A viability assay for *Candida albicans* based on the electron transfer mediator 2, 6 – Dichlorophenolindophenol (DCIP)

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Short title: Viability assay for *Candida albicans* using DCIP

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Abstract

Candida albicans is an opportunistic fungal pathogen with a comparably high respiratory activity. Thus, we established a viability test based on 2,6 dichlorophenolindophenol (DCIP), a membrane permeable electron transfer agent. NADH dehydrogenases catalyze the reduction of DCIP by NADH, and the enzymatic activity can be determined either electrochemically via oxidation reactions of DCIP or photometrically. Among the specific respiratory chain inhibitors only the complex I inhibitor rotenone decreased the DCIP-signal from *C. albicans* leaving a residual activity of approximately 30 %. Thus, the DCIP-reducing activity of *C. albicans* was largely dependent on complex I activity. *C. albicans* is closely related to the complex I-negative yeast *Saccharomyces cerevisiae*, which had previously been used in DCIP – viability assays. Via comparative studies, in which we included the pathogenic complex I-negative yeast *Candida glabrata*, we could define assay conditions, which allow a distinction of complex I-negative and -positive organisms. Basal levels of DCIP turnover by *S. cerevisiae* and *C. glabrata* were only 30 % of those obtained from *C. albicans*, but could be increased to the *C. albicans* level by adding glucose. No significant increases were observed with galactose. DCIP reduction rates from *C. albicans* were not further increased by any carbon source.

Keywords: complex I activity, respiratory chain inhibitors, *Candida glabrata*, *Saccharomyces cerevisiae*, NADH dehydrogenases, metabolic activation

Introduction

One of the most frequent human fungal pathogens is *Candida albicans*. It is the cause of oral and vaginal thrush as well as of severe mucosal and systemic infections in immuno-compromised individuals [1]. Environmental safety concerns and needs in medical diagnosis to rapidly identify pathogenic organisms and their susceptibilities to antibiotics have prompted the development of a variety of new diagnostic methods [2, 3]. Molecular methods have the drawback that they do not distinguish between viable and non-viable organisms so that the infectious risk may be overestimated [2]. However, classical methods for the determination of viable microorganisms are based on the capacity of the organism to multiply and to form colonies, which has been questioned due to long analysis times, and the development of assays that rely on the metabolic activity rather than cell growth has been suggested [4]. Respiration [5] and electron transfer reactions [6-9] were considered suitable indicators of the metabolic activity of cells, as they are essential for proliferation of aerobic organisms.

In eukaryotic organisms the respiratory chain is located in mitochondria where more than 90% of the total oxygen is consumed via the electron transport chain (ETC). The classical respiratory chain (CRC) in eukaryotes comprises three large protein complexes: NADH-ubiquinone oxidoreductase (complex I), ubiquinol: cytochrome *c* oxidoreductase (complex III or cytochrome bc_1 complex) and cytochrome *c* oxidase (complex IV) (Fig. 1). In each complex electron transport is coupled to proton translocation, with the resultant proton motive force being used for ATP synthesis. Each complex can specifically be inhibited, e.g. complex I by rotenone, complex III by antimycin and myxothiazol and complex IV by cyanide. Electron transfer from succinate to ubiquinone without pumping of protons is done by succinate dehydrogenase (complex II) [10], of which thenoyltrifluoroacetone (TTFA) is a

