Direct Electrochemical Determination of *Candida albicans* Activity

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Running title: Bio-electrochemistry of *Candida albicans*

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Abstract

Despite advances made in the field, rapid detection methods for the human pathogen *Candida albicans* are still missing. In this regard, bio-electrochemical systems including electrochemical sensors and biosensors satisfy the increasing demand for rapid, reliable, and direct microbial analyses. In this study, the bioelectrochemical characteristics of *C. albicans* were investigated for use in an analytical system that determines the viability of the organisms. The electrochemical responses of viable and non-viable cells of *C. albicans* and *Saccharomyces cerevisiae* were monitored. Cyclic voltammograms (CV) showed an irreversible oxidation peak at about 750 mV that accounts for viable cells. The peak current increased at viable cell numbers ranging from 3×10^5 to 1.6×10^7 cells/ml, indicating that the amount of viable cells can be accurately quantified. To elucidate the underlying electron transfer processes, the influences of electron transfer chain (ETC) – inhibitors on the electrochemical behavior of the two organisms were investigated. Inhibition of the function of classical respiratory chain (CRC) led to a decrease in the electrochemical response, whereas the oxidation current increased when the alternative oxidase (AOX) pathway was blocked by salicylhydroxamic acid (SHA). Blocking the AOX pathway improved the electrochemical performance, suggesting an involvement in the CRC, with cytochrome c oxidase (COX) as a relevant protein complex. Mutants, in which components of COX were deleted, showed a lower electro-activity than the wild-type strain. Particularly, deletion of subunit COX5a almost completely abolished the electrochemical signal. We believe that this work can be utilized for the development of early detection assays and opens the door for new technological developments in the field of *C. albicans*.

Keywords: Bioelectrochemistry of *Candida albicans*; Pathogen detection; mitochondrial respiratory chain; Cytochrome c oxidase

Introduction

Candida albicans is a polymorphic fungus that causes a broad spectrum of diseases in humans, ranging from localized mucosal infections to systemic candidiasis. The most important virulence factor of *C. albicans* is the morphological switch between yeast and filamentous forms (Pitarch, Nombela et al. 2006; Mayer, Wilson et al. 2013). Since it is an opportunistic pathogen, mainly patients with a compromised immune system are at risk to develop systemic candidiasis. As detection of microorganisms usually requires several days, in intensive care units of hospitals antimycotics are frequently given prophylactically to prevent *C. albicans* infection. Yet, this could be prevented with simple and rapid diagnostic tools.

The classical detection methods of viable microorganisms rely on the cultivation and growth of the organisms on agar plates or in liquid culture. Subsequently, colony forming units (CFUs) or cell numbers, the dry cell weight or the turbidity of samples is assessed (Keer and Birch 2003).

PCR technique is extremely sensitive and may also allow further characterization of the pathogens (Gilbride, Lee et al. 2006), (Bekal, Brousseau et al. 2003). However, both molecular methods cannot distinguish between viable and nonviable organisms, so that the infectious risk may be overestimated (Girones, Ferrus et al. 2010). Measuring the efficiency of microbial respiration and the activity of the electron transport chain (ETC) are considered to be suitable indicators of cellular activity, because they are essential for the replication of aerobic organisms. In eukaryotes, the respiratory chain is located in mitochondria where more than 90% of the total oxygen is consumed via the ETC. Therefore, the oxygen uptake rate of a microbial culture is a direct indicator of the respiratory activity (Wesolowski, Hassan et al. 2008). Moreover, redox compounds, such as tetrazolium salts (Tsukatani, Oba et al. 2003; Tsukatani, Suenaga et al. 2008; Tsukatani, Higuchi et al. 2009), resazurin (Alamar blue) (Byth, McHunu et al. 2001; Watanabe, Manefield et al. 2009; Mendoza-Aguilar, Almaguer-Villagran et al. 2012), and various quinoid compounds, can be used in colorimetric assays to assess viability and/or proliferation of living cells. However, these assays are not suitable for routine laboratory use due to the long incubation time of the redox mediators in the microbial culture and interference with the optical density measurement.

Alternatively, electron transfer processes from microorganisms towards electrodes in bio-electrochemical systems can be exploited for the development of microbial fuel cells (Schaetzle O 2008) or diagnostic tools (Kang, 2012 ; Lovley, 2012) that provide a rapid assessment of microbial activity (Marsili, Rollefson et al. 2008; K. Junil 2012). In bio-electrochemical systems, either the direct interaction between microorganisms and electrodes can be used, or the microbe-electrode communication can be induced with natural (Wang, Tsujimura et al. 2007) or artificial redox mediators (Roller SD 1984; Heiskanen A 2004; Gottschamel, Richter et al. 2009). Electrochemical detection methods that are based on redox mediators are applicable when the reduced electron acceptor can be oxidized at electrodes (K. Junil 2012; Congdon, Feldberg et al. 2013). Since these compounds have to be added to the sample. The most simplistic approach is a mediator-less electrochemical system, which relies on the transfer of electrons from an electrochemical active microorganism to the electrode surface (Bullen, Arnot et al. 2006). Mediator-less system for the detection of microorganisms are known since the late 1970s (Matsunaga, Karube et al. 1979; Matsunaga and Namba 1984; Lovley 2012).

