Electroactivity of *Yarrowia lipolytica* and bioprocess improvement

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Submitted in fulfilment of the requirements for the degree of
Master of Science in Biomedical Engineering

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May 2023

Declaration

I hereby, declare that this manuscript, entitled “Electroactivity of *Yarrowia lipolytica* and bioprocess improvement”, is the result of my own work except for quotations and citations which have been duly acknowledged.

I also declare that, to the best of my knowledge and belief, it has not been previously or concurrently submitted, in whole or in part, for any other degree or diploma at Nazarbayev University or any other national or international institution.

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May 2023

Abstract

Electrofermentation is known to mitigate redox imbalance in anaerobic growth, thus increasing growth and key metabolite production in yeast. In the first part of this work, I investigated growth, biofilm formation, and oil degradation under electrofermentation conditions of *Yarrowia lipolytica* through laboratory-scale bioelectrochemical cells. Two commonly used redox mediators, 2-hydroxy-1,4-naphthoquinone (2-HNQ) and potassium ferricyanide ($K_3[Fe(CN)_6]$) were tested to enhance the bio-electrochemical process. Results show that $K_3[Fe(CN)_6]$ leads to better planktonic growth and biofilm formation compared to 2-HNQ. In the second part of the thesis, I investigated the possibility of using sunflower oil as an additional carbon source in a laboratory-scale electrofermentation process, to stimulate lipase production, a key group of enzymes used in the biofuel production and upcycling of waste oil. While I could not directly measure lipase expression, the electrofermentation experiments resulted in approximately 47 % higher cumulative charge output in presence of 5% of sunflower oil, suggesting that electrofermentation increases lipase production and oil metabolism. This work lay the basis for the development of an efficient waste oil degradation process under electrofermentation conditions.

Acknowledgments

Firstly, I would like to express my uttermost gratitude towards my supervisors Professor Tri Pham and Professor Boris Golman. It has been their supervision and direction throughout the duration of my studies which has allowed me to successfully complete this Master's program. I am appreciative for all the hours of discussion they have offered me, especially in the areas of electrofermentation and imaging. Secondly, I would like to express my gratitude to Dr. Obinna Ajunwa and two PhD candidates Neda Eghtesadi and Kayode Olaifa for their amazing assistance with numerous aspects of my research. Additionally, I would like to show my indebtedness to the administration staff at Nazarbayev University whose efforts a lot of the time go unnoticed.

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List of Abbreviations

Abbreviation	Definition
EF	Electrofermentation
AEF	Anodic EF
CEF	Cathodic EF
CA	Chronoamperometry
DPV	Differential pulse voltammetry
FDA	Food and Drug Administration
GRAS	Generally Recognized As Safe
CDW	Cell dry weight
$K_3[Fe(CN)_6]$	Potassium Ferricyanide
2-HNQ	2-hydroxy-1,4-naphthoquinone
YPD	Yeast Extract–Peptone–Dextrose
OD	Optical density
ORP	Oxidation reduction potential
SEM	Scanning electron microscope
CEF	Cathodic EF
AEF	Anodic EF
SCP	Single-cell protein
WE	Working electrode
EC	Electrochemical cells
DMSO	Dimethyl sulfoxide

Chapter 1 – Introduction

1.1 Background

Yarrowia lipolytica is an ascomycete yeast that belongs to the Saccharomycetes class and demonstrates obligate aerobic characteristics. *Y. lipolytica* is part of the healthy human microbiota and in most cases does not cause any disease, thus is considered as non-pathogenic and safe by various world organizations including GRAS and FDA (Groenewald et al., 2013). Due to its minimal nutritional requirements, as well as high productivity, it is widely used in industry to produce a variety of metabolites, including single-cell protein, single-cell oil, enzymes, minerals, vitamins, amino acids, etc. (Jach & Malm, 2022). Numerous industrial applications exist regarding *Y. lipolytica*, including the synthesis of citric acid and other valuable metabolites, as well as the decomposition of hydrocarbons. This microorganism is widely recognized for its capacity to store large quantities of lipids in the cytoplasm, reaching up to 30-50% of the cell's dry weight (CDW). Through genetic engineering, lipid accumulation in obese *Y. lipolytica* cells can reach up to 90% of CDW (Madzak, 2021). Lipid-laden yeast can be used for biofuel production (Chattopadhyay & Maiti, 2021) and potentially for human or animal feeding (Shurson, 2018).