specific inhibitor. However, *C. albicans* has a more complex respiratory chain structure due to the presence of three respiratory pathways: In addition to the classical respiratory chain (CRC), an alternative oxidase [10-12] and a parallel electron transport chain (PAR) [13] were described, as shown in Fig. 1. The CRC comprises all four already described enzymatic complexes [10, 14], leading to coupling of proton translocation and NADH oxidation. The alternative respiratory pathway is generally conferred by a cyanide-insensitive alternative oxidase located on the matrix side of the inner mitochondrial membrane and encoded by two nuclear genes, *AOX1a* and *AOX1b*. *AOX1a* is constitutively expressed, whereas expression of AOX1b is induced, for example by the presence of inhibitors of complexes of the CRC. These alternative oxidases catalyze the direct oxidation of ubiquinol by oxygen without proton translocation and, thus, bypass complexes III and IV and enable respiration even in the presence of downstream CRC inhibitors. Due to the lack of a proton gradient energy storage from NADH oxidation is less efficient than in the CRC. AOX are inhibited by salicylhydroxamic acid (SHA) [12, 15]. The PAR is only activated when both the CRC and the AOX pathways are totally blocked, allowing electron flux to be redirected upstream of complex III, in parallel to the CRC [13]. In other fungi, such as the pathogenic yeast *Candida glabrata* and the non-pathogenic yeast *Saccharomyces cerevisiae*, complex I is absent and so-called external and internal NADH dehydrogenases catalyze the oxidation of NADH without generation of a trans-membrane proton gradient. For simplicity we call these yeasts complex I – negative yeasts, to distinguish them from yeasts, which possess all four complexes of the CRC, such as *Candida albicans*, which we call complex I – positive yeasts. Complex I – negative yeasts are usually rotenone-insensitive [16, 17].

One direct indicator of the respiratory activity of microorganisms is the oxygen consumption rate, and we had previously shown that *C. albicans* has a higher respiratory activity than *S. cerevisiae* [5]. Alternatively the activity of electron transfer reactions can be evaluated by

artificial electron acceptors, and tetrazolium salts [8, 9, 18, 19], resazurin (AlamarBlue) [7, 20] and various quinoid compounds [6, 21, 22, 23] have been used to detect the viability of microbial and mammalian cells via color changes, which are usually related to the reduction of these compounds. However, also electrochemical detection methods are applicable, when the reduced electron acceptor can be oxidized at electrodes.

2,6-dichlorophenolindophenol (DCIP) is one of the compounds allowing both spectrophotometric [22, 23] and voltammetric [24] detection of cellular reactions. It is known as photometric pH and redox indicator [25]. The reduced form is colorless, whereas the oxidized form has a dark blue color in solutions with pH > 6 and is pink in solutions with lower pH [25]. The reduced form of DCIP ($DCIPH_2$) contains two oxidizable groups, namely the 2,6-dichloro-4-quinone imino group and the phenolic ring. The oxidation of the quinone imino group is considered to be a reversible two-electron + two-proton transfer reaction and usually procedes at potentials below 0.1 V (vs. Ag/AgCl reference electrode) [26]. In acidic solutions the formal potential is shifted to more positive values [25], and at a glassy carbon electrode even a clear separation of the anodic and the cathodic peak was observed [26]. Oxidation of the phenolic ring occurs at potentials higher than 0.5 V and is an irreversible reaction [26]. It is assumed that potentials higher than 0.4 V lead to electropolymerization of DCIP with preservation of the redox properties for NADH [26, 27]. Most electrochemical assays with DCIP focus on the low potential range and use DCIP for mediated NAD(P)H detection [26, 27]. DCIP easily penetrates cell membranes and reacts with the intracellular NAD(P)H pool. This reaction is catalyzed by NAD(P)H dehydrogenases and DCIP is used as an indicator of NADH dehydrogenase activities, in particular of the activity of complex I [22 - 24]. However, DCIP was also used in a toxicity assay based on the complex I – negative yeast S. *cerevisiae*, as the absorbance of DCIP at 600 nm decreased with increasing metabolic

activity of *S. cerevisiae* [22]. Thus there are also NADH-DCIP reductase activities, which are independent of complex I.

We established a viability assay for *C. albicans* based on the electron transfer capabilities of DCIP and the electrochemical analysis of DCIP in the extended potential range from –0.25 V to +1.0 V. We were interested in an assay, which supported the analysis of structures of electron transport chains and of the mode of action of respiratory chain inhibitors in yeasts. Thus, we performed comparative studies between *C. albicans* and the complex I – negative yeasts *C. glabrata* and *S. cerevisiae*. This enabled us to define assay conditions, which allow a distinction of complex I - positive and –negative yeasts. Moreover, the relevance of metabolic activation could be studied.