In this study, we have focused on the bio-electrochemistry of *C. albicans* (target organism). *C. albicans* offers the opportunity to explore factors that play a key roles in the interaction of the microorganism with electrodes, as the structure of its respiratory chain is significantly different from that of *S. cerevisiae* (model organism) (Joseph-Horne, Hollomon et al. 2001). *C. albicans* has three respiratory pathways (Fig. 1): The classical respiratory chain (CRC), an alternative oxidative pathway (AOX) (Huh and Kang 2001; Joseph-Horne, Hollomon et al. 2001; Veiga, Arrabaca et al. 2003) and a parallel electron transport chain (PAR) (Ruy, Vercesi et al. 2006). The CRC comprises all four enzymatic complexes including the proton pumping complex NADH dehydrogenase (complex I), which is missing in *S. cerevisiae.* The AOX catalyzes the direct oxidation of ubiquinol by oxygen and, thus bypass complexes III and IV which enable respiration even in the presence of downstream CRC inhibitors. 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, parallel to the CRC (Ruy, Vercesi et al. 2006).

(Figure 1)

In our previous study, we have developed a mediated bioelectrochemical system for the detection of *C. albicans's* viability in which electrons are shuttled from the NADH-dehydrogenases of ETC (Hassan, 2011). Recently the early detection of *C. albicans* biofilms at porous electrodes has been reported (Congdon, 2013). Intriguingly, the direct electron transfer capability of *C. albicans*, which provides a better mean to determine the biological functions of respiratory pathways of *C. albicans*, has not been reported yet.

Therefore, we investigated the direct bio-electrochemistry of the target organism as well as the involvement of the respiratory pathways in the electrochemical signals. Voltammograms from suspensions of *C. albicans* and *S. cerevisiae* showed an irreversible oxidation peak at around 750 mV, indicating that electrons were transferred from the cells to the electrode surface. Since non-viable cells do not exhibit any electrochemical activity, the generated oxidation current reflects the cell viability status. The peak height correlated with the cell numbers and increased with the cultivation time. To obtain a more detailed understanding of the relevant electron transfer step, ETC-inhibitors were used. The peak current decreased when the CRC pathway was inhibited. In addition, the electrochemical activities of COX mutants of *S. cerevisiae* were significantly lower than those of the wild type. In particular, the deletion of subunit *COX5a* almost completely abolished the electrochemical response. This confirms the essential role of COX (complex IV) in the interaction between yeasts and electrodes.

2. Materials and methods

2.1. Materials and instruments

Potassium phthalate mono basic was purchased from Riedel-de Haen, and a 100 mM solution was adjusted to pH 7 by KOH. YPD broth, antimycin A from *Streptomyces sp*., and rotenone were purchased from Sigma. YPgal medium contained yeast nitrogen base with amino acids from Sigma, peptone from Roth, and galactose from Merck. Salicylhydroxamic acid (SHA) was from Aldrich. Synthetic carbon powder was obtained from Sigma-Aldrich and paraffin oil from Fluka.

All OD measurements were performed in 180 µl sample volumes with the microtiter plate reader µQuant (BioTek Instruments GmbH, Bad Friedrichshall, Germany), and fluorescence was determined with the microtiter plate reader Cytofluor, Reader Series 4000 (PerSeptive Biosystems, Framingham, USA). The electrochemical measurements were carried out with a computer-controlled Gamry Potentiostat / Galvanostat / ZRA G750 (Gamry, Pennsylvania, USA)**,** which was connected to a three-electrode electrochemical cell with a carbon paste working electrode, a Pt disc auxiliary electrode, and a KCl saturated Ag/AgCl reference electrode.

2.2. Preparation of microbial samples

2.2.1 Standard cultivation conditions

The following fungal strains were used: *C. albicans* strains CAF2-1 and *S. cerevisiae* BY4741 and its single gene deletion mutants *COX5a*, *COX5b*, *COX7*, *COX8*, and *COX12* (Euroscarf, Frankfurt, Germany). Fungi were cultivated in YPD medium overnight to stationary phase at 30 °C with shaking (160 rpm). A pre-culture was prepared by diluting the overnight culture to an optical density (OD_{620}) of 0.2 in YPD medium and further cultured for 3 hours to reach exponential growth phase. The working culture was prepared by diluting the pre-culture to an OD_{620} of 0.l in YPD. After cultivation for another 3 hours, the OD_{620} was recorded. The cells were harvested by centrifugation (Eppendorf centrifuge 5804R) at 5000 rpm at room temperature for 5 min and resuspended in 1 ml phosphate buffered saline (PBS).

2.2.2 Electrochemical response of dead cells

Non-living cells of *C.-albicans* were obtained from the working culture after 3 hours cultivation time and autoclaving at 121 °C for 20 min. The cells were harvested and resuspended as described above.

2.2.3 Electron transfer chain inhibitors

Microorganisms were treated with the electron transport chain (ETC) inhibitors using the final concentrations given in Tab. 2, Supplementary Information-(SI). The inhibitors were added to the working cultures. The microorganisms were allowed to grow in the presence of the inhibitors at 30-°C for 3 hours, harvested and resuspended as described above.

2.3. Preparation of carbon paste electrodes and voltammetric procedure

1 g synthetic carbon powder (1-2 micrometers) was thoroughly mixed with 0.4 ml paraffin oil. A portion of the paste was packed into the tip of the electrode assembly with a surface area of 0.5 cm^2. When the working electrode was regenerated or cleaned, a small part of the paste was cut off and polished with wetted filter paper. Before use, the working electrode was activated in 0.1 M phthalate buffer, pH 7 by cyclic scans from 0 - 1 V with scan rates of 50 mV/s. A 1 ml microbial suspension in PBS was introduced into the electrochemical cell, which contained 24 ml 0.1 M phthalate buffer, pH 7. The cells were mixed with the buffer and allowed to adapt for approximately 5 min. Linear sweep voltammograms (LSV) and cyclic voltammograms (CV) were recorded in the potential range of 0 – 1 V with scan rates of 50 mV/s. All-electrochemical experiments were carried out without stirring at room temperature.

