

Succinate Dehydrogenase Activity Assay *in situ* with Blue Tetrazolium Salt in Crabtree-Positive *Saccharomyces cerevisiae* Strain

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Summary

A spectrophotometric method for determining succinate dehydrogenase (SDH) activity assay in azide-sensitive yeast *Saccharomyces cerevisiae* has been developed. The permeabilization of yeast cells by 0.05 % digitonin permitted to study yeast enzymatic activity *in situ*. The reduction of blue tetrazolium salt (BT) to blue tetrazolium formazan (BTf) was conducted in the presence of phenazine methosulphate (PMS) as an exogenous electron carrier, and sodium azide (SA) as an inhibitor of cytochrome oxidase (Cyt) pathway. Various factors such as type of substrate, BT concentration, cell number, temperature and time of incubation, and different Cyt pathway blockers were optimized. In earlier studies, dimethyl sulfoxide (DMSO) had been selected as the best solvent for extraction of BTf from yeast cells. The linear correlation between permeabilized yeast cell density and amount of formed formazan was evidenced in the range from $9 \cdot 10^7$ to $5 \cdot 10^8$ cells per sample solution. Below the yeast cell concentration of 10^7 the absorbance values were too low to detect formazans with good precision. This standardized procedure allows the estimation of SDH activity in whole cells, depending on vitality level of yeast populations. Significant increases of succinate dehydrogenase activities were observed in sequential passages as the result of the increase of activity of the strain and adaptation to cultivation conditions.

Key words: succinate dehydrogenase (SDH), *Saccharomyces cerevisiae*, enzymatic activity *in situ*, blue tetrazolium

Introduction

Detection of microorganisms in different biotechnological processes is frequently carried out using the classical plate method. However, this method has many methodological problems connected with cell clumping, long incubation time, labour intensity and inability of cells to form colonies due to cell injury and the presence of viable but non-culturable cells. Other methods of measuring cell growth and activity include dye exclusion techniques, adenosine triphosphate (ATP) measurements or cellular dehydrogenase activity assay.

There is a wide range of tetrazolium salts commonly used in the field of microbiology from the classical ones to the new generation of their derivatives. Among them are: 2,3,5-triphenyltetrazolium chloride (TTC) (1), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (2–4), 5-cyano-2,3-ditolyltetrazolium chloride (CTC) (5), 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) (6), 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate (WST-1) (2,7), 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) (8) or 2,2'-dibenzothiazolyl-5,5'-bis-[4-di(2-sulphoethyl)-

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carbamoylphenyl]-3,3'-(3,3'-dimethoxy-4,4'-biphenylene) ditetrazolium, and disodium salt (WST-5) (9).

Reduction of various tetrazolium salts by transfer of electrons from dehydrogenases in metabolically active cells leads to production of formazans, highly coloured end products. This reaction is used in a wide range of biological assays including tests of viability due to the fact that dehydrogenases play a crucial role in raising the energetic state of the cell. The reduction process is mediated by exogenous electron carriers like phenazine methosulphate (PMS), 1-methoxyphenazine methosulphate (MPMS) or Meldola Blue (MB). This reaction may be compromised by oxidoreductases of mitochondria, endoplasmic reticulum or plasma membrane (10).

In recent years quantitative histochemical procedures have been proved to be powerful research tools, especially in microphotometric assessment *in situ* of the specific activity of dehydrogenases in individual cells. These assays are a simple and valid alternative to conventional biochemical techniques. Techniques *in situ* can provide the cellular resolution necessary to determine enzyme-specific activities not only in whole cell preparations but also in distinct subcellular compartments (11–13).

Succinate dehydrogenase is the component of complex II of the respiratory chain that catalyses the oxidation of succinate to fumarate in the Krebs cycle (8). Flavin adenine dinucleotide (FAD) is also part of the succinate dehydrogenase (SDH) active enzyme complex. The oxidation of succinate to fumarate is the only Krebs cycle reaction that takes place in the inner membrane itself, as opposed to the other reactions that are catalyzed by soluble enzymes.

Succinate is the most efficient energy source, so the SDH activity assay is an important method for measurement of the yeast vitality with the scope of controlling different fermentation processes (14). In Crabtree-positive yeasts, cyanide, azide or antimycin should be used in the SDH assay *in situ* to prevent interference from cytochrome oxidase.