Materials and methods

Chemicals and reagents

Synthetic carbon powder and 2,6-dichlorophenolindophenol (DCIP) sodium salt were obtained from Sigma-Aldrich. A stock solution of 0.1 M of DCIP was prepared by dissolving the appropriate amount of DCIP in MQ water. The final concentration in the electrochemical cell was 40µM. YPD medium, antimycin A from *Streptomyces sp*., and rotenone were purchased from Sigma, and paraffin oil and 2-thenoyltrifluroacetone (TTFA) were obtained from Fluka. NADH and KCN were obtained from Roth (Karlsruhe; Germany) and Merck (Darmstadt, Germany), respectively. All chemicals were of analytical grade and used without further purification.

Microorganisms and growth conditions

The following microbial strains were used: *C. albicans* CAF2-1 [28], which was derived from *Candida albicans* SC5314 (ATCC MYA-2876), and *S. cerevisiae* BY4741. Yeast cells were cultivated overnight in 250 ml flasks in 50 ml YPD medium (yeast extract (10 g/l), peptone (20 g/l), and glucose (20 g/l)) at 30° C. A pre-culture was prepared by diluting the overnight culture to an optical density (OD_{620}) of 0.2 in 25 ml YPD and the yeast cells were allowed to grow for 3 hours so that they reached the exponential growth phase. The OD was determined in 180 µl sample volume with the microtiter plate reader µQuant (BioTek Instruments GmbH, Bad Friedrichshall, Germany). The working culture was prepared by diluting the pre-culture to an OD_{620} of 0.2. After cultivation for another 3 hours, the OD_{620} was recorded and the whole suspension was taken into a falcon tube and cells were harvested by centrifugation (Eppendorf centrifuge 5804R) at room temperature at 5000 rpm for 5 min, and washed carefully three times with PBS. The washed cells were resuspended in 1 ml PBS.

To test the electrochemical activity of non-living organisms (dead cells), the working culture was autoclaved at 121°C for 20 min. The cells were harvested, washed and resuspended by the same procedure as described above.

Voltammetric procedure and preparation of carbon paste electrode

All electrochemical measurements were performed using a computer controlled Gamry Potentiostat/Galvanostat/ZRA G750, which was connected to a three electrode system comprising a carbon paste working electrode, a Pt disc auxiliary electrode and an Ag/AgCl/3M KCl reference electrode. The carbon paste electrode was prepared by thoroughly mixing 1 g of synthetic carbon powder 1-2 micron with 0.4 ml paraffin oil in a small hand mortar. The hollow electrode (5mm) was filled with the carbon paste. For regeneration and cleaning a small part of the paste was cut off and the electrode surface was polished with a wetted filter paper. The working electrode was electrochemically activated prior to measurements by applying ten cyclic scans from 0.0 to 1.0 V with a sweep rate of 50mV/s in PBS buffer (pH 7) as a supporting electrolyte. Then 1 ml of the washed yeast cells were introduced into the electrochemical cell containing 24 ml PBS buffer with 40µM DCIP. All electrochemical experiments were carried out at room temperature. The incubation time of the cell suspension with DCIP usually was 5 minutes before linear sweep voltammograms (LSV) or cyclic voltammograms (CV) were obtained. Anodic peak currents were determined as absolute values, i.e. they were not related to a baseline resulting from other oxidation reactions occurring at lower potentials.

Spectrophotometric assay of the viability of yeasts

For validation of the electrochemical data, also spectrophotometric DCIP assays with the chosen yeasts (*C. albicans*, *C. glabrata* and *S. cerevisiae)* were performed. In each well of a

96-well plate (BD Falcon, New Jersey, USA) 180 µL of the cell suspension with OD_{620} of 0.5 was mixed with DCIP (in PBS) to result in a final concentration of 40 µM. The plate was incubated on a plate shaker at 30 °C for 20 min and the DCIP color at 600nm was determined using the microtiter plate reader µQuant (Biotek Instruments). The color change was calculated according to equation 1:

(1) DCIP color change (%) = 100 - (A_{600}(20 min) / A_{600}(0 min))*100)

Oxygen determination of yeasts

The consumption of oxygen was monitored during the cultivation of yeast cells in YPD medium according to a previously described procedure [5]. Oxygen was determined with round-bottomed OxoPlates (PreSens, Regensburg, Germany) following the procedure given by the manufacturer. Briefly, the fluorescence of the indicator and the reference dye were determined from the bottom of the plates at excitation wavelengths λ_{ex} of 530 nm and emission wavelengths λ_{em} of 620 nm for the indicator dye and λ_{ex} 530 nm and λ_{em} 590 nm for the reference dye. Calibration was performed with water saturated with air (100%) and with an aqueous Na_2SO_3 solution (10 g/l) (0%) according to the supplier's protocol.

