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EVALUATION OF YEAST CELL VITALITY USING DIFFERENT FLUORESCENT DYES

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Vitality of different industrial yeast strains was investigated using four standard fluorescent dyes by studying changes in microscopic evaluation of yeast nuclei and cell membranes. Fluorescent dyes were: acridine orange, DAPI, trypan blue and primuline. Comparison of the results obtained by fluorescent techniques with these dyes showed good correlation of yeast cell vitality measurements. Simultaneous staining with fluorochromes provides a powerful way to demonstrate culture heterogenity and changes within yeast cells during long cultivation.

1. Introduction

Vitality and viability represent two aspects of yeast cell condition. Vitality is a function of the physiological capabilities of the cell, while viability is a term used to describe if a cell is alive or dead. Viability refers to percentage of live cells, whereas vitality reflects the metabolic function of the cells [1, 2].
Assessment of the yeast cell state is very important in many areas of industrial microbiology [3]. Traditional methods of viability assay are based on counting colony forming units on agar plates, but these methods are very slow, requiring 24 to 48 hours of incubation [4]. The long time necessary for culturing the agar plates makes this method impossible to use the results for current control of process. In addition, the sensitivity of this methods tends to be very low, because the losses of cell activities are shown in percentages, while the dilution procedure before inoculation of plates is logarithmic. Accurately assessing how many cells

in a population are alive is sufficient to enable decisions on yeast quality, but the vital condition of yeast cells is also very important [5].

Existing staining methods can be grouped into three categories according to what is measured: loss of replication, cell damage or loss of metabolic activity. The yeast cell membrane is affected by the stresses that occur during fermentative processes or during storage. Starvation, the presence of ethanol, temperature or aging cause damage to the cell membrane, which, in turn may result in reduced yeast growth, loss of viability and a decrease in metabolic activity [6, 7].

Current recommended techniques for determination of cells vitality are based on brightfield stains, especially methylene blue, but they are known to have many limitations [8, 9]. The methylene blue assay is subjective and tedious, and operators can quickly suffer fatigue with further impact on the accuracy of the test. An alternative for studying the vitality of yeast cells can be a vital unfluorescent dye, Phloxine B (tetrabromotetrachlorofluorescein, also named Acid Red 92 or Magdala Red). All yeast cells absorb the dye, but metabolically active cells are able to pump it out and remain colorless [10].

The most interesting staining methods are fluorescence techniques because they provide high sensitivity, but there is no absolute method. Fluorescence is a process where a substance after having absorbed light (photons) emits radiation of a wavelength (color) that is longer that of the absorbed light. This emission stops immediately after termination of excitation. Fluorescent microscopy and its applications is based on this phenomenon. The wide used fluorescent stain is acridine orange (AO). This dye can freely enter dead or alive cells and bind to nucleic acids. AO interacts with DNA and RNA by intercalation or electrostatic attraction respectively. DNA intercalated AO fluoresces green (525 nm) and RNA electrostatically bound AO fluoresces red (>630 nm). It can distinguish between quiescent and active cells. However, AO assay is also subjective and tedious because, depending on DNA/RNA ratio, we can obtain differently stained cells from yellow through orange to green. Therefore microbiologists should identify the most appropriate fluorophore for studying cell viability [1, 11, 12]. In the laboratory to distinguish between metabolically active and dead fungal cells fluorochrome FUN1 is utilized. This fluorescent vital dye can be used for observation of cylindrical intravacuolar structures within the vacuoles of metabolically active yeast cells. FUN-1 staining, which begins as a diffuse pool of fluorescent cytoplasmic stain, uses an unknown endogenous biochemical processing mechanism to compact and form orange-red cylindrical intravacuolar structures within the cell vacuole [13]. For better visualization of these structures a second fluorochrome - Calcofluor can be used. However, this technique isn't helpful for routine, rapid studies in yeast industry because it requires two expensive fluorochromes, the complex two-step staining procedure and special multipass filter.

In this study three alternative fluorescent dyes were evaluated for measurement of vitality of different industrial yeast strains. These fluorochromes are relatively cheap and easy for application in yeast industry. Staining of yeast cells was done with 4',6-diamidino-2-phenylindole (DAPI), primuline (P) and trypan blue (TB). DAPI was used to specifically stain nuclei while P and TB were used to visualize permeabilized or damaged yeast cell membranes. However DAPI can stain yeast cells only after crossing trough the cell plasma membranes. The results obtained from these fluorescent techniques for different yeast populations were compared with results for standard, wide used procedure with acridine orange (AO) staining

2. Experimental details

2.1. Yeast strains

Strains used in this study are shown in Table 1.