For metabolic activation of *C. albicans* and *S. cerevisiae*, the phthalate buffer was supplemented with 20 g/l glucose or galactose. A 1 ml microbial suspension of *C. albicans* or *S. cerevisiae* in PBS was incubated in 24 ml of carbon source-containing phthalate buffer for 30 min at 30 °C. Then the electrochemical performance was measured.

2.4. Oxygen determination and viability tests

Oxygen consumption was monitored during the cultivation of *S. cerevisiae* in YPgal medium instead of YPD medium to avoid fermentative metabolism. Oxygen was determined in round-bottomed 96-well OxoPlates® (PreSens, Regensburg, Germany) following the procedure given by the manufacturer. Briefly, the fluorescence of the indicator and the reference dye were determined at the excitation wavelength of 530 nm and the emission wavelengths of 620 nm for the indicator dye and emission wavelengths of 590 nm for the reference dye. Calibration was performed with water saturated with air (100%) and aqueous Na_2SO_3 solution (10 g/l) (0%) (Wesolowski, Hassan et al. 2008).

Cell viability was determined with the WST assay [2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium]) (Roche). 10 µl of the WST reagent was added to each well of a 96-well microtiter containing 170 µl of yeast cell suspension in YPD culture medium. After 20 min incubation at room temperature, the absorbance was measured at 450 nm using plate reader (Hassan and Bilitewski 2011).

3. Results and discussion

3.1 Electron transfer reaction from *C. albicans*

The capability of microorganisms to transfer electrons to electrodes without the addition of exogenous mediators has been known for a long time (Busalmen, 2008; Huang, 20089). For bacteria, the direct electron transfer was shown to be achieved either by the production of soluble small-molecule that acted as electron shuttles (Rabaey, Boon et al. 2005; Marsili, Baron et al. 2008) or by creating a conductive layer in the outer-membranes (Xiong, 2006, Bond, 2003). For eukaryotes such as *C. albicans*, a direct electron transfer has not been demonstrated so far.

In this study, we demonstrated that cyclic voltamograms of suspensions of *C. albicans* (Fig. 2a) and *S. cerevisiae* (Fig. 2b) showed an anodic peak at around 750 mV versus Ag/AgCl. There was no corresponding cathodic peak in the reverse scan suggesting that the electrochemical reaction was an irreversible process. As the anodic peak potentials (*Ep*) were in the same range for both organisms, they likely share the same electro-active component. Since no electrochemical response was observed when dead cells of *C.*

albicans were injected into the voltammetric cell, the measureable oxidation current is a reliable indicator of cell viability.

(Figure 2)

3.2. Electrochemical monitoring of growth rate

Next, the electron transfer from a *C. albicans* suspension to electrodes was exploited to determine the relationship between cell density and the oxidation peak current. An increase of the peak current with increasing numbers of *C. albicans* was found in the range of 3 x 10^5 to 1.6 x 10^7 cells/ml, as shown in Fig. 3a**.** Additionally, the proposed assay was used to monitor the growth rate of *C. albicans* over the long incubation period. The results showed a strong correlation with the classical microbiological methods, OD620 and WST test, as shown in Fig. 3b.

(Figure 3)

3.3 Analysis of the reaction mechanism

The major source of electrons in living organisms is the respiratory chain. To avoid interferences from medium constituents or secreted metabolites, the microorganisms were washed with PBS prior to activation of the respiratory chain by adding different carbon sources to the buffer, or its inhibition with chemical inhibitors or usage of single gene deletion mutants.

3.3.1. Activation of the respiratory chain

In the living organisms, the electron transfer processes are dependent on the metabolic activity. In yeast system, based on the available carbon source in the cultivation medium, the metabolic activity could either originate from aerobic respiration or fermentation (Wesolowski, Hassan et al. 2008; Wesolowski, Hassan et al. 2010). Therefore, the suspension cells were supplemented with a fermentable carbon source (glucose), and a non-fermentable sugar (galactose) before the electrochemical activities were tested.

As a result, in *C. albicans*, the addition of carbon sources significantly increased the oxidation current in comparison to buffer (Fig. 4). However, only galactose increased the electrochemical activity of *S. cerevisiae*, while glucose had almost no effect (Fig. 4). These data demonstrated that the activation of the mitochondrial respiratory chain regulates the electron transfer efficiency from *C. albicans* to the electrode surface.

(Figure 4)

3.3.2 Influence of electron transport chain inhibitors (ETC)

To determine the impact of respiratory chain on the electrochemical activity of *C. albicans* and *S. cerevisiae*, the activity of individual complexes of the mitochondrial respiratory chain were inhibited by treating both microorganisms with the ETC-inhibitors, rotenone, antimycin A (AA), and cyanide. Rotenone inhibits the activity of complex I, whereas antimycin A interferes with the electron flow in the complex III. Cyanide generally affects all metalloenzymes, and was used because of its binding potential to Fe^{3+} in the heme groups of complex IV. We additionally used TTFA, which is a complex II inhibitor, and SHA, an inhibitor of the AOX pathway. As *S. cerevisiae* lacks complex I and AOX, rotenone and SHA were not applied to this organism.

As shown in Fig. 5, cells treated with CRC-inhibitors produced a lower oxidation current than untreated cells. On the contrary, blocking the alternative oxidative pathway (AOX) by SHA enhanced the electrochemical performance of *C. albicans*. As a conclusion; blocking the branched respiratory pathway (AOX) directs the whole electron flow to the CRC, thus enhancing the electrochemical signal.

