Polish Journal of Microbiology
2006, Vol. 55, No 2, 127–131

Novel Yeast Cell Dehydrogenase Activity Assay *in situ*

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Received 14 October 2005, revised 2 January 2006, accepted 4 January 2006

This article is devoted to the memory of the late Prof. W.J.H. Kunicki-Goldfinger on the tenth anniversary of his passing away

A b s t r a c t

The aim of this research was to develop a suitable method of succinate dehydrogenase activity assay *in situ* for different industrial yeast strains. For this purpose different compounds: EDTA, Triton X-100, sodium deoxycholate, digitonin, nystatin and β-mercaptoethanol were used. The permeabilization process was controlled microscopically by primuline staining. Enzyme assay was conducted in whole yeast cells with Na-succinate as substrate, phenazine methosulfate (PMS) as electron carrier and in the presence one of two different tetrazolium salts: tetrazolium blue chloride (BT) or cyanoditolyl tetrazolium chloride (CTC) reduced during the assay. In comparabile studies of yeast vitality the amount of intracellular ATP was determined according to luciferin/luciferase method. During the succinate dehydrogenase assay in intact yeast cells without permeabilization, BT formazans were partially visualized in the cells, but CTC formazans appeared to be totally extracellular or associated with the plasma membrane. Under these conditions there was no linear relationship between formazan color intensity signal and yeast cell density. From all chemical compounds tested, only digitonin was effective in membrane permeabilization without negative influence on cell morphology. Furthermore, with digitonin-treated cells a linear relationship between formazan color intensity signal and yeast cell number was noticed. Significant decreasing of succinate dehydrogenase activity and ATP content were observed during aging of the tested yeast strains.

K e y w o r d s: yeasts cells permeabilization, succinate dehydrogenase, formazans

Introduction

Succinate dehydrogenase (SDH) plays a crucial role in the energy supply for physiological activity of every living cell, included microorganisms (Samokhvalov *et al*., 2004). Therefore SDH activity assay is an important method for measurement of the yeast vitality in scope to control of different fermentation processes.

Reduction of various tetrazolium salts by dehydrogenases of metabolically active cells leads to production of highly colored end products – formazans. Tetrazolium salts commonly used in microbiological applications include: 2-(p-iodophenyl)-3(p-nitrophenyl)-5-phenyltetrazolium chloride (INT) (Saliola *et al*., 2004), 2,3,5-triphenyl tetrazolium chloride (TTC) (Rosa and Tsou, 1963), and more recently, new generation tetrazolium salts: 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) (Bernaś and Dobrucki, 1999), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Berridge *et al*., 1996; Stowe *et al*., 1995; Freimoser *et al*., 1999), 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) (Kuhn *et al*., 2003) or 4-[3-(4-idophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST1) (Berridge *et al*., 1996; Tsukatani *at al*., 2003).

Enzymatic reactions in yeasts are usually studied in cell-free extracts which requires disruption of cells and as consequence, inactivation of particular enzymes often can be observed. In the case of enzymatic reaction conducted *in situ* the plasma membrane forms a barrier with low degree of penetration. Therefore, cell permeabilization is recommended as an alternative method for the study of intracellular enzyme activities.

The aim of this research was to develop a suitable method of yeast cells permeabilization in succinate dehydrogenase activity assay *in situ* which can be used for different industrial *Saccharomyces cerevisiae* strains.

Experimental

Materials and Methods

Yeast strains used in this study. Five yeast strains *Saccharomyces cerevisiae* (races: Bc16a, Jaa, IIa, Ja 64 and baker's industrial strain) used in this study orginated from the International Collection LOCK 105 of the Institute of Fermentation Technology and Microbiology, Technical University of Łódź.

Media and cultivation. Yeast strains were cultivated in 50 ml of worth broth (Merck) at 30ºC for 20 hours or 7 days with shaking at 220 rpm/min.

Yeast cells permeabilization. Different compounds: 1% Triton X-100 (Sigma-Aldrich), 0.1% sodium deoxycholate (Sigma-Aldrich), 0.05% digitonin (Fluka), 185 mg/100 ml EDTA (Sigma-Aldrich), 0.39 g/10 ml β-merkaptoetanol (POCh) were used to treated tested yeast strains before the assay. The permeabilization process was controlled microscopically by staining with 0.01% primuline (Sigma-Aldrich) (Duffus *et al*., 1984).