Table 1

Yeast strains characteristics

Yeast strain	Symbol	Appropriation	Source
Saccharomyces cerevisiae	Bc 16a	distillery	LOCK 105*
S. cerevisiae	Jaa	distillery	LOCK 105
S. cerevisiae	IIa	distillery	LOCK 105
S. cerevisiae	Ja 64	bakery	LOCK 105
S. cerevisiae	B**	bakery	Polish yeast factory
Debaryomyces occidentalis	Y500/5	amylolytic strain	LOCK 105

*)LOCK105: The Industrial Microorganisms Collection, Technical University of Lodz
**) fresh pressed baker's yeast obtained from Polish yeast factor

2.2. Media and growth conditions

The strains were cultivated in 50 mL of worth broth (Merck, Germany) at 30 ºC until 7 days on the shaker (220 rpm/min). The culture volume did not exceed 25% of the flask capacity.

2.3. Fluorescent dyes

Acridine and primuline orange were from Sigma-Aldrich (UK), DAPI from Sigma-Aldrich (Germany), while trypan blue was from Merck (Germany).

2.4. Fluorescence microscopy

After cultivation yeast cells were collected by centrifugation (2200×g, 10 min, room temperature), washed twice and resuspended in Ringer solution. Standardized cell suspensions contained about 1×10^8 cell/mL were transferred to the glass tubes and stained in appropriate conditions shown in Table 2. For image acquisition was used the fluorescence microscope Olympus BX 41 equipped with a digital camera and analysis software Image J 1.34n (National Institute of Health, USA).

Table 2

Yeast cells staining conditions

Fluorochrome	Acridine orange	Trypan blue	DAPI	Primuline
Concentration in sample / %	0.01	0.04	0.01	0.01
Incubation time / min	3-5	3-5	15-20	3-5
Staining temperature / ºC	22	22	37	22
Excitation wavelength / nm	502	530-550	340	410
Emission wavelength / nm	526	675	488	550
Filter used / nm	470-490	530-550	360-370	360-370

2.5. Statistical analysis

To determine the number of stained/unstained cells for each strain and each fluorescent technique at least 300 cells from three independent experiments were evaluated. To multiple comparisons of the results obtained by four different fluorescent methods the One Way Analysis of Variance (ANOVA test), that compares means by using estimates of variance, was used.

3. Results and discussion

Results of different fluorescence methods of staining for tested yeast populations from early stationary phase - after 24 hours of growth are presented in Table 3. Comparison of the results obtained by using four different fluorescent probes with different mechanisms of action showed very good correlation

between them. At the 0,05 significance level, one-way ANOVA revealed no statistically significant difference between the means of results for each fluorescent stain. Therefore it can be concluded that for each strain, the means obtained using each fluorescence procedure are not significantly different.

Table 3

Comparison of yeast strains vitality measured by fluorescence staining with different dyes

Yeast strain	Fluorophore	Vitality /%			Mean
		Experiment 1	Experiment 2	Experiment 3	
Bc16a	DAPI	64.7 ± 4.5	64.3 ± 3.2	66.0 ± 9.8	**65.0 ± 5.7**
	Primuline	64.0 ± 4.0	72.3 ± 2.3	67.7 ± 6.8	**68.0 ± 5.5**
	Trypan blue	72.0 ± 5.6	74.3 ± 0.6	66.7 ± 7.5	**71.0 ± 5.8**
	Acridine orange	69.3 ± 7.8	68.0 ± 3.4	63.7 ± 6.5	**67.0 ± 5.9**
Jaa	DAPI	99.0 ± 3.0	99.3 ± 5.0	98.7 ± 4.0	**99.0 ± 3.6**
	Primuline	97.3 ± 4.0	95.0 ± 2.6	98.7 ± 4.2	**97.0 ± 3.6**
	Trypan blue	95.0 ± 3.0	95.3 ± 5.0	94.7 ± 4.0	**95.0 ± 3.6**
	Acridine orange	97,7 ± 4,2	97.0 ± 6.0	96.3 ± 5.1	**97.0 ± 4.5**
IIa	DAPI	33.0 ± 7.0	34.7 ± 9.4	31.3 ± 8.5	**33.0 ± 7.4**
	Primuline	29.0 ± 8.0	26.0 ± 7.2	32.0 ± 8.7	**29.0 ± 7.4**
	Trypan blue	30.0 ± 7.0	31.7 ± 9.4	28.3 ± 8.5	**30.0 ± 7.4**
	Acridine orange	38.0 ± 7.0	39.7 ± 9.4	36.3 ± 8.5	**38.0 ± 7.4**
Ja 64	DAPI	89.0 ± 3.0	89.3 ± 5.0	88.7 ± 4.0	**89.0 ± 3.6**
	Primuline	91.0 ± 4.0	89.3 ± 3.0	92.7 ± 4.0	**91.0 ± 3.6**
	Trypan blue	90.0 ± 3.0	90.3 ± 5.0	89.7 ± 4.0	**90.0 ± 3.6**
	Acridine orange	93.0 ± 4.0	91.3 ± 5.0	94.7 ± 4.2	**93.0 ± 3.6**
B	DAPI	51.0 ± 8.0	53.0 ± 10.0	49.0 ± 7.8	**51.0 ± 7.9**
	Primuline	45.0 ± 9.0	41.7 ± 8.3	48.3 ± 9.8	**45.0 ± 8.4**
	Trypan blue	45.0 ± 8.0	43.0 ± 10.6	47.0 ± 9.6	**45.0 ± 8.4**
	Acridine orange	40.0 ± 8.0	42.0 ± 10.6	38.0 ± 9.6	**40.0 ± 8.4**
Y500/5	DAPI	45.3 ± 5.5	45.7 ± 7.1	44.0 ± 6.2	**45.0 ± 5.5**
	Primuline	44.0 ± 6.0	42.3 ± 4.2	45.7 ± 6.1	**44.0 ± 5.0**
	Trypan blue	51.0 ± 5.0	52.0 ± 7.2	50.0 ± 6.2	**51.0 ± 5.5**
	Acridine orange	46.7 ± 5.6	45.6 ± 8.4	48.5 ± 5.3	**46.9 ± 5.8**