(Figure 5)

3.3.3 Bio-electrochemistry of COX mutants

Our data above revealed that the undisturbed function of the classical respiratory chain was essential for electrochemical activity of the tested organisms. As a complementary approach, we investigated the effects of single gene deletions. We focused on the final

complex of the electron transport chain, cytochrome *c* oxidase (COX) because we observed the most significant decrease of the peak current with the COX inhibitor KCN. This protein complex takes part in the transfer of electrons from cytochrome *c* through several steps to the terminal binuclear site involving heme a_3 and Cu_B (heme a_3–Cu_B), where catalysis of the reduction of O_2 to water takes place (Barrientos, Gouget et al. 2009). Subunits of COX, which are encoded by nuclear genes, contribute to the assembly of the whole enzyme and to the intramolecular electron transfer rates (Barrientos, Gouget et al. 2009). We used *S. cerevisiae* mutants with single deletions in *COX5a*, *COX5b*, *COX7*, *COX8*, and *COX12* to elucidate the relevance of the different subunits for the electrochemical signal. As shown in Fig. 6a, the signal was reduced for all mutants. The strongest effect was observed for the *COX5a* mutant, that oxidation peak current was reduced to almost zero.

(Figure 6)

3.3.4 The respiration efficiency regulates the electrochemical activity

To confirm the relevance of subunits *COX5a* and *COX5b* for the respiratory activity, oxygen consumption was measured for the respective mutants cultivated in YPgal medium. As visible in (Fig. 6b), the respiratory activity was reduced in the *COX5a* and *COX5b* mutants. This effect was more pronounced in the *COX5a* mutant. *COX5a* is described as an aerobic gene, whereas *COX5b* is a hypoxic gene, meaning it is only expressed under low oxygen concentrations (Burke, Raitt et al. 1997). During our cultivation conditions, the expression levels of the hypoxic gene *COX5b* might not have been high enough to fully compensate for the deletion of the aerobic gene *COX5a*, thereby leading to the strong effect on the respiratory activity.

Overall, these data agree with the observations obtained with the ETC-inhibitors, as the oxidation peak current increased with the increasing respiratory activity, and any interference by the deletion of a regulatory gene led to a reduction of signals.

4. Conclusion

In the current study, we focused on the use of direct bio-electrochemistry of *C. albicans* as a simple tool for monitoring ~~the~~ cell~~s~~ viability. Our results demonstrated that viable cells transfer electrons to the electrode surface. The electron transfer capacity was strongly controlled by the mitochondrial respiratory chain efficiency, since inhibition of CRC pathway, either chemically by using ETC-inhibitors, or genetically by knocking out specific genes, led to a decrease in the oxidation current. However, blocking the AOX pathway enhanced the electrochemical responses of *C. albicans*, which confirms the importance of CRC in the process of the electron transfer. Although this direct bio-electrochemical method is a simple mean for measuring the cell viability of yeast such as *C. albicans*, the mechanism is still unclear. Therefore, we seek to clarify how *C. albicans* interacts with the electrode.

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Figure Captions

Figure 1: Respiratory chain structure of *C. albicans.* NADH is oxidized by NADH-dehydrogenases leading to reduced coenzyme Q. This is also produced from the oxidation of succinate by succinate-dehydrogenase. Reduced coenzyme Q is oxidized again either by oxygen through the alternative oxidase, or by cytochrome c in the cytochrome bc_1 complex. Cytochrome *c* is finally oxidized by cytochrome *c* oxidase. These latter reactions can also be catalyzed by components of the parallel pathway PAR.

Figure 2a: Cyclic voltammogram of *C. albicans*. The oxidation current in *C. albicans* versus blank phthalate buffer, using a scan rate of 50 mVs^{-1} and a final concentration of cells in the electrolyte of 3.8 x 10^6/ml).

Figure 2b: Linear sweep voltammograms of *C. albicans, S. cerevisiae*, and dead cells of *C. albicans*. The oxidation current in *C. albicans, S. cerevisiae* and dead cells of *C. albicans* was measured with cell counts of 3.8 x 10^6/ml, 9.15 x 10^6/ml, and 2.2 x 10^6/ml, respectively.

Figure 3a: Relationship between cell numbers of *C. albicans* and the anodic peak current in LSVs using scan rates of 50 mV/s.

Figure 3b: Growth curve of *C. albicans* as determined by optical, calorimetrical and electrochemical methods. Growth of *C. albicans* was determined by OD_{620} nm (turbidity), viability test (WST) (OD_{450} nm), and the electrochemical method (current in LSV). *C. albicans* was cultivated in YPD; the working culture was cultured for 45 h.

Figure 4: Influence of the metabolic activity of cells. Glucose or galactose was added to the phthalate buffer for the metabolic activation of the yeasts. Galactose was used as a non-fermentable carbon source, whereas glucose can also be consumed by fermentative pathways in *S. cerevisiae*.

Figure 5: Effects of electron transport chain (ETC) inhibitors on the anodic peak currents of *S. cerevisiae* and *C. albicans.* Inhibitor concentrations were chosen on the basis of inhibitory effects on oxygen consumption: rotenone (41 µg/ml), thenoyltrifluoroacetone (TTFA) (5 µg/ml), antimycin A (1.5 µg/ml), KCN (5 µg/ml), and salicylhydroxamic acid (SHA) (14 µg/ml) were added.

Figure 6: Impact of cytochrome c oxidase in *S. cerevisiae* on the oxidation current and oxygen uptake rate.

(a): Anodic peak currents of *S. cerevisiae* (WT) and single gene deletion mutants of cytochrome c oxidase, using 2.3 x 10^6 cells/ml, 2.3 x 10^6 cells/ml, 2.3 x 10^6 cells/ml, 2.71 x 10^6 cells/ml, 2.6 x 10^6 cells/ml, and 2.4 x 10^6 cells/ml, for wild-type (WT) and *COX5a*, *COX5b*, *COX7*, *COX8*, and *COX12* mutants, respectively).