Formazans formation. Two different tetrazolium salts blue chloride BT (Sigma-Aldrich) or cyanoditolyl tetrazolium chloride CTC (Polysciences) were used in the enzyme assay at concentration 0.68 mM and 4 mM respectively and were reduced during assay to form crystals of formazan, which were observed microscopically. BT formazans in the bright-field microscopy were an opaque blue intracellular deposits and the CTC formazans formed red, highly fluorescent crystals with fluorescence emission primarily in the red region, when excitation wavelength was in the region 360–370 nm.

Succinate dehydrogense SDH assay. This enzyme activity was assayed in whole cells. The cells were collected, washed and resuspended in Ringer solution. Standardized cell suspensions with 9×10^7 to 5×10^8 cell/ml were transferred to the tubes and centrifuged (10 min, 2100×g). Supernatants were discarded and 0.3 ml 0.05 M Na-succinate solution (Merck), one small crystal of PMS (Sigma-Aldrich) and 3 ml of solution contained 50 mg BT, 185 mg EDTA, 300 mg sodium azide (Sigma-Aldrich) in 100 ml were added to the biomass. The mixture was incubated for 60 min at 37°C and then the reaction was stopped with 0.4 ml of 37% formaldehyde (POCh) and the samples were centrifuged as before (10 min, 2100×g). Supernatants were discarded and the pellets were resuspended in DMSO (Sigma-Aldrich) for extraction of formazan crystals formed in yeast cells during the assay. Extraction was conducted with three sequenced volumes of 3, 2 and 2 ml DMSO, then extracts were pulled and the final absorbance was measured at 540 nm by spectrophotometer Specol 210 (Carl Zeiss, Jena, Germany). Each experiment was performed in triplicate and each data was the mean of three measurements. One SDH activity unit was equal to 1 µmol of formazan formed by 1×10^8 yeast cells at 37°C in 60 minutes.

ATP content. Intracellular ATP content was determined by luciferin/luciferase method using Hy-Lite2 luminometer (Merck) as relative light units (RLU). (De Luca *et al*., 1979).

Results

In microscopic studies for SDH activity assay of yeast cells without permeabilitzation, BT formazans (BTf) can be seen partially in the cells, but CTC formazans (CTCf) appeared to be totally extracellular or associated with the plasma membrane (Fig. 1 Ia, b; Fig. 2 Ia, b). Under these reaction conditions no simple linear correlation was observed between formazan absorbance and cell density (Fig. 3). Microscopic observations after primuline staining showed that the yeast plasma membrane was the barrier for tetrazolium salt in SDH activity assay *in situ*. In the case of cell permeabilization, when different active agents were added, such treatment changed the morphology of cells. Only digitonin was effective in membrane permeabilization without negative influence on cell morphology (Fig. 4). After digitonin treatment visible

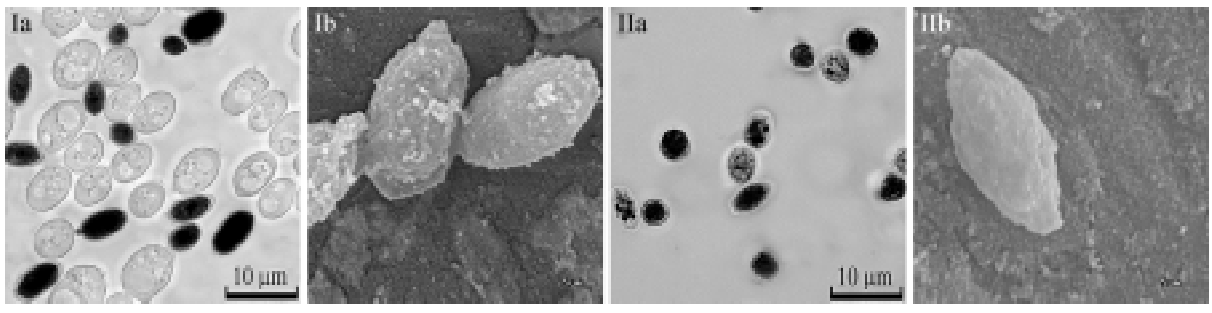

Fig. 1. BTf crystals.
I) outside yeast cells; II) inside yeast cells; a) bright-field microscopy; b) electron microscopy