To increase cell densities of oleaginous yeast and promote higher lipid synthesis, several fermentation modes such as batch, fed-batch, and continuous culture can be used in conjunction with a multistage strategy. However, like all industrial processes, they have a variety of drawbacks, including poor productivity, as well as cell and product dilution. Novel fermentation techniques, such as electrofermentation (EF), have been developed recently to enhance and expand on conventional fermentation processes in order to address these deficiencies. It affects extracellular electron transfer, which refers to the extracellular exchange of the electrons generated inside the cell as a result of intracellular metabolic activity. During EF this exchange occurs between the cell and the artificial electrode, altering oxidation reduction potential (ORP) and increasing metabolism inside the cell (Gong et al., 2020). The anode serves as an electron acceptor and a cathode as an electron donor, it controls the rates of electron transfer during microbial metabolic reactions and aids in reducing the redox imbalances observed during fermentation processes. Depending on whether the cathode or anode is utilized as the working electrode, it is classified as cathodic EF (CEF) or anodic EF (AEF) (Bhagchandanii et al., 2020). Thus, through electrochemistry, it is possible to accelerate

the redox reactions occurring inside the metabolic environment of a microorganism. Some electroactive microorganisms such as Shewanella and Geobacter spp., naturally perform extracellular electron transfer, exchanging electrons directly with minerals/artificial electrodes. *Y. lipolytica* is not considered an electroactive microorganism, thus, to mediate electron transfer from electron-rich compounds to electron-depleted compounds (or electrodes) and vice-versa, it needs to be supplied with a redox mediator. Examples of soluble redox mediators include neutral red, riboflavin, potassium ferricyanide ($K_3[Fe(CN)_6]$), 2-hydroxy-1,4-naphthoquinone (2-HNQ), etc. (Olaifa et al., 2021).

1.2 Goals & Objectives

The main goal of this research is to grow *Y. lipolytica* as a biofilm on solid electrodes under EF conditions. The secondary goal is to use vegetable oil as an additional carbon source under EF conditions. To achieve these goals the following objectives must be met:

1. Identifying a suitable mediator for EF of *Y. lipolytica*.
2. Identifying optimal parameters for EF of *Y. lipolytica* by electrochemical analysis using chronoamperometry (CA), cyclic voltammetry (CV).
3. Testing the effect of sunflower oil on the growth and electrochemical output of *Y. lipolytica* biofilms.

Chapter – 2 Literature review

2.1 *Y. lipolytica* and its industrial application

Y. lipolytica (former *Candida lipolytica*) demonstrates the highest growth rate at 29 °C but is capable of tolerating a wide range of temperatures from 20 and up to 40 °C (Sekova et al., 2019). The optimal pH for *Y. lipolytica* is 5.5, and it can be cultivated in nutrient media such as F (Jach et al., 2018). *Y. lipolytica* shows dimorphic growth, which implies it has two distinct phenotypes. Round and spherical cells are the most common shape of the cells. The cells might transform into a filamentous growth form when exposed to various environmental stressed conditions, including low or high temperature, unsuitable pH, mechanical damage, etc. (Heard & Fleet, 1999).

Due to its ability to manufacture metabolites at high yields, utilize a variety of substrates, and be genetically adaptable, *Y. lipolytica* has drawn interest from both academia and industry. It possesses several essential qualities that are required for the production on an industrial scale, including safety, resilience, effective and stable genetic alterations, the ability to employ a range of substrates, and the potential to build extremely high cell densities. (Park & Ledesma-Amaro, 2022).

Biotechnological applications of this fungus include the production of single-cell protein, single-cell oil, enzymes, vitamins, amino acids, etc. (Jach & Malm, 2022). Fermentation of *Y. lipolytica* has been used to produce various value-added products. For example, it can produce a water-soluble biopolymer called liposan, which is composed of 83% polysaccharides and 17% protein (Sajna et al., 2015). It has also been used for the production of omega-3, cis-5,8,11,14,17-eicosapentaenoic acid, which are essential fatty acid for human organism, which benefits all organ systems, especially cardiovascular and immune systems (Xie et al., 2016). Extracellular lipases and SCPs have also been produced at large rates concurrently using high-density fed-batch fermentation (Pan et al., 2023).