Effects of electron transfer chain inhibitors

C. albicans cells were treated with electron transport chain (ETC) inhibitors using the final concentrations 41 µg/ml rotenone, 5 µg/ml 2-thenoyltrifluoroacetone (TTFA), 14 µg/ml salicylhydroxamic acid (SHA), 1.5 µg/ml antimycin A (AA) and 5 µg/ml cyanide (KCN). The concentrations were chosen for each compound on the basis of the inhibitory effects on oxygen consumption and reactive oxygen species (ROS) induction (data not shown). These inhibitors were added to the medium of the working culture (YPD). The yeast cells were

allowed to grow in the presence of the inhibitors at 30 °C for 3 h and were harvested and re-suspended as described above.

Metabolic activation

The metabolism of yeast cells (*C. albicans*, *C. glabrata* and *S. cerevisiae*) was activated by the addition of glucose or galactose to the PBS buffer. 2% glucose or galactose solutions were prepared in PBS. Sterilization was done by autoclavation. 1 ml of the washed yeast cells was transferred into a flask with 24 ml of the carbohydrate buffered solution and left on the shaker for 30 min at 30 ^{o}C. Then the electron transfer activities of the organisms to DCIP were electrochemically and photometrically determined by the addition of DCIP and incubation for 5 min and 20 min, respectively. Signals from cultures without carbohydrate supplement were used as control.

The rotenone-insensitive DCIP - reducing activity of the yeasts in the presence or absence of different C-sources was determined photometrically in 96 well plates. Each well of a 96-well plate was filled with 140 µl of PBS supplemented with or without C-source and with or without rotenone (41 µg/ml) or the appropriate volume of solvent (DMSO). 20 µl of the cell suspension were added to each well, and the plate was incubated at 30 ^{o}C on the plate shaker for 30 min. Finally, 20 µL of DCIP were added to a final concentration of 40 µM. The change of the blue color was monitored at 600 nm after 20 min.

Determination of NADH

S. cerevisiae and *C. albicans* were grown in YPD at 30 ^{o}C for 3 h and the corresponding OD_{620} was measured (working culture). The cells were collected by centrifugation, washed and re-suspended in PBS as described before. The supernatant was transferred into a new falcon tube. NADH was quantified in both the cell suspension and the supernatant. 5 µl of the

cell suspension or the supernatant were placed in a 96 well plate (Costar, New York, USA) and 45 µl of PBS were added to obtain a final volume to 50 µl. The fluorescence intensity was quantified by a plate reader (Synergy 4, BioTek Instruments GmbH) at the excitation wavelength λ_{ex} 370 nm and the emission wavelength λ_{em} 460 nm. An NADH calibration curve was obtained from a dilution series of NADH standard solutions.

Determination of reactive oxygen species (ROS)

The amounts of reactive oxygen species (ROS) produced in the yeast cells were measured by a fluorometric assay using H_2DCFDA,SE (2',7'-dichlorodihydrofluorescein diacetate, succinimidyl ester) (Invitrogen) as a Reactive Oxygen Species (ROS) indicator as previously described [29]. The working culture was prepared by diluting the pre-culture to an OD_{620} of 0.2 in fresh YPD medium. After cultivation for another 3 hours, the OD was measured, cells were harvested by centrifugation for 5 min at 5000 rpm at room temperature, and carefully washed with MQ water three times. Washed cells were resuspended in sterile water to an OD_{620} of 0.5. H_2DCFDA, SE was added from a stock solution of 2 mg/ml in DMSO to a final concentration of 40 µg/ml. After 30 min of incubation at 30 °C, the stained cells were collected by centrifugation and re-suspended again in sterilized MQ water. 120 µl of the stained cells were taken into a 96 well plate containing 60 µl of rotenone (final concentration was 41 µg/ml). The fluorescence intensity was quantified after 2 h using a microtiter plate reader (Synergy 4, BioTek Instruments GmbH) at the excitation wavelength λ_{ex} 485 nm and the emission wavelength λ_{em} 535 nm.