In the present study, modification of a BT assay for measurement of SDH activity *in situ* in Crabtree-positive, azide-sensitive *Saccharomyces cerevisiae* cells is described.

Materials and Methods

Strain, media and culture conditions

Saccharomyces cerevisiae Bc16a, a distillery strain from Collection LOCK 105 of the Institute of Fermentation Technology and Microbiology, Technical University of Lodz used in this study, was stored on wort agar slants under standard laboratory conditions. Yeast cells used in experiments were cultivated in 50 mL of wort broth (Merck, Germany) at 30 °C during 24 h on rotary shaker at 20 rpm. Cell concentrations were determined by counting in a Thoma counting chamber under a light microscope.

Chemical reagents

Tetrazolium salt solution was composed of 100 mg of tetrazolium blue chloride (Sigma, Japan), 1.5 mL of

N,N-dimethylformamide (Sigma, USA), 650 mg of sodium azide (Sigma, Germany) and 370 mg of EDTA calcium disodium salt (Aldrich, the Netherlands) dissolved in 200 mL of redistilled water. This solution was sterilized by filtration (0.45 µm) and stored frozen in aliquots; additionally, 2 mg/200 mL of antimycin A (Sigma, Germany) as Cyt pathway blocker was used.

Water solution of 0.5 M sodium succinate (Merck, Germany) or 0.5 M glucose (POCh, Poland) used as substrate in SDH activity assay was sterilized by filtration (0.45 µm) and stored frozen in aliquots.

Phenazine methosulphate (Sigma, Germany), hydrazine hydrate (POCh, Poland), formaldehyde (POCh, Poland) and DMSO (Sigma-Aldrich, Germany) were used in SDH activity assay as the electron carrier, reducing reagent, inactivation reagent and organic solvent, respectively.

Succinate dehydrogenase activity assay

SDH activity was measured in the *in situ* assay with whole cells. Yeast cells were collected from cultivation medium by centrifugation (10 min, 2100×g, room temperature) and washed twice with Ringer solution in the same manner. Standardized cell suspensions in Ringer solution containing $9 \cdot 10^7$ – $5 \cdot 10^8$ cells/mL were transferred to the tubes and centrifuged as before. Supernatant was discarded and to the biomass were added: 0.3 mL of 0.5 M substrate dissolved in water, 3 mL of 0.68 mM tetrazolium salt dissolved in water and one small crystal of PMS. The mixture was then incubated at constant temperature of 37 °C and the reaction was stopped after 60 min by the addition of 0.4 mL of 37 % formaldehyde. The samples were centrifuged as before and supernatants were discarded but the pellets were resuspended in 7 mL of undiluted DMSO for extraction of formazan crystals formed in yeast cells during the assay. The final absorbance of DMSO extracts was measured at 540 nm (Specol 210, Carl Zeiss, Jena, Germany) and calculated for BTf in µmol per sample. Each experiment was performed in triplicate and each data was the mean of three measurements.

BT formazan crystals were obtained by chemical reduction of 0.68 mM BT salt at 100 °C for 30 min. A series of samples was made up by taking 0, 0.3, 0.5, 0.7, 0.9 and 1.0 mL of BT solution and making up to 1.0 mL with redistilled water. As a reducing agent, 40 µL of 80 % hydrazine hydrate (POCh, Poland) were used. Formazan crystals were dissolved in 7 mL of undiluted DMSO. The final absorbance of DMSO extracts was measured at 540 nm and calculated for BTf in µmol per sample.

Results and Discussion

Blue tetrazolium formazan absorption spectrum

Among different organic solvents used in the experimental assays, the best solubility of blue tetrazolium formazan (BTf) was noticed with DMSO (data not shown). The absorption spectrum of BTf solubilized in DMSO was determined in a wide wavelength range of 480–600 nm with maximal absorbance seen at 540 nm (Fig. 1).