Lipase is a key enzyme produced by *Y. lipolytica*. Lipases have several uses in the food, dairy, detergent, and pharmaceutical sectors (Houde et al., 2004). In humans, lipase is primarily produced by the pancreas and aids in the breakdown of lipids into free fatty acids and glycerol. In most animal species, lipases play critical functions in the metabolism, transportation, and

breaking down of dietary lipids (Gupta et al., 2004). Lipase-producing microbes have been isolated from industrial waste, vegetable oil processing plants, dairies, oil-contaminated soil, oilseeds, and decaying food (Andualema & Gessesse, 2012). Due to their propensity to produce extracellular enzymes that facilitate the extraction of the fermentation medium, fungi are the perfect microorganisms for manufacturing industrial lipases. Lipase biosynthesis may be controlled by varying physiological conditions, and a high yield can be obtained by employing a fed-batch fermenter (Bharathi et al., 2018). *Aspergillus, Penicillium, Rhizopus*, and *Candida* are all major lipase-producing fungus taxa (Singh & Mukhopadhyay, 2011). Microbial lipases are used in the manufacturing of a broad spectrum of goods, including oil, food, soaps & detergents, cosmetics, paper, leather, textiles, and biodiesel. They are also utilized in the biocatalytic resolution of pharmaceutical intermediates, esters and amino acid derivatives, manufacturing of fine chemicals, and agrochemicals. Lipase found application as a component of biosensors, in bioremediation processes and in the preparation of cosmetics and perfumes (Hasan et al., 2006).

2.2 Electrofermentation and its working mechanism

Lipase production by *Y. lipolytica* can be performed through numerous conventional methods, including fed-batch fermentation and high-throughput fermentation (Back et al., 2016). For example, a study conducted this year performed fed-batch fermentation of mutant *Y. lipolytica* strain to produce extracellular lipases and SCPs at high levels (Pan et al., 2023). However, metabolic processes in conventional fermenters are frequently redox unbalanced. This may result in the buildup of byproducts and a decrease in the production of yield. The buildup or absence of intracellular reducing equivalents might obstruct the flow of metabolic energy toward the product or result in thermodynamic restrictions (Gong et al., 2020). Such shortcomings can be compensated electrochemically by the recent technique called electrofermentation (EF). To accelerate the metabolism of substances, EF allows controlling electron transfer by altering ORP and NADH/NAD+ ratio (Gong et al., 2020). It also improves the overall biomass yield and supports the production of a specific product by stimulating carbon chain elongation or breakdown (Bhagchandanii et al., 2020). Based on the direction of electron flow, EF is classified as anodic, where generated electrons are accepted by an anode, or cathodic, where oppositely the cathode as a working electrode act as an electron donor. During the EF of microbes, the presence of working electrodes acts as a driving tool and alters

their metabolic pathway, which results in a higher rate of intracellular activity (Moscoviz et al., 2016).

EF of naturally electroactive species such as bacteria that belongs to the family Shewanellaceae usually does not require additional chemicals that mediate redox reactions. In this case, electron transport is performed by redox proteins, such as cytochromes and nanowires, or by molecules like riboflavin, which is produced by the microorganism itself. If a microorganism lacks such mechanisms, an artificial redox mediator is added to the electrochemical system. Redox mediators enhance extracellular electron transfer through oxidation/reduction by the microorganism and electrode (Gemünde et al., 2022). There are a number of redox mediators that have been identified in the literature, but due to their toxicity, only few of them are employed routinely. Some examples of redox mediators include $K_3[Fe(CN)_6]$, veratrole alcohol, violuric acid, 2-Methoxy-10H-phenothiazine, 3-hydroxyanthranilic acid, 2-HNQ, N-hydroxyacetanilide, phenol red, dichlorophenol red, syringaldehyde and acetosyringone (Husain & Husain, 2007).

2.3 Previously conducted research outcomes

So far, there has been no published research on the EF of *Y. lipolytica* yet. Although the research described below did not involve *Y. lipolytica*, the methodology of the experiments as well as the results of the studies served as the foundation for the present research.

The study by Arbter et al. (Arbter et al., 2019) aimed to improve lipid production of oleaginous yeast *Rhodosporidium toruloides* via EF. This study is the first publication where authors conducted an in-silico assessment to determine potential growth of lipid production and metabolic alterations in yeast caused by EF. The platinized titan electrodes as well as redox mediator Neutral Red were used. Subsequently, CEF and a AEF were experimentally tested at various oxygen levels. Even under completely aerobic settings, CEF permitted the artificial reduction of the extracellular redox potential to less than 200 mV. Even though *R. toruloides* is also strictly an aerobic yeast, the oxygen supply was restricted after 34-36 h and the environment shifted to microaerobic, which resulted in a doubling in production yield. The authors concluded that despite increasing microbial lipid production and leading to higher experimental triacylglycerol (TAG) yield and productivity, it is still not clear if higher biofilm production was caused by AEF or stress. It also resulted in the synthesis of lipids towards a higher degree of saturation and relatively higher amounts of polyunsaturated fatty acids.