(b): Oxygen consumption of *S. cerevisiae* (WT) and of the *COX5a* and *COX5b* single gene deletion mutants was performed by cultivation in YPgal medium, and cell numbers of 5.6 x 10^6/ml, 5.9 x 10^6/ml, and 6.2 x 10^6/ml for *S. cerevisiae* (WT), *COX5a*, and *COX5b*, respectively.

Figure 1

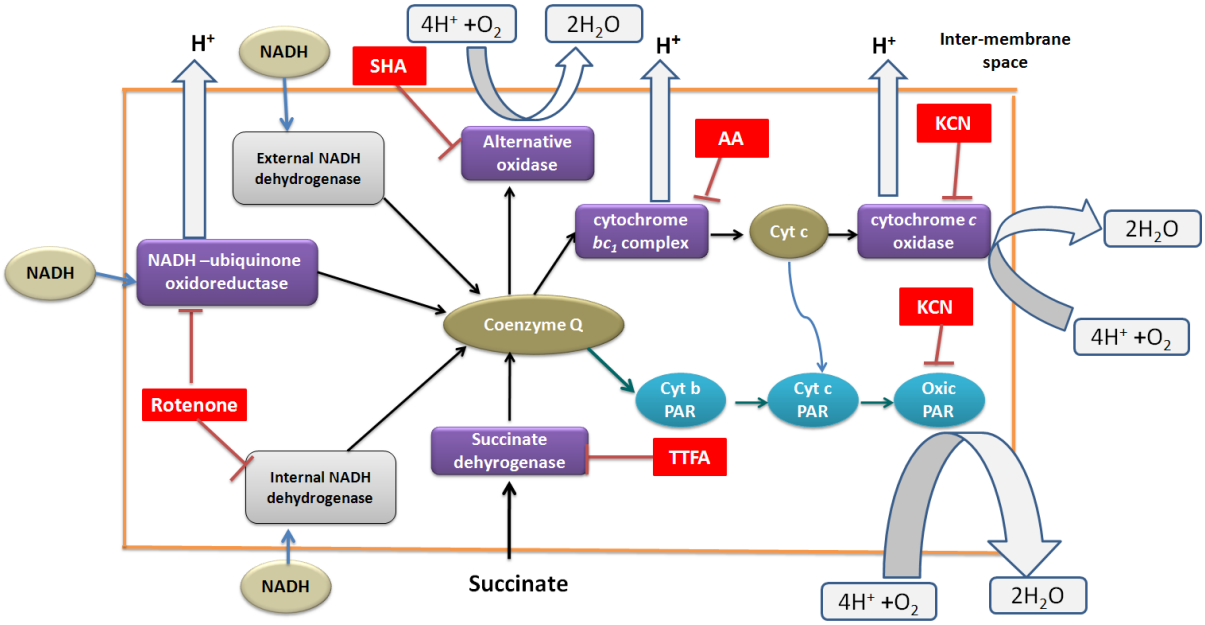

H+
NADH
SHA
4H+ +O2
2H2O
H+
H+
Inter-membrane space
External NADH dehydrogenase
Alternative oxidase
AA
KCN
cytochrome bc1 complex
Cyt c
cytochrome c oxidase
2H2O
NADH
NADH –ubiquinone oxidoreductase
Coenzyme Q
KCN
4H+ +O2
Rotenone
Cyt b PAR
Cyt c PAR
Oxic PAR
Succinate dehyrogenase
TTFA
Internal NADH dehydrogenase
NADH
Succinate
4H+ +O2
2H2O

Figure 2a

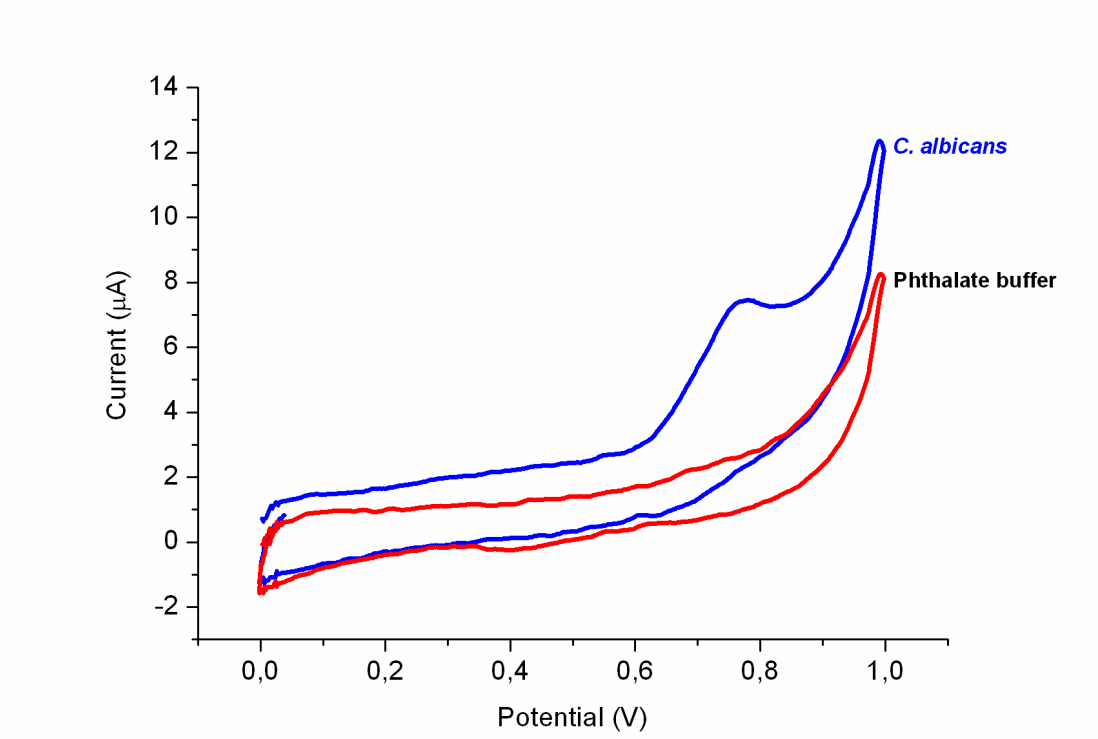


Figure 2b:

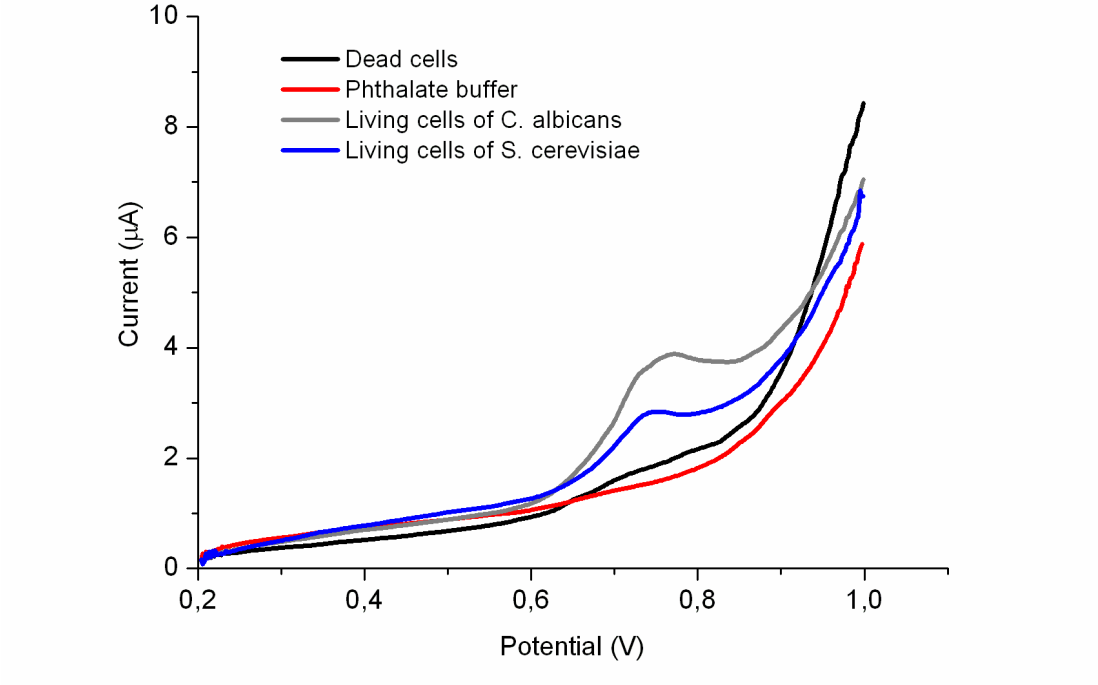


Figure 3a

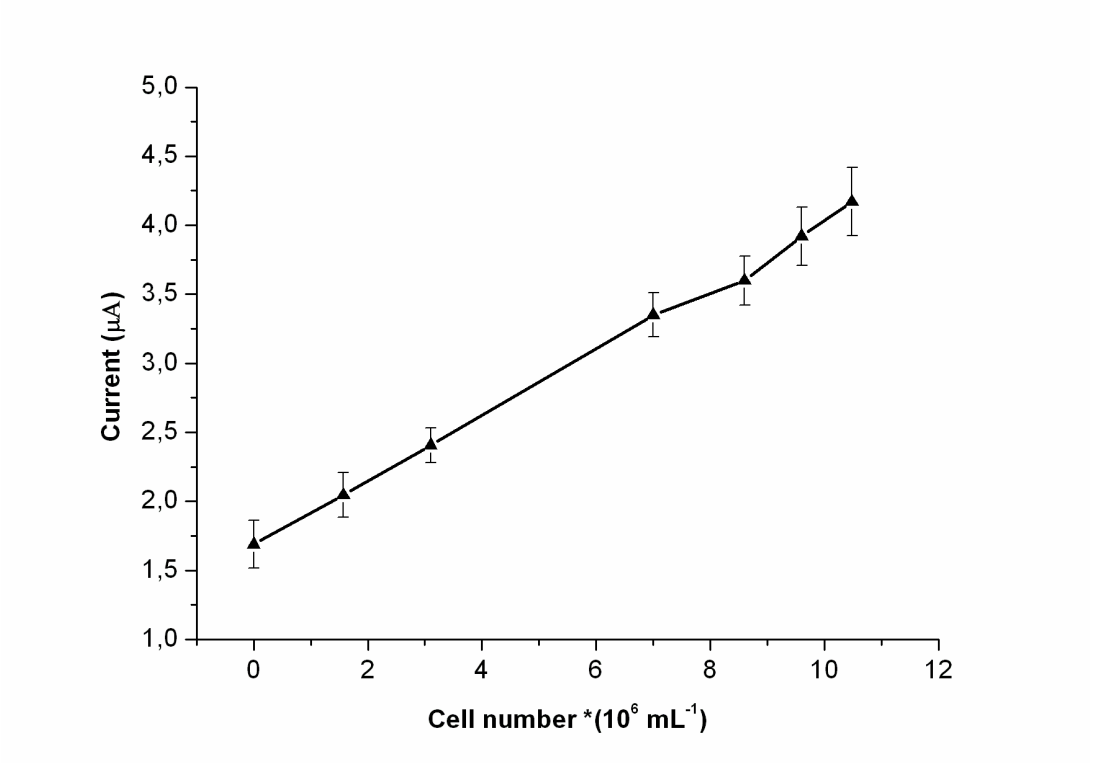


Figure 3b

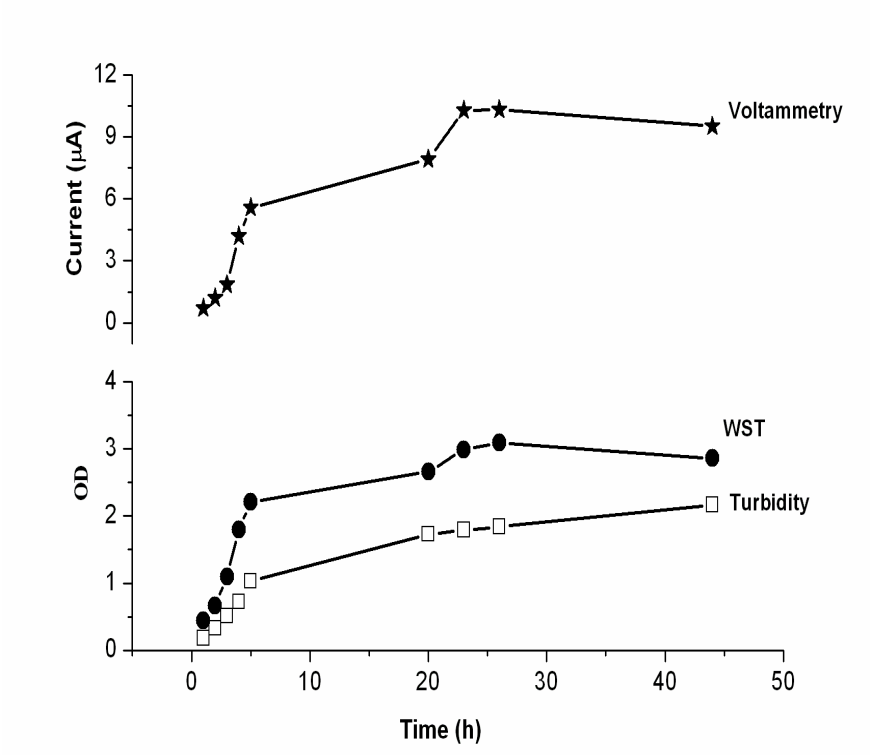


Figure 4

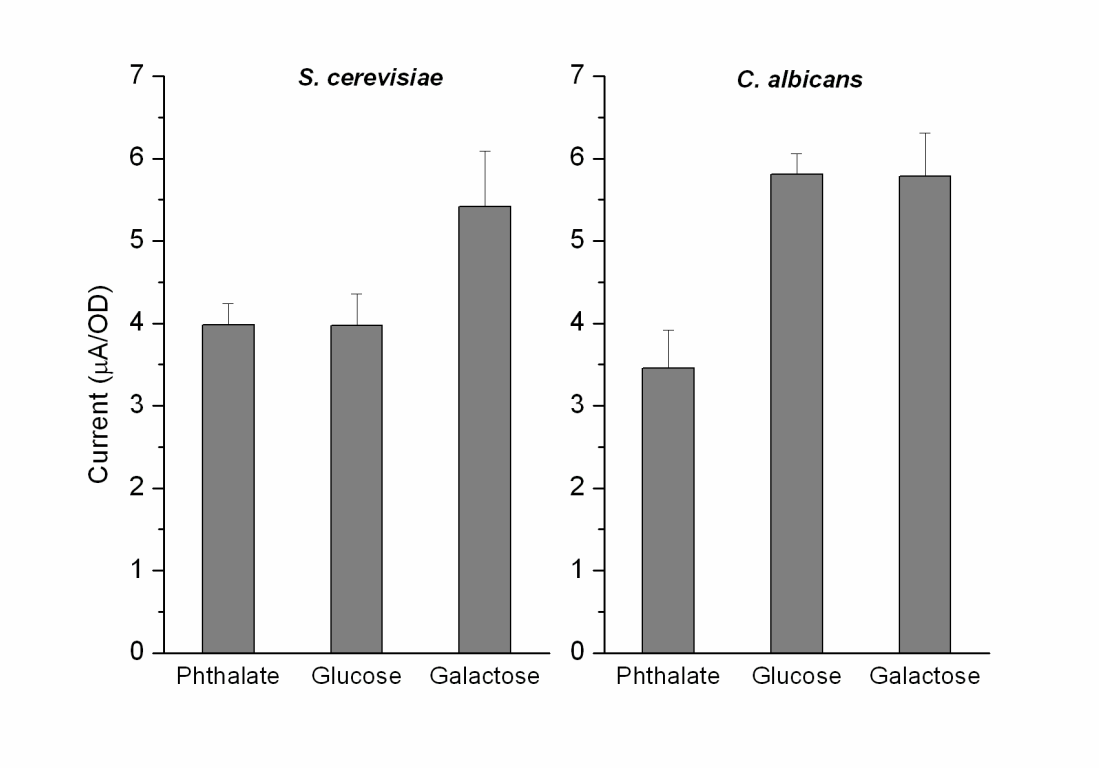

S. cerevisiae
C. albicans
Current (μA/OD)
0
1