Results and discussion

Electrochemical reaction of DCIP with yeasts

The cyclic voltammogram of DCIP showed two clearly defined oxidation peaks at +0.1 V and +0.5 V, and a reduction peak at +0.05 V, indicating the reversible nature of the first oxidation step (Fig. 2a) [26]. The addition of *C. albicans* led to a spontaneous increase of both oxidation peak currents. Additionally, the first peak became broader, and the second peak shifted by almost 0.1 V to +0.6 V (Fig. 2b). With the extension of the reaction time of *C. albicans* with DCIP (electrode switched off) to 15 min the first peak became less sharp and developed to a wave, of which the steady state current was almost identical to the original peak current (Fig. 2). In contrast, the peak form corresponding to the second oxidation step did not change and the peak current increased with reaction time at least comparable to the increase of the steady state level of the first oxidation step. These changes were also observed, when NADH was co-incubated with DCIP (Fig. 2c). After addition of DCIP the above mentioned anodic and cathodic peaks from DCIP oxidation (+0.1 V; +0.5 V) and reduction (+0.05 V) appeared in addition to the peak from NADH oxidation (+0.4 V). However, after a reaction time of 5 min the NADH peak was disappeared and the second DCIP oxidation peak was shifted to +0.6 V. As the second peak remained sharp and well defined in all experiments and the peak current also was an indicator of the reaction of the yeasts with DCIP, in following investigations also linear sweep voltammetry in the potential range from 0.2 V to 1.0 V was used.

Fig. 3 shows the linear sweep voltammograms of *C. albicans* and *S. cerevisiae* with DCIP in the anodic potential range at different incubation times of the yeast cells with DCIP (0, 5, 10 and 15 min). As observed in cyclic voltammograms (Fig. 2b) an anodic peak was observed at 600 mV. The oxidation peak current increased by more than 50 % with increasing the incubation time of DCIP with *C. albicans* to 15 min (from approximately 16.5 µA to approximately 29 µA), whereas only a minor increase in the oxidation peak current of DCIP incubated with *S. cerevisiae* was observed (approximately 10 % in 15 min). Thus, *S. cerevisiae* cells were significantly less capable to reduce DCIP.

No electrochemical response was observed when dead cells of *C. albicans* were used. Therefore, the anodic peak current at +0.6 V was obviously an indicator for the reduction of DCIP by viable *C. albicans* cells.

Peak current correlated with cell numbers and growth rate of C. albicans

The detection of reduced DCIP via the anodic peak current at 0.6 V provides a good bio-electrochemical system for monitoring the growth and viability of *C. albicans*. Figure 4 shows the dependence of the peak current on the number of viable cells. The current increased in the range from 2.6 x 10^6 to 4.5 x 10^7 cells/ml. Thus, a growth curve could be recorded during a cultivation period of 6h showing the three typical growth phases, i.e. the lag phase, the exponential or log phase and the stationary phase. In contrast to the estimation of cell numbers from OD_{620} – measurements, these electrochemical data indicate the increase of numbers of only viable cells.

Assay specificity

DCIP is reduced by a number of different organisms, ranging from mammalian cells and yeasts to bacteria, which is usually observed via the decolorization of the blue dye. It is assumed that enzymes of the respiratory chain, and in particular NADH dehydrogenases, are involved in this reaction. Thus, we used the common inhibitors of the classical respiratory chain, namely rotenone, TTFA, antimycin A, and cyanide, and also SHA as inhibitor of the alternative oxidase (Fig. 1) to identify the relevant electron transfer steps**.** Of these inhibitors only rotenone, the complex I – inhibitor, decreased the signals from reduced DCIP. Residual anodic peak currents of approximately 30 % and residual absorbance changes of almost 50 % were observed (Table 1). Thus, in *C. albicans* the major complex involved in the reduction of

DCIP was the rotenone-sensitive complex I, whereas the other complexes of the classical respiratory chain did not contribute.