In 2016 the journal “Biotechnology for Biofuels and Bioproducts” described a study involving obligate aerobic bacteria *Pseudomonas putida* in a bioelectrochemical system, where electrode and redox chemicals as extracellular electron sinks were provided. A titanium mesh was used as a cathode electrode. Despite being promising organisms for industrial production, its strict requirement of oxygen became a huge obstacle to its widespread use. As a result of an applied bioelectrical system, bacteria could grow under anaerobic conditions and produce over 90 % yield. $K_3[Fe(CN)_6]$, riboflavin, and five more chemical compounds at 1 mM concentration were added as a redox mediator, but only few of them with redox potentials greater than 0.207 V interacted with the cells. Cells possessed a higher adenylate energy charge, indicating that they could produce energy by employing the anode as a terminal electron acceptor (Lai et al., 2016).

The electrochemical analysis of yeast was made in 2021 for *Candida albicans* biofilms. The article discusses the importance of analyzing Candida biofilms and rapidly assessing antifungal therapy in clinical practice. Existing biochemical approaches for assessing Candida biofilms are time-consuming, costly, and need expert interpretation. The authors propose electroanalysis as a suitable real-time tool for antifungal testing of *C. albicans* biofilms. The procedure is less cost - effective and faster than existing molecular techniques, and it does not need professional data analysis. Potassium ferricyanide was used as a redox mediator at a concentration of 0.1 mM. During EF, three different antifungal drugs (Flz, AmB and complex Ag3) were tested on *C. albicans*, showing enhanced antifungal activity due to produced oxygen radicals as the product of oxidizing electrode potential at the electrode. Antifungal compounds demonstrated an increased inhibitory effect against biofilm formation, which has great potential for further studies in antifungal drug development (Olaifa et al., 2021).

Chapter – 3 Methodology

3.1 Materials

For bio-electrochemical experiments, graphite screen-printed electrodes (SPE Ref. C110, DropSens, Spain) with 4 mm diameter graphite working electrode (WE), graphite auxiliary electrode and silver (Ag) pseudo-reference electrode were used. A VSP multichannel potentiostat (Bio-Logic, France) was used for all experiments. A 10 mL volume electrochemical cells (EC) with a working volume of 8 mL were used. 2-HNQ was dissolved in dimethyl sulfoxide (DMSO) at 10 mM concentration. $K_3[Fe(CN)_6]$ solution at the same concentration was prepared using distilled water. *Y. lipolytica* W29 cultures were kindly provided by the research group of Dr. Isabella Pisano, University of Bari, Italy. The cultures were prepared using a YPD medium which consisted of yeast extract (20 g L^{-1}), glycerol (10 g L^{-1}), peptone (10 g L^{-1}), and dextrose (30 g L^{-1}). These and other materials used, including crystal violet, acetic acid, $K_3[Fe(CN)_6]$, and 2-HNQ, were obtained from Sigma Aldrich and Thermo Fisher Scientific. Fluorescent images of SPEs were obtained with ZEISS Axio Zoom.V16.

3.1 Planktonic growth curves

The toxicity of 2-HNQ to planktonic *Y. lipolytica* cells was measured in 48-well plates at varying concentrations of 2-HNQ (5 – 500 µM). Wells with no 2-HNQ serve as a control. *Y. lipolytica* cells were injected into fresh YPD media and cultured overnight. Resulted broth was then diluted into fresh YPD medium to a final optical density OD_{600} = 0.1. YPD medium for consisted of yeast extract (1%), peptone (2%), and dextrose (2%). The pH of the medium was 5.6 without adjustment, which is suitable for the microorganism of interest (Sekova et al., 2019). Using Gen5TM Microplate Reader and Imager Software, OD600 measurements were made while the cells were cultured at 29 °C for 48 hours.

In 48-well plates, the K3[Fe(CN)6] toxicity to planktonic *Y. lipolytica* cells was evaluated at various doses. $K_3[Fe(CN)_6$ was added at varied concentrations of 5 - 500 µM from a freshly made stock solution after overnight cultures were diluted into fresh medium to a final optical density OD_{600} 0.1. Wells with no $K_3[Fe(CN)_6]$ serve as a control. Microtiter plates were incubated at 29 °C for 48 h.

3.2 Biofilm formation

The effect of different mediators on the biofilm formation of *Y. lipolytica* was measured using 48-well plates and crystal violet essay for biofilm quantification.

2-HNQ at different concentrations (5 – 500 µM) were added to five rows of two microtiter plates containing overnight cultures with optical density OD_{600} of 0.1. The sixth row was filled with control. There were six replicates for each concentration. Plates were kept at 29 °C temperature in a static condition for 48 h. After the culture media was removed, cells were stained with 0.1% crystal violet dye for 30 min. When the wells were completely dry, acetic acid (30% v/v) was added. Then the plates were placed on a laboratory shaker for 15 min to achieve complete dissolution of the dye in the acid. The OD_{600} was recorded using Gen5TM Microplate Reader and Imager Software. The same procedure was repeated for $K_3[Fe(CN)_6]$ with one biological replicate. The incubation time was 48 h, while the temperature was kept at 29 °C in a static condition (Figure 5).