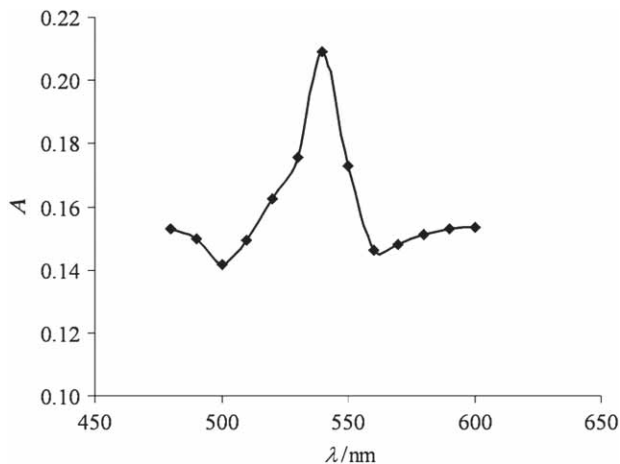


Fig. 1. Absorption spectrum of 0.68 M BTf in DMSO

Optimization of SDH activity assay conditions

Sodium succinate (0.5 M) and glucose (0.5 M) were compared as the substrates for SDH activity measurements. According to the earlier studies, SDH reaction is repressed by glucose and derepressed on respiratory carbon sources (8). This fact was also confirmed in our study, which indicated that unspecific reduction of tetrazolium was lowered in the presence of glucose (Fig. 2a).

BT used in a concentration range from 0.137–1.37 mM did not have any significant influence on BTf formation (Fig. 2b), and therefore the standardized BT salt concentration was set at 0.68 mM. However, it cannot be excluded that the response of strains to different tetrazolium concentrations may be strain- and tetrazolium salt-dependent, as it had previously been shown for *Candida* strains and XTT sodium salt (6).

The BT formazan product is water insoluble, but readily diffuses out of yeast cells after solubilization in DMSO. However, we have noticed that after one-step solubilization with 3 mL of DMSO there was a slight residual blue or pink discoloration of the cell pellets. This suggests that some residue of BTf remains bound to certain cell components or is entrapped inside the cells. Therefore, it was necessary to conduct the next two steps of solubilization of formazan with the volume of 2 mL of DMSO for each one. In this way BTf may be fully removed from the yeast cells.

Process of different tetrazolium salts reduction (4) and earlier data suggested that another tetrazolium salt, TNBT, reduced by SDH, produced at least two kinds of formazans, more or less resistant to organic solvents (15). A similar observation was noticed in a preliminary study with BT formazans. Our observations also suggest that BT, reduced by dehydrogenases and stored in the presence of light and air, produces formazan, which is resistant to solubilization in DMSO, but is soluble in *N,N'*-dimethylformamide. Therefore, 30 μ L of *N,N'*-dimethylformamide were added to 3 mL of total SDH assay mixture for better solubilization of BT in the water. Addition of formamide and incubation of cells in this mixture did not have any significant influence on the measurement of respiratory activity of yeast (data not

shown). However, taking into consideration toxicity and chemical aggression of *N,N'*-dimethylformamide, we decided to use DMSO in the SDH assay for health and safety reasons.

Fig. 2c shows BT formazan formation during 60-minute incubation of yeast cells and tetrazolium salt solution at different temperatures between 30 and 60 °C. The maximum of BTf synthesis during 60 min of incubation was seen at 50 °C. However, due to the expected sensitivity of yeast cells to high temperatures, the incubation temperature of 37 °C was chosen as the standard.

As shown in Fig. 2d, there was continuous and linear increase of BTf formation during 1 h of incubation at two different temperatures, 37 °C (standard) and 50 °C (maximum). The standardized incubation time was set at 60 min.