However, in previous reports DCIP was used as indicator of the metabolic activity of the complex I-negative yeast *S. cerevisiae* [22]. Thus, for comparison and further investigations on the specificity of the assay we included *S. cerevisiae* and the pathogenic complex I - negative yeast *C. glabrata* [17] in our studies. As previous toxicity assays were based on photometric detection, which could deliver different results by color changes due to pH changes, we established the photometric assay for *C. albicans* for comparison. Moreover, we investigated the respiratory activity of the yeasts by analysis of the oxygen consumption rates.

Incubation of DCIP with yeast cells led to a decolorization of the blue color of DCIP. It was observed that the blue color of DCIP (oxidized form) was consumed very fast and turned into the colorless (reduced form) in the presence of *C. albicans*, however the rates of blue color change were very low when DCIP was incubated with *C. glabrata* or with *S. cerevisiae* (Fig. 5A). Similar ratios were obtained for the electrochemical signals from the different yeasts, as shown in Fig. 5B. Thus, we could show that indeed the presence of complex I is not an essential prerequisite for the reduction of DCIP, even though reaction rates were the highest in the complex I - positive yeast *C. albicans.*

As we had observed a higher DCIP - reducing activity for *C. albicans* than for *C. glabrata* and *S. cerevisiae,* we analyzed the respiratory activities of the yeasts by measuring the oxygen uptake rates [5]. *C. albicans* consumed most of the dissolved oxygen during the first 20 min, whereas after the same incubation time still more than 60 % and 80 % dissolved oxygen was left when *C. glabrata* and *S. cerevisiae* were cultivated (Fig. 6), indicating a significantly higher respiratory activity of *C. albicans*. However, the cultivation medium YPD is a glucose-rich medium, in which Crabtree–positive yeasts, such as *S. cerevisiae* and *C. glabrata,* may

use fermentation pathways even in the presence of oxygen, so that oxygen consumption rates were reduced.

Metabolic activation of the yeasts

The incubation of the yeasts with DCIP was performed in an electrolyte solution, which did not contain carbon sources, so that the metabolic activity of the suspended yeasts relied on internal nutrient stores. In order to test the effects of metabolic activation by addition of different carbon sources on the DCIP reduction rate, we supplemented the incubation buffer with glucose or galactose. Data are summarized in Table 1. For *C. albicans*, the oxidative peak current was almost independent of the addition and types of carbon sources. Effects were slightly stronger, when DCIP reduction was followed photometrically, as the colour change rate of 22 % increased to 35 % after the addition of glucose. However, the signals from *S. cerevisiae* and *C. glabrata* increased to the 3- to 4-fold value in the presence of glucose, reaching almost the same level as the signals from *C. albicans*. The addition of galactose had no or minor effects on the DCIP re-oxidation peak currents from *S. cerevisiae* and *C. glabrata*.

The activity of DCIP-NADH reductases, among which is complex I, in *C. albicans* obviously reached almost the maximum value independent of the additional stimulation of metabolism by carbon sources. However, only the presence of the fermentable carbon source glucose activated the DCIP-NADH reductase activity in the Crabtree-positive and complex I - negative yeasts *S. cerevisiae* and *C. glabrata*, whereas the non-fermentable carbon source galactose had significantly smaller effects. Thus, in these yeasts DCIP reduction by the NADH pool was not strongly related to the activity of the respiratory chain, but may be due to the increased cellular NADH levels resulting from less efficient NADH oxidation during fermentation.

Effect of rotenone after metabolic activation

The DCIP-NADH reductase activity of *C. albicans* without metabolic activation could be inhibited by rotenone to approximately 30 %. Metabolic activation of *S. cerevisiae* and *C. glabrata* by glucose increased the DCIP reductive activity to a similar level as in *C. albicans*. Therefore, we investigated the effects of rotenone on the DCIP reducing activity of all yeasts after addition of glucose.