3.3 Sunflower oil as an additional carbon source

Both, 5% (w/v) of sunflower oil and 1% (w/v) of TWEEN80 were added into freshly prepared YPD media. The solution was then mixed using Vortex and in case of solidifying of fats was put into an incubator at 37 °C for 5-10 min. After that, overnight incubated *Y. lipolytica* broth was added to reach OD_{600} = 0.1. $K_3[Fe(CN)_6]$ was added accordingly to various final concentrations (5, 10, 20, 50, 100, 200, and 500 µM). The 48-well plate was filled with free fatty acids (FFAs) containing broth and kept at 29 °C in the incubator without shaking.

3.4 Electrochemical system

The 8 mL broth with *Y. lipolytica* cells at OD_{600}= 0.1 was placed in 5 cm electrochemical cells with 50 µM $K_3[Fe(CN)_6]$ as a redox mediator. SPE electrodes were sterilized two times with ethanol before use. The electrochemical cells were kept at 29°C in a steel beads dry bath. For 48 hours CA, and DPV were performed consecutively. The parameters for CA include three potential values E_i = 200, 400, and 600 mV vs. Ag, running at 0, 24, and 48 h. For CV E = −0.3 V vs. Ag, E_{if} = 0.4 V vs. Ag pseudo-reference electrode was used. Obtained data was illustrated and analyzed by EC Lab and OriginPro 8.5.

Chapter – 4 Results

4.1 Effect of redox mediators on planktonic growth and biofilm formation

In images obtained with SEM, only the spherical phenotype of *Y. lipolytica* was observed (Figure 1). The filamentous form, which mostly appears as a result of alteration in pH of the media or mechanical damage, was not detected at conditions used in this research.

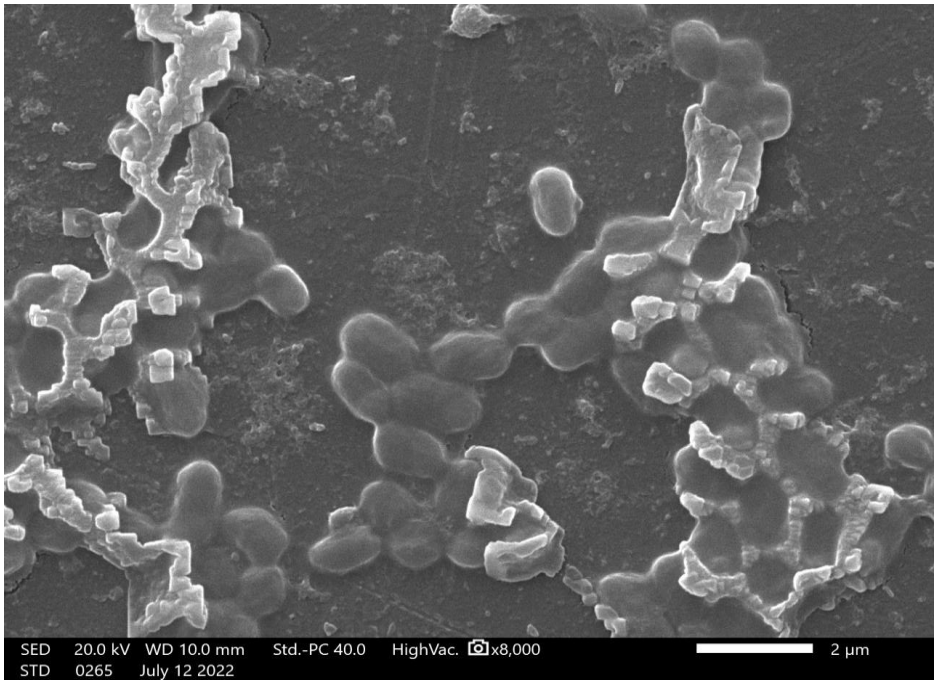

Figure 1. Image of *Y. lipolytica* obtained using scanning electron microscopy (SEM). Cells were cultivated in a YPD medium at 29°C. Spherical phenotype is expressed.