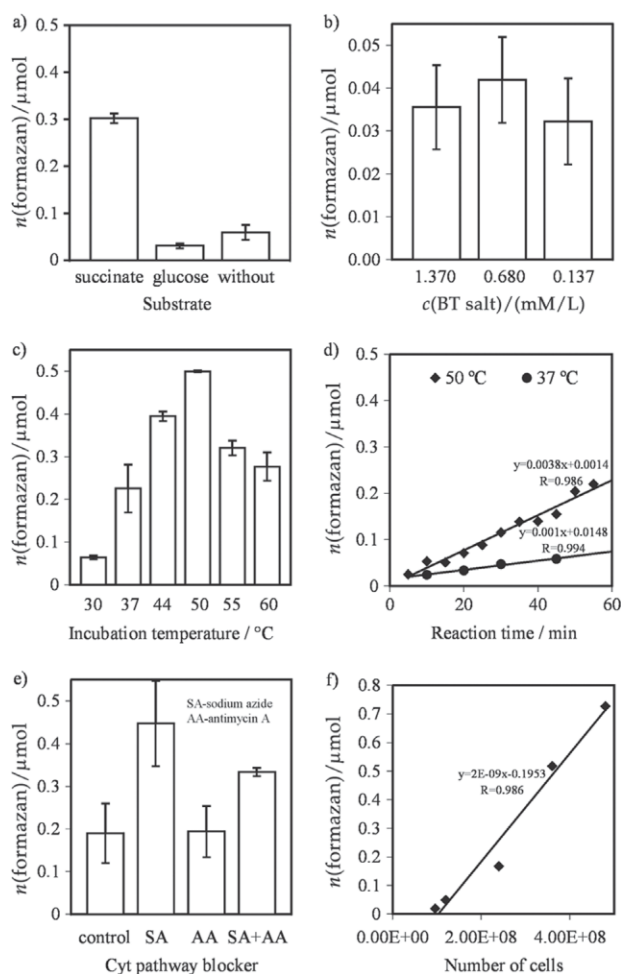


Fig. 2. Optimization of SDH activity assay conditions

Effect of mitochondrial blockers

The effects of sodium azide (SA) and antimycin A (AA), the cytochrome oxidase (Cyt) inhibitors, on the SDH activity were studied (Fig. 2e). In *Saccharomyces cerevisiae* strain formazan production was dependent on the presence of SA, but the effect of AA or the mixture of AA and SA on SDH activity was not observed. The yield of the reaction (formazan production) was unex-

pectedly high in the presence of azide. Interpretation of this fact is difficult and there is no satisfactory explanation of this interesting phenomenon. Earlier studies on different tetrazolium salts show that the addition of electron transport blockers may reduce the flow of electrons to the terminal electron acceptor O_2 . With some tetrazolium salts, this dramatically increases the production of coloured formazans (16).

Effect of yeast cell density

For a wide range of total cell numbers used in the SDH assay, there was no simple linear correlation between the cell number and the final absorption of formed BTf in DMSO extract (17). This lack of proportionality was also pointed out in the earlier works conducted with different tetrazolium salts and yeasts (6). There is no doubt that the plasma membrane can be a barrier for BT salts and PMS in the SDH activity assay conducted with intact cells, so the reduction of the membrane impermeable salts may be partially carried out outside the cell by active dehydrogenases linked to plasma membrane (5).

Our previous studies have shown that mild permeabilization of the yeast cell membrane may increase penetrability and this appeared to be an important step in effective BTf formation during SDH assay with yeast cells (17,18). Upon binding to membrane sterols, digitonin induced some deformation of the membranes, which in turn increased their permeability for tetrazolium salt and electron carrier (19). However, the inner mitochondrial membrane contained active SDH and had only a low level of sterols, so it was resistant to digitonin treatment. Yeast cell permeabilization with digitonin treatment in a dose-dependent manner had previously been tested for the concentration range from 0.01 to 0.1 % using incubation time of 10–30 min (20,21). In our studies with digitonin-treated yeast cells, a very good linear correlation ($R=0.99$) between the cell number and BTf absorbance of DMSO extract was observed. Measurements of BTf absorbance were proportional to the cell number in the assay in the range from $9 \cdot 10^7$ to $5 \cdot 10^8$ cells (Fig. 2f). However, for lower yeast cell concentrations, below 10^7 cells/assay, the absorbance values were too low to detect formazan formation with good precision.

The detailed protocol of optimized SDH activity assay *in situ* with yeast cells is described schematically in Fig. 3.

SDH activity assay in *Saccharomyces cerevisiae* during sequential passages

Significant increases of succinate dehydrogenase activities were observed during sequential passages of yeast cells to fresh wort broth as the result of increased vitality of the strain stored under standard laboratory conditions and its progressive adaptation to the culture conditions (Fig. 4). Our earlier studies showed that significant decrease of SDH activity was observed during aging of the tested strains (17). Additionally, the present results confirm that the SDH reaction with BT tetrazolium salt can be used in a wide range of biological assays including tests of cell vitality.