The resolution of microscopic images with different fluorophores for yeast cells in early stationary phase and in old 7 day populations are shown in Table 4 and Photo1. Inside each yeast strain from stationary phase different results of staining were observed, indicating of different degree of membrane damage or membrane permeabilization occurring during yeast cultivation. For older, 7-days populations the results were consistent across all three fluorophores (DAPI, P, TB) used for staining. This suggests that the dynamic of aging process is individual feature of each yeast strain.

Table 4

Changes of yeast cell vitality measured with different fluorophores in young (24 hours) and old (7 days) populations

Yeast strain	Fluorophore	Vitality / %	
		24 hour culture	7 day culture
Bc16a	DAPI	65	0
	Primuline	68	0
	Trypan blue	71	0
	Acridine orange	(green 67; yellow 33)	(orange 12; yellow 88)
Jaa	DAPI	99	0
	Primuline	97	0
	Trypan blue	95	0
	Acridine orange	(green 97; orange 3)	(orange 7; yellow 93)
IIa	DAPI	33	0
	Primuline	29	0
	Trypan blue	30	0
	Acridine orange	(green 38; orange 62)	(yellow 100)
Ja 64	DAPI	89	0
	Primuline	91	0
	Trypan blue	90	0
	Acridine orange	(green 93; orange 7)	(yellow 100)
B	DAPI	51	0
	Primuline	45	0
	Trypan blue	45	0
	Acridine orange	(green 40; orange 60)	(yellow 100)
Y500/5	DAPI	45	0
	Primuline	44	0
	Trypan blue	51	0
	Acridine orange	(green 46,9; orange 0,3; yellow 52,8)	(yellow 100)

Acridine orange staining procedure didn't give univocal results due to the fact that the yeast cells of the same strain were differently stained: from green to yellow. Another observation is that from comparison of vitality measurement

with three fluorochromes: DAPI, P and TB, the only yeast "green cells" are fully vital without damage of plasma membranes.