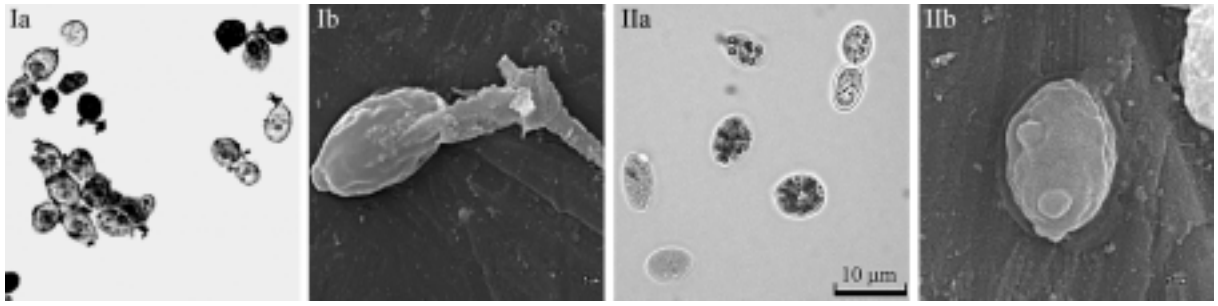

Fig. 2. CTCf crystals.
I) outside yeast cells; II) inside yeast cells; a) bright-field microscopy; b) electron microscopy

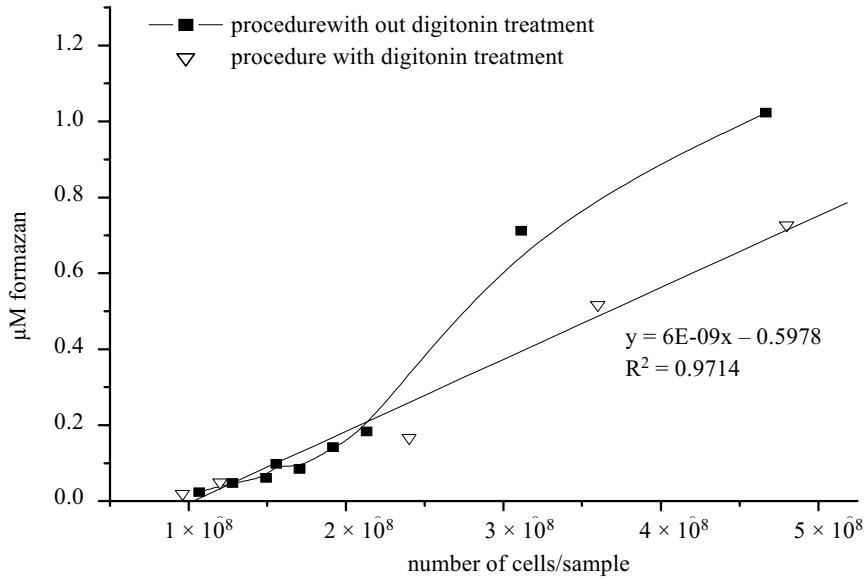

Fig. 3. Yeast cells density and BTf signal

formazan crystals were observed inside the yeast cells but not outside them (Fig. 1 IIa,b; Fig. 2 IIa,b). Good correlation ($R^2 = 0{,}97$) between BTf absorbance intensity after DMSO extraction and number of cells was seen. Linear correlation was observed in the concentration range of yeast cells from 9×10^7 to 5×10^8 per sample. For yeast cell concentrations below 1×10^7 per sample the formazan color intensity signals were too low to detect with good precision (Fig. 3).

The results obtained for SDH activity were in good agreement with ATP content in yeast cells. Significant decreasing of succinate dehydrogenase activity and ATP content were observed during aging of tested strains (Table I).

Table I
SDH activity and ATP content in different populations of *Saccharomyces cerevisiae* strains

Strain	20 h culture		7 day culture	
	ATP content [fM/cell]	SDH activity [µM/10^8 cells]	ATP content [fM/cell]	SDH activity [µM/10^8 cells]
S. cerevisiae Bc16a	95.9787	0.028	0.0991	0.0019
S. cerevisiae Jaa	115.9787	0.0973	5.3479	0.0166
S. cerevisiae IIa	1.6037	0.0146	0.1241	0.0029
S. cerevisiae Ja64	201.7287	0.1069	0.9979	0.0071
S. cerevisiae industrial strain	86.1037	0.0631	0.1848	0.0025
	Correlation factor $R^2 = 0.72$		Correlation factor $R^2 = 0.97$	