We observed no inhibitory effect of rotenone on the DCIP reaction with *C. glabrata* and *S. cerevisiae*, irrespective of the presence of glucose, while the rotenone inhibitory effect was significant for *C. albicans* (Tab. 1). However, in the absence of glucose the rotenone-sensitive contribution to the overall color consumption rate was 70 - 50 %, whereas in the presence of glucose it was only approximately 30 %.

Investigations of correlations to NADH concentrations in cells and supernatants

As the reducing agent of DCIP in the living cells is NADH, we investigated the correlation between NADH concentrations and the electrochemical signals. As already shown in Figure 2c, also a direct, non-catalyzed reduction of DCIP by oxidation of NADH is possible and we wondered whether the presence of cells was required or whether secreted NADH might be the source of the observed signals. We determined extra- and intra-cellular concentrations of NADH in *C. albicans* and *S. cerevisiae* via NADH fluorescence, and found a significantly higher NADH fluorescence in the supernatants, i.e. extracellular NADH-concentrations for both *S. cerevisiae* and *C. albicans* were higher in the supernatants than in the cell suspensions (Tab. 2). However, the consumption rates of the DCIP blue color (reduction of DCIP) were faster by the cell suspensions than by the supernatants, in particular by *C. albicans* (Tab. 2). Hence, it can be concluded, that the redox reaction of DCIP with living organisms is not only

related to NADH availability, but is controlled by the enzymatic activity of NADH dehydrogenases.

Discussion

In this study, we used DCIP to probe the viability of yeasts. In particular we analysed the redox activity of the complex I – positive yeast *Candida albicans* and of the complex I – negative yeasts *S. cerevisiae* and *Candida glabrata.* DCIP was reduced by all three yeasts and we determined the reduction of DCIP photometrically, as the reduced form of DCIP is colorless, and voltammetrically via the anodic peak current at approximately 0.6 V. Results from both detection methods correlated very well and both signals could be used as indicators for the interaction of DCIP with the yeast cells.

We showed that the DCIP - reducing activity of viable cells was higher than of the chemical reduction of DCIP by NADH. In *C. albicans* it was controlled by the catalytic activity of a DCIP-NADH – dehydrogenase, which was largely rotenone-sensitive, i.e. dependent on the activity of complex I of the classical respiratory chain. Electron transport chain inhibitors of other complexes had no inhibitory effect.

We also observed the catalyzed electron transfer from *S. cerevisiae* and *C. glabrata* to DCIP, but at much lower rates, and these redox reactions were rotenone - insensitive. Both yeasts lack the mitochondrial complex I, thus alternative DCIP – NADH reductases have to be present. The signals from *S. cerevisiae* and *C. glabrata* could significantly be increased by the addition of glucose, and not by the addition of galactose. Glucose is utilized in *S. cerevisiae* and *C. glabrata* (Crabtree - positive yeasts) by respiratory and fermentative pathways even in the presence of oxygen, whereas galactose is only utilized by respiration and not by fermentation. This indicated that in these yeasts respiratory pathways were not essentially involved in DCIP reduction.

The addition of either glucose as fermentable carbon source or of rotenone as inhibitor of complex I allowed the distinction between Crabtree – positive and – negative yeasts, and between complex I - positive and -negative yeasts. Moreover, in combination with other assays this differential test (+/- addition of compound of interest) could also be used for the characterization of inhibitors of the electron transport chain. In rich media inhibitors of the four major complexes of the respiratory chain do not affect the growth of yeasts but inhibit the consumption of oxygen. Among inhibitor candidates the DCIP test subsequently allows the identification of complex I – inhibitors, when it is performed with complex I - positive yeasts, such as *C. albicans*, without the supplementation of glucose.

Acknowledgements

R.Y.A.H. was supported by the Egyptian Government via the Egyptian Ministry of Higher Education and Scientific Research, which is gratefully acknowledged.

Table 1: Influence of the addition of glucose or galactose and of the complex I – inhibitor rotenone (41 µg/mL) on the DCIP reduction rates of the different yeasts; determined via the DCIP - oxidation peak currents (electrochemistry, after 5 min) or via absorbance changes (after 20 min). Values were normalized with respect to the OD_{600} of the cell suspension to consider different cell densities.