Figure 2 shows a planktonic growth curve of *Y. lipolytica* in 48 wells plates with different concentrations of the redox mediators. Both mediators have been previously reported in electroactive bacteria and yeast. While $K_3[Fe(CN)_6]$ is usually regarded as mildly toxic under active growth conditions, 2-HNQ is considered biocompatible at low concentrations (< 100 µM). However, the effect on planktonic growth is species-dependent, thus an initial characterization was required as there are no previous studies on the effect of these two redox mediators on the planktonic growth of *Y. lipolytica*. The results show that 2-HNQ slightly decreases the yield at concentrations < 100 µM and is toxic at higher concentrations. At 200 µM, 2-HNQ decreased the planktonic growth 43%, while 500 µM led to an almost 90% growth reduction. In contrast, $K_3[Fe(CN)_6]$ as a redox mediator, did not affect the planktonic growth of *Y. lipolytica* at all concentrations.

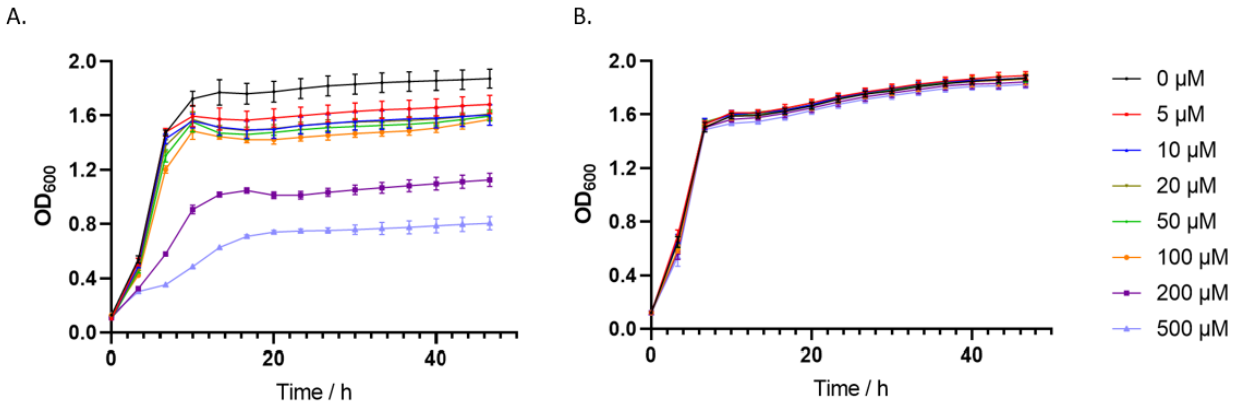

Figure 2. Effect of (A) 2-HNQ and (B) $K_3[Fe(CN)_6]$ on planktonic growth of *Y. lipolytica* at 29 °C under static conditions. Initial OD_{600} = 0.1. The experiments were carried out with 12 pooled replicates each, over two plates with 6 well replicates for each mediator.

Since EF is a bio-interface phenomenon that is correlated with the quantity of biofilm generated, it is crucial to maximize biofilm production. To collect and quantify the formed biofilm, the crystal violet staining method was used. It shows the level of cell adhesion on the surface of the plate. Figure 3 shows the result of biofilm formation using 2-HNQ and $K_3[Fe(CN)_6]$ as redox mediators. It demonstrates that 2-HNQ does not significantly affect the biofilm formation of *Y. lipolytica* at concentrations <200 µM and suppress at higher values. $K_3[Fe(CN)_6]$, on the other hand, leads to thicker layers of biofilm formation, reaching its highest optical density at 50 and 100 µM.