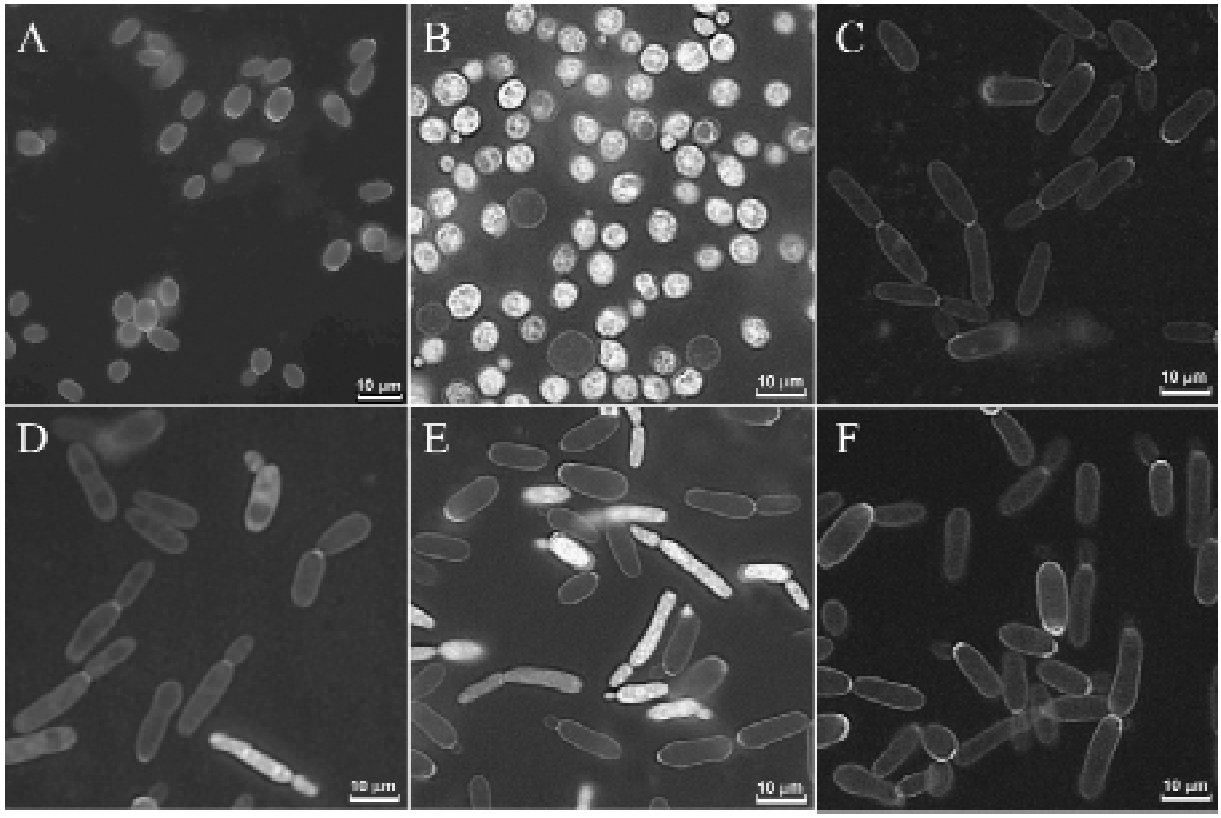

Fig. 4. Yeast cells after permeabilization and primuline staining.
A) native cells; yeast cells after: B) digitonin, C) EDTA, D) Triton, E) deoxycholate, F) β-mercaptoethanol.

Discussion

The earlier research described in the literature and conducted with different tetrazolium salt and yeast genera showed that colorimetric signal of extracted formazans was not proportional to the cell number (Kuhn *et al.*, 2003; Tsukatani *at al*., 2003). In our study the plasma membrane was the barrier to study SDH activity *in situ*. This fact was confirmed by microscopic studies and primuline staining. This may explain the lack of linear relationship between cell number and formazan absorbance in our previous experiments. Gentle permeabilization of yeast cell membrane, increasing its penetrability for tetrazolium salt, appeared to be an important step for BT formazan signal and SDH assay in yeast cells (Caldwell, 1987). In earlier studies on permeabilization, this process was conducted with digitonin at concentration from 0.01% to 0.1% during 10–30 min (Alamäe and Järviste, 1995; Freire *et al*., 1998; Samokhvalov *at al*., 2004). For digitonin-treated yeast cells linear relationship between cell number and BTf absorbance was observed.

The reaction with tetrazolium salt can be used in a wide range of biological assays including tests of cell viability. The developed method of SDH assay can be used in the study not only of yeast cell activity but to detect respiring cells in different ecosystems: water, food matrices, activated sludge or biofilms.

Acknowledgements. We gratefully acknowledge financial support of UE 6PR Grant NMP3-CT-2003- 504937 PERCERAMICS

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