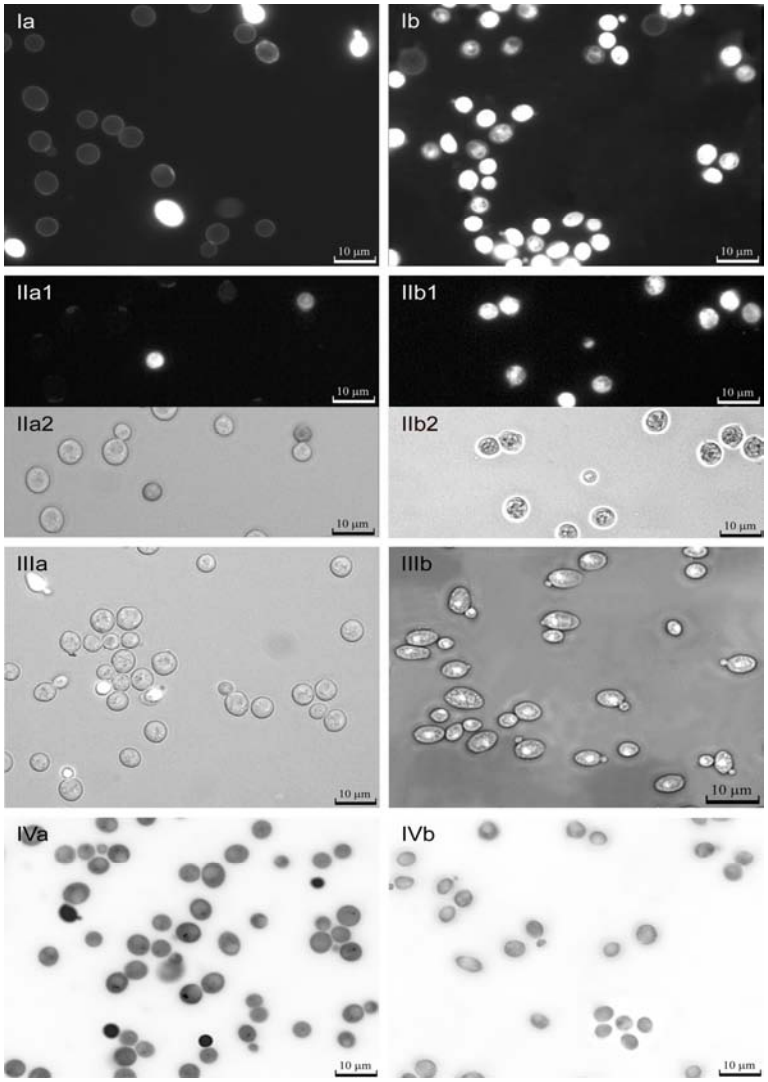

Photo 1. The results of fluorescent staining for young (24-hours) and old (7-days) yeast Bc16a populations. a) 24-hours culture; b) 7-days culture; I-primuline; II-trypan blue; III-DAPI; IV-acridine orange. For trypan blue staining cells were observed using fluorescence (1) and visible light (2)

Primuline and trypan blue are typical cell wall damage/permeability indicators. The use of the fluorochrome primuline for microscopically discriminating between viable and nonviable microorganisms was suggested by Meissel *et al.* [14], but no corroborative evidence accompanied this claim. Meissel argued that stained cells, i.e. fluorescent, were nonviable and unstained cells, i.e. nonfluorescent, were viable. It was shown that the yeast suspension were related to the relative percentage of viable cells as determined by the primuline technique [15]. In old populations, the transformation of cell wall began by the disappearance of the internal layer, then the mannans of external layers are in turn attacked [16]. As a consequence cell wall becomes thin, but rich in 1-3-β-glucosyl residues which are stained by primuline [17].

The reactivity of trypan blue (TB) is based on the fact that the dye is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, all the cells which exclude trypan blue are viable.

DAPI is known to form fluorescent complexes with natural double-stranded DNA, showing a fluorescence specificity for AT, AU and IC clusters. Because of this property DAPI is a useful tool in various cytochemical investigations, especially nuclear cytology, eg. in evaluation of apoptotic somatic cells. When DAPI binds to DNA, its fluorescence is strongly enhanced, what has been interpreted in terms of a highly energetic and intercalative type of interaction. However there is also evidence that DAPI binds to the minor groove, stabilized by hydrogen bonds between DAPI and acceptor groups of AT, AU and IC base pairs. Therefore DAPI is useful fluorophore for yeast cells only after crossing trough the membranes. So, yeast plasma membrane has not only been proved useful in determining viability, but also vitality of yeast industrial strains.

4. Conclusions

Our results have shown fluorescent techniques with the use of DAPI, primuline or trypan blue are simple, rapid and reliable for characterization of physiological state of yeasts. All of three tested fluorescence staining techniques can be used in rapid methods for control of biotechnological processes where the physiological conditions of yeast cells plays an essential role.

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OZNACZANIE ŻYWOTNOŚCI KOMÓREK DROŻDŻY Z ZASTOSOWANIEM RÓŻNYCH BARWNIKÓW FLUORESCENCYJNYCH

Streszczenie

Badano żywotność komórek drożdży w oparciu o barwniki fluorescencyjne wykrywające zmiany mikroskopowe jądra i membran komórkowych. Zastosowano: oranż akrydyny, DAPI, błękit trypanowy i prymulinę. Analiza porównawcza wyników uzyskanych z zastosowaniem różnych fluorochromów wykazała wysoki stopień ich korelacji. Barwienie fluorescencyjne stanowi przydatne narzędzie dla oznaczenia zarówno żywotności komórek drożdży, jak i zróżnicowania populacji w czasie procesu fermentacyjnego.

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