Yeast	Without carbon sources				Glucose			Galactose [µA]
	Electrochemistry		Absorbance			Absorbance		
	Control [µA]	(+) Rotenone [µA]	Control [%]	(+) Rotenone [%]	Control [µA]	Control [%]	(+) Rotenone [%]	
C. albicans	7,2 ± 0,7	1,7 ± 0,1	22 ± 4	11 ± 3	9 ± 2	35 ± 5	23 ± 1	7,5 ± 0,5
C. glabrata	2,3 ± 0,6	--	11 ± 3	14 ± 1	7,3 ± 0,9	31 ± 5	32 ± 5	4,3 ± 0,3
S. cerevisiae	2,2 ± 0,7	--	9 ± 3	9 ± 2	8,7 ± 0,8	32 ± 4	35 ± 5	1,7 ± 0,3

Table 2: Relationship between NADH concentrations and DCIP reduction rates: NADH concentrations in the supernatants were higher than in the cells, however, the DCIP reduction rates determined from the yeast suspensions were higher than from the supernatants, showing the catalytic effect of the DCIP – NADH reductases.

Candida albicans				***Saccharomyces cerevisiae***			
Cells		Supernatant		Cells		Supernatant	
NADH (mM)	Color change (%)	NADH (mM)	Color change (%)	NADH (mM)	Color change (%)	NADH (mM)	Color change (%)
0.1	**14**	**0.2**	**4**	**0.03**	**6.4**	**0.25**	**4**

Figures captions

Figure 1. Scheme of the respiratory chain structure of *C. albicans.* NADH is oxidized by any of the NADH-dehydrogenases, in particular by the proton-pumping NADH-ubiquinone oxidoreductase (complex I), leading to reduced coenzyme Q. Coenzyme Q is also produced from the oxidation of succinate by succinate-dehydrogenase (complex II). Reduced coenzyme Q is oxidized either by oxygen through the alternative oxidase, or by cytochrome c in the cytochrome bc_1 complex (complex III). Cytochrome *c* is oxidized by oxygen by cytochrome *c* oxidase (complex IV). These latter reactions can also be catalyzed by components of the parallel pathway PAR. Inhibitors are known for each of the major enzyme complexes; SHA: salicylhydroxamic acid; AA: antimycin A; KCN: cyanide; TTFA: Thenoyltrifluoroacetone

Figure 2. a) Cyclic voltammogram of DCIP (40 μM) in PBS. The electrode was immersed in the DCIP solution for 5 min before the voltammogram was recorded. b) Cyclic voltammogram of DCIP with *Candida albicans.* A cell concentration of 2.5 x 10^6 cells/ml was used. The voltammograms were recorded after the indicated incubation times, with the electrode being switched off during inubation. c) Cyclic voltammogram of NADH (40 μM) incubated with DCIP in PBS. In all experiments the scan rate was 50 mV/s and representative voltammograms of repeated independent experiments are shown.

Figure 3. Linear sweep voltammograms of DCIP (40μM) incubated with *C. albicans* and *S. cerevisiae* after different incubation times (0, 5, 10, and 15 minutes) in PBS. The scan rate was 50 mV/s, the OD_{620} of each microbial suspension in the electrochemical cell was 0.5.

Figure 4. Increase of the peak currents due to electrochemical DCIP re-oxidation during growth of *C. albicans.* The relationship of cell numbers to the oxidation peak current of DCIP (40 μM) is also shown.

Figure 5. (A) Colorimetric assay of the DCIP-reducing activity of *C. albicans, C. glabrata* and *S. cerevisiae*, the incubation time of the yeasts with DCIP in the 96-well plate was 20 min. (**B)** Electrochemical detection of reduced DCIP after incubation with the different yeasts for 30 min.

Figure 6. Oxygen consumption of *C. albicans*, *C. glabrata* and *S. cerevisiae* incubated in YPD medium. Oxygen concentrations are given as % air saturation. Starting OD_{620} of *C. albicans* and *C. glabrata* was 0.05. For *S. cerevisiae* an OD_{620} of 0.1 was used.

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