Fig. 3) and the methods based on staining cells (Figs 5–7).

![Fig. 6. Cell viability estimated with PI and FDA. WT and $\Delta sod1$ cells after incubation with the oxidants were suspended in PBS and stained with both 5 $\mu$g mL$^{-1}$ PI and 10 $\mu$g mL$^{-1}$ FDA for 20 min in the dark at RT. FDA/PI fluorescence was examined under the fluorescence microscope at $\lambda_{ex} = 480$ nm from at least 400 cells in each experiments. The results are presented as relative values (%) compared to the control for the wild-type strain (a). (b) a–green-fluorescent viable cells; b–red-fluorescent dead cells. Bars indicate SD; n = 3; *P < 0.05, **P < 0.01, ***P < 0.001 for the comparison of the results obtained through the CFU method vs. the FDA/PI method. Letters a, b, and c on the graph indicate differences between WT vs. $\Delta sod1$ cells at P < 0.05, P < 0.01, and P < 0.001, respectively.](https://academic.oup.com/femsyr/article-abstract/14/7/1068/531490)

![Fig. 7. Cell viability determined using Cell Counting Kit-8. WT and $\Delta sod1$ cells after incubation with oxidants were suspended in a 100 mM phosphate buffer with pH 7.0, containing 0.1% glucose and 1 mM EDTA, and mixed with the sodium WST-8 salt. The absorbance of the cell suspension was measured after 3 h using the microplate reader at $\lambda = 450$ nm. The amount of formazan produced was directly proportional to the number of living cells. The results are presented as relative values (%) compared to the control for the wild-type strain. Bars indicate SD; n = 3; *P < 0.05, **P < 0.01, ***P < 0.001 for the comparison of the results from the CFU method vs. the WST-8 method. Letters a, b, and c on the graph indicate the differences between WT vs. $\Delta sod1$ cells at P < 0.05, P < 0.01, and P < 0.001, respectively.](https://academic.oup.com/femsyr/article-abstract/14/7/1068/531490)
observed differences do not undermine the use of any of the compared methods; rather, they point out to the differences in the methodology and interpretation of the results. In the CFU method, percent viability (marked on the x-axis) includes only the cells capable to reproduce. In the staining method, the same parameter (percent viability) covers both live cells and cells capable to reproduce as well as cells that are alive but not capable to reproduce.

The presented methods may be used to assess viability of yeast cells, which in practice means determination of percentage of dead cells in a population. These methods, however, do not characterize the physiological state of cells after exposure to different types of stress. In our opinion, this parameter is very important, especially when rather than observing dead cells we want to focus on cells that are unable to reproduce. In such a case, determination of cell vitality in the sense of physiological capabilities of cells may help explain the mechanism of action of different types of stress. We propose three different methods, which are dependent on one another and which together provide comprehensive information on the physiological state of the cells. First of all, we estimated the

![Image](https://academic.oup.com/femsyr/article-abstract/14/7/1068/531490)

Fig. 8. Metabolic activity determined with FUN-1 stain. WT and $\Delta$Sod1 cells after incubation with oxidants were suspended in a 10 mM Na-HEPES buffer with pH 7.2, containing 2% glucose, and estimated with 0.5 $\mu$M FUN-1. The fluorescence of the cell suspension was measured after 15 min using the microplate reader at $\lambda_{\text{ex}} = 480$ nm and $\lambda_{\text{em}} = 500–650$ nm. The metabolic activity of cells was expressed as a change in the ratio of red ($\lambda = 575$ nm) to green ($\lambda = 535$ nm) fluorescence (a); graphs b and c show emission spectrum. The result can be analyzed also by fluorescence microscopy and presented as a percentage of metabolically active cells (d). Metabolically active cells a contain cylindrical, red-fluorescent structures in their vacuoles (e), while b and c – dead cells or cells with little or no metabolic activity – exhibit green cytoplasmic fluorescence and lack fluorescent intravacuolar inclusions (f). The results are presented as relative values (%) compared to the control for the wild-type strain (a and d). Bars indicate SD; $n = 3$; letters a, b, and c on the graphs indicate the differences between WT vs. $\Delta$Sod1 cells, at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

![Image](https://academic.oup.com/femsyr/article-abstract/14/7/1068/531490)