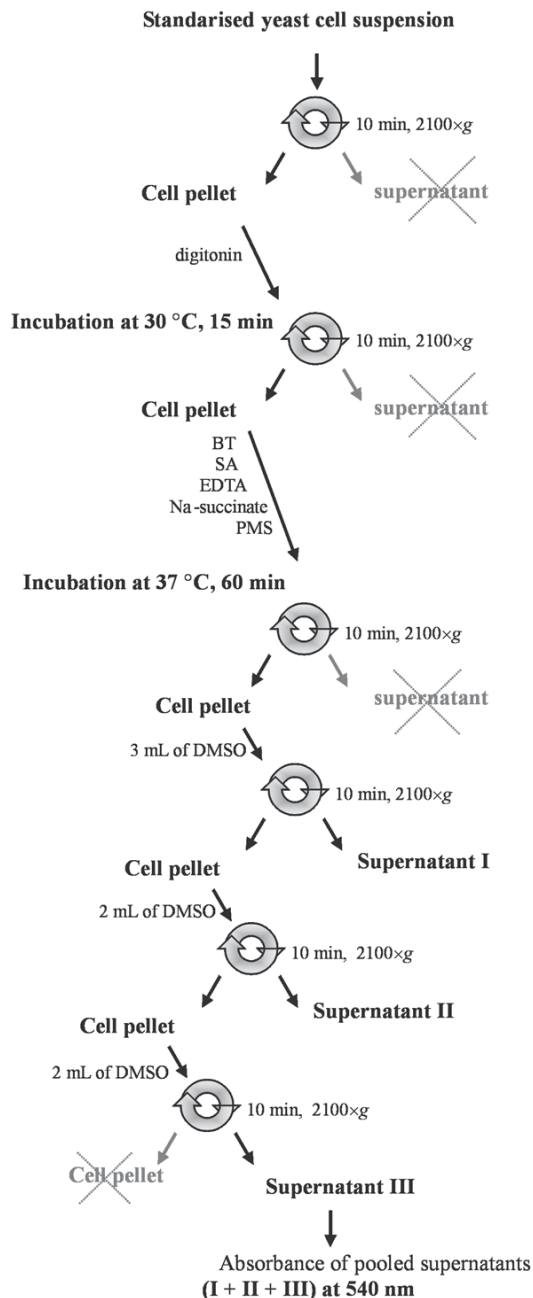


Fig. 3. Detailed protocol of optimized SDH activity assay *in situ*

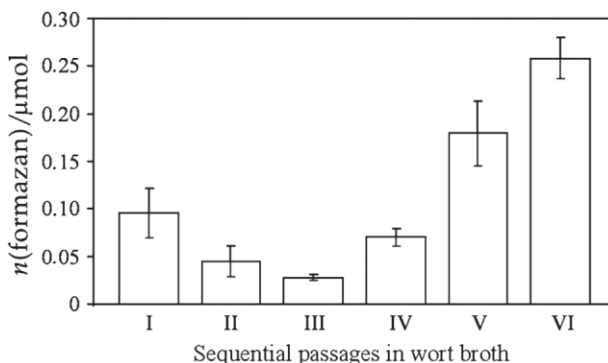


Fig. 4. SDH activity *in situ* measurement for *Saccharomyces cerevisiae* Bc16a strain during sequential passages

Conclusions

SDH activity assay conducted *in situ* with whole cells is a powerful research tool for evaluation of the physiological state of microorganisms. Blue tetrazolium BT salt fulfills a number of criteria of the assay: low price, a suitable reaction speed and easiness to read, so it may be widely used for detecting growth and physiological state of yeast cells. It is known that different yeast strains metabolize this particular substrate with different capabilities (6). This is also true for dehydrogenases of metabolically active yeast cells which have different subcellular origins. Therefore, for Crabtree-negative, cyanide-insensitive cells, this assay should take into consideration blocking of alternative oxidase pathway by salicylhydroxamic acid (SHAM) (22,23). Modification of SDH assay *in situ* conducted under strictly standardized conditions gives the opportunity to measure physiological activity and energy potential of cells.

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