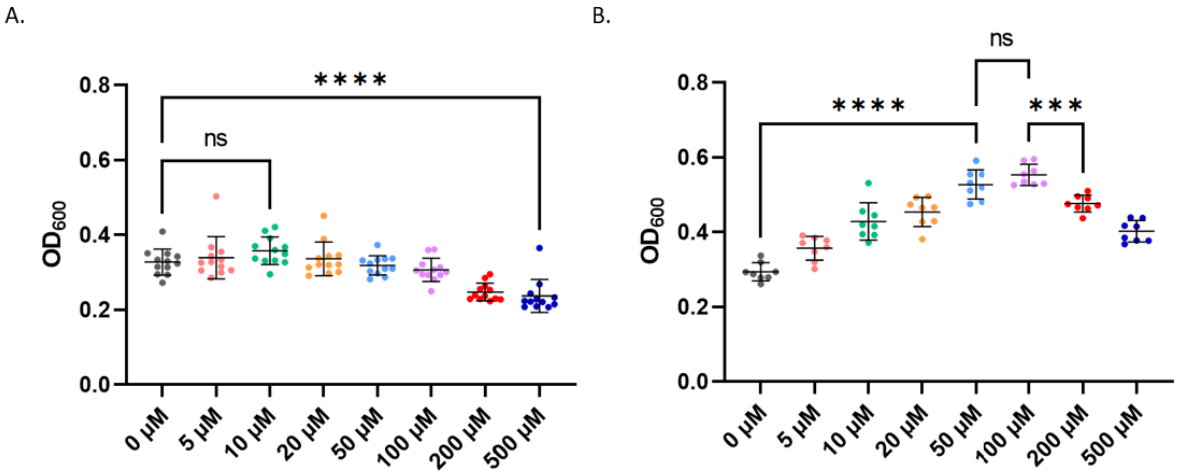

Figure 3. Effect of (A) 2-HNQ and (B) $K_3[Fe(CN)_6]$ on biofilm growth of *Y. lipolytica* on 48-well plates over 48 h with an initial OD_{600} = 0.1. The experiments were carried out with 12 pooled replicates each, over two plates with 6 well replicates for each mediator.

4.2 Electrochemical experiments on *Y. lipolytica*

Based on the results obtained from planktonic and biofilm growth experiments, $K_3[Fe(CN)_6]$ at 50 µM concentration was selected as a more suitable redox mediator for further electrochemical experiments. Since there has been no information found on the electrochemistry of *Y. lipolytica*, three potential values (200, 400 and 600 mV vs. Ag) were tested to see which give the best result (Figure 4). The first two values have been consistently reported in the EF literature, while the third was chosen to test the resistance of *Y. lipolytica* to electrochemical potential. Peaks observed through DPV demonstrated that the growth of the biomass/biofilm is partly inhibited at 600 mV, as also shown by the CA. More distinct peaks were observed after 48 hours at V= -250 and V= 300 mV. A peak at 300 mV obtained from the sample with 400 mV applied potential slightly shifted to the negative side which seems an electron transfer mechanism is happening inside the yeast. The total charge amount obtained from the sample with the highest applied potential is twice as high as 400 mV, reaching ±108.4 mC. The total charge amount after 48 hours is shown in Figure 7.

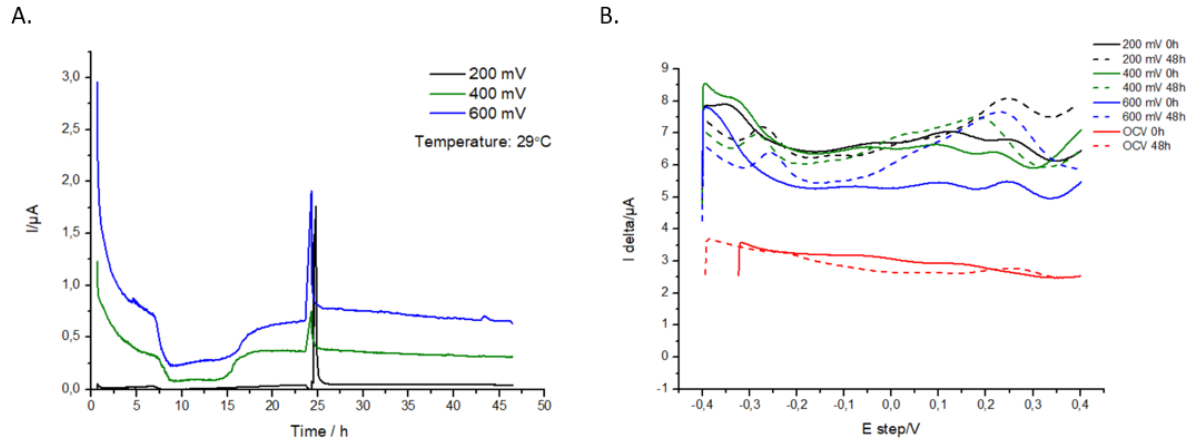

Figure 4. (A) The current output and (B) DPV curves of *Y. lipolytica* biofilm over 48 h with different potential values and $K_3[Fe(CN)_6]$ at 50 µM. The culture medium is YPD.

4.3 Effect of sunflower oil on the growth and electrochemical output of *Y. lipolytica* biofilms

It was previously reported that oil and free fatty acids (FFA) can enhance the growth of *Y. lipolytica* (Colacicco et al., 2022). Figure 5 shows the planktonic and biofilm growth results with $K_3[Fe(CN)_6]$ after adding 5% of sunflower oil to the YPD medium. The standard deviation of each group of values is higher compared to the values obtained in media without oil, which

can be a result of decreased surface adhesion caused by the *Y. lipolytica* growing at the water/liquid interface due to the formation of the emulsion with TWEEN80. At 50 and 100 µM of $K_3[Fe(CN)_6]$ the highest biofilm formation was observed, which shows consistency with previous biofilm formation results. It also proves that *Y. lipolytica* can use sunflower oil as an additional carbon source and may have an increased amount of lipase secreted to break it down. Planktonic growth and biofilm formation experiments were repeated with 10% sunflower oil, the results are shown in Appendix A. Yeast demonstrated minimum to no planktonic growth and mild biofilm formation with 10% sunflower oil in culture media, which means that at higher oil concentrations cells experience suppression of planktonic growth.