2
3
4
5
6
7
Phthalate
Glucose
Galactose

Figure 5

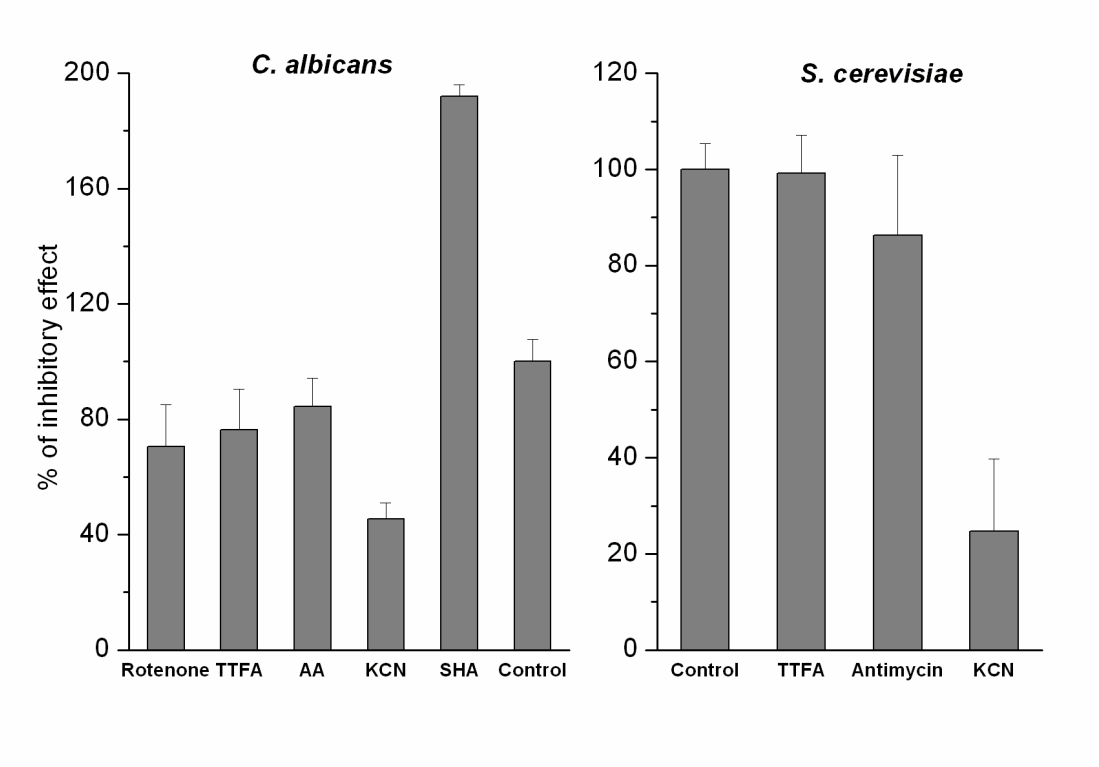
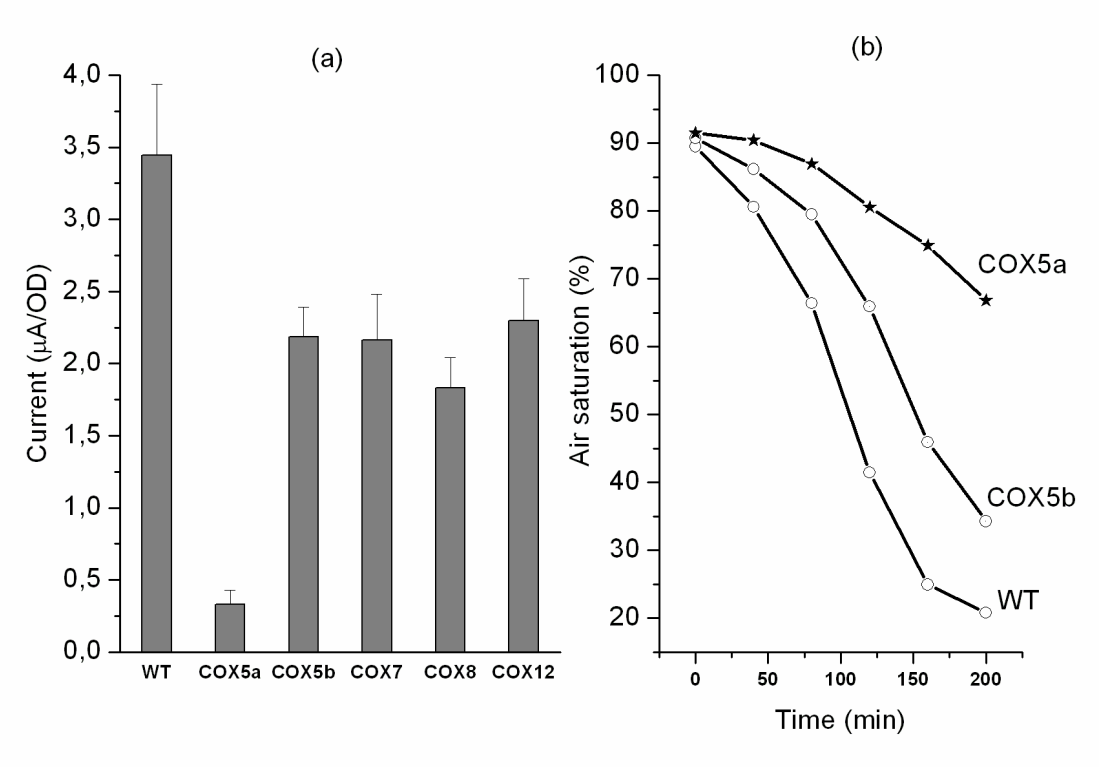

C. albicans
% of inhibitory effect
200
160
120
80
40
0
Rotenone
TTFA
AA
KCN
SHA
Control
S. cerevisiae
120
100
80
60
40
20
0
Control
TTFA
Antimycin
KCN

Figure 6:


(a)
Current (μA/OD)
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
WT
COX5a
COX5b
COX7
COX8
COX12
(b)
Air saturation (%)
100
90
80
70
60
50
40
30
20
0
50
100
150
200
Time (min)
COX5a
COX5b
WT