Fig. 9. ATP content determined using BactTiter-Glo Microbial Cell Viability Assay. WT and $\Delta$Sod1 cells after incubation with the oxidants were suspended in 100 mM phosphate buffer, pH 7.0, containing 0.1% glucose and 1 mM EDTA. One hundred microlitre cell suspension with density $10^6$ cells mL$^{-1}$ was used for determination purposes. Luminescence was recorded after 5 min using the microplate reader. The luminescent signal was proportional to the amount of ATP present, which was directly proportional to the number of cells. The results are presented as relative values (%) compared to the control for the wild-type strain. Bars indicate SD; $n = 3$; the letters a, b, and c indicate the differences between WT vs. $\Delta$Sod1 cells at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.
metabolic activity of yeast cells with the FUN-1 stain (Fig. 8). FUN-1 is a chlorinated cyanine dye which penetrates cell membranes. Cells with high metabolic activity contain cylindrical red-fluorescent structures in their vacuoles. Dead cells or cells with little or no metabolic activity exhibit diffuse green fluorescence from the whole cytoplasm and contain no fluorescent intravacuolar inclusions (Millard et al., 1997). The results obtained through this method may be read using both the fluorescent plate reader and the fluorescence microscope. An analysis of the red to green fluorescence ratio is recommended mainly to determine the direction of changes in the metabolic activity of cells (Fig. 8a–c). The differences between the wild-type strain and the Δsod1 mutant are statistically significant only for the nontreated sample; in other cases, the differences are not statistically significant. However, complementation of these results with the microscopic analysis is more informative and allows for characterization of cells and determination of cell percentage with different levels of metabolic activity (Fig. 8d–f). In the case of the wild-type strain, we observed a strong decrease in metabolic activity after incubation of cells with H2O2 and allyl alcohol (c. 36% and 11%, respectively), and a slight decrease after incubation with menadione (93%). We noted an almost complete lack of metabolic activity of Δsod1 mutant cells after incubation with all tested oxidants, in both cases compared to the control for the wild-type strain (Fig. 8d). For all tested oxidants, the differences between the wild-type strain and the Δsod1 mutant are statistically significant. We also observed that incubation of Δsod1 mutant cells in buffer without addition of oxidants (control condition) resulted in a significant decrease in their metabolic activity compared with the wild-type strain (Fig. 8a). This effect was shown in our previous paper (Kwolek-Mirek et al., 2011a). The level of metabolic activity is closely related to the cell ATP content. One of the methods to estimate this parameter is BacTiter-Glo™ Microbial Cell Viability Assay (Fig. 9). This particular method is based on the reaction of luciferin with ATP in the presence of luciferase, Mg2+ ions, and oxygen, resulting in emission of light. The luminescent signal is proportional to the cellular ATP content, which is dependent on the number of living cells in the suspension (Junker & Clardy, 2007). We have observed the ATP content of c. 35%, 46%, and 51% in the case of the wild-type strain, and c. 26%, 60%, and 66% in the case of the Δsod1 mutant after incubation of cells with H2O2, allyl alcohol, and menadione, respectively; in both cases, the results were compared to the control for the wild-type strain (Fig. 9). The differences between the wild-type strain and the Δsod1 mutant were statistically significant only for the allyl alcohol-treated
sample. Differences in the cellular ATP content between the wild-type strain and the Δsod1 mutant in control conditions may be caused by different cell volumes (respectively, c. 38 and 57 μm³, data not shown). The fact that H₂O₂ strongly reduces the level of ATP in the yeast cells compared to menadione was previously shown by Osorio et al. (2003) Under nonfermentative growth, ATP is produced by yeast cells primarily in mitochondria. Therefore, changes in mitochondrial morphology and activity are strictly linked to the cellular ATP content. For detecting changes in the respiratory activity of the yeast cells incubated with oxidants, we used cell-permeate fluorescent dyes rhodamine 123 and rhodamine B probes (Fig. 10a and b). Both dyes are used for analysis of changes in the mitochondrial membrane potential. Rhodamine 123 is a cationic green-fluorescent dye which is readily sequestered by active mitochondria (Ludovico et al., 2001), and rhodamine B is a hexyl ester red-fluorescent dye (Marchi & Cavalieri, 2008). Both dyes give similar results and therefore can be used independently. In this study, we used both dyes to compare the achieved effects. After incubation with oxidants, we observed generally significant changes in mitochondrial network as well as in the activity of the mitochondrial membrane potential for both dyes. The yeast cells exposed to H₂O₂ and allyl alcohol show a greater decrease in the mitochondrial membrane potential than the cells exposed to menadione. These changes are more noticeable in the case of the Δsod1 mutant compared to the wild-type strain (Fig. 1a and b).

The wide range of methods for the assessment of both viability and vitality of yeast cells presented in this work may be of significant assistance when selecting the most appropriate way to assess the state of the cells in toxicological studies, but also in industrial microbiology. There are certain types of industrial processes where microorganisms are used. The yeast strains for these processes are carefully selected in respect of their viability and capacity to produce some end products. It seems that an in-depth characterization of cells not only in terms of their viability but also in terms of their vitality could be helpful during modification and optimization of industrial processes where microorganisms are used.

As we have shown, the results obtained by these methods in many cases are not the same, as viability and vitality represent two different aspects of yeast cell biology. We suggest therefore using not merely one but a few selected methods from both presented groups, especially for the study of the influence of different toxic factors on yeast cells (Fig. 1). In this way, we will obtain full and comprehensive results that will help explain the mechanism of action of these factors.

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