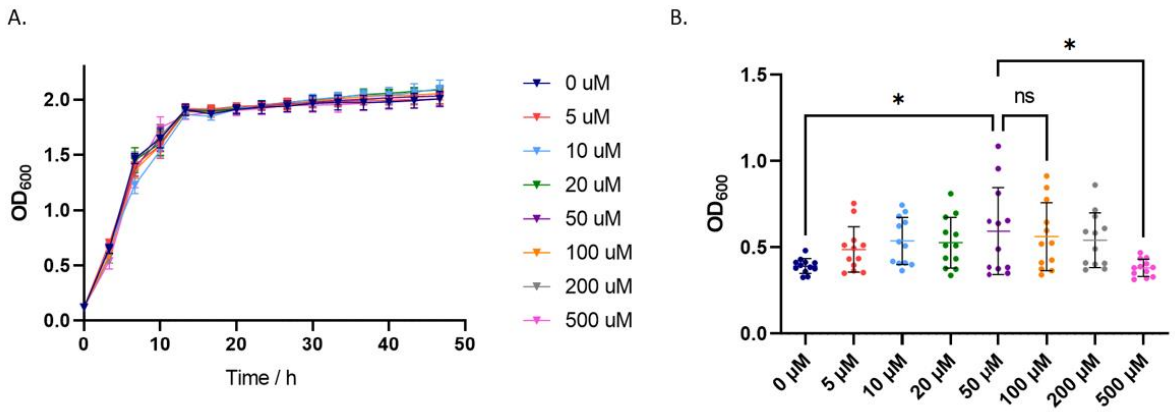

Figure 5. Planktonic (A) and biofilm (B) growth of *Y. lipolytica* with 5% of sunflower oil. The culture medium is YPD. $K_3[Fe(CN)_6]$ as a redox mediator was tested at various concentrations (0-500 µM).

To test the electroactivity of the yeast in the media with oil, conventional electrochemical experiments were performed. To keep the experiments consistent, three potential values were applied as in the previous experiments (Figure 6). With 5% oil yeast cells produced more charge output at E=600 mV applied potential, compared to lower potential values. However, results from DPV showed that cells in 5% oil gave higher peak intensity at 200 mV with a small peak at 100 mV, which slightly shifted to the right after 48 hours. The positive shift was observed in all four samples, including open-circuit voltage.

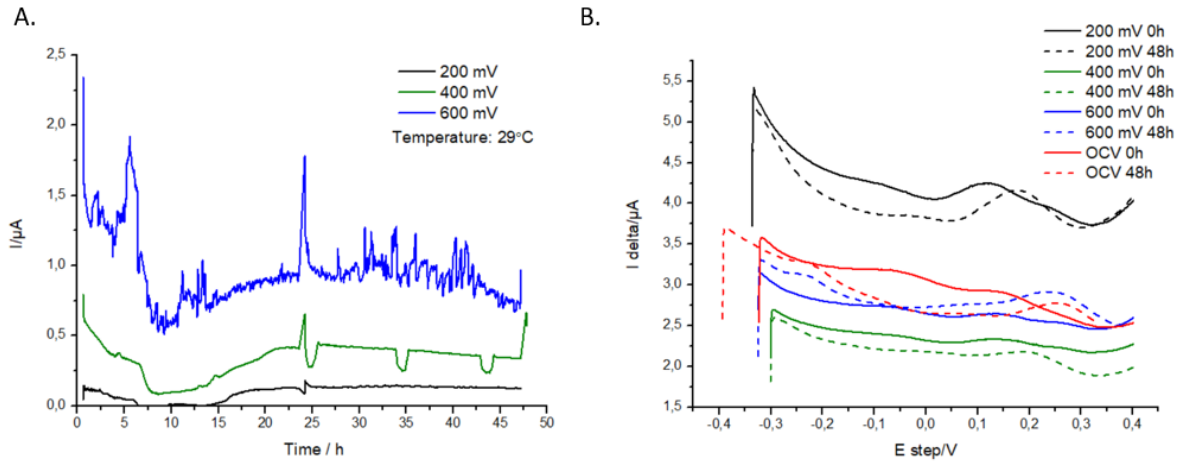

Figure 6. (A) The current output and (B) DPV curves of *Y. lipolytica* biofilm in media with 5% sunflower oil over 48 h with different potential values and $K_3[Fe(CN)_6]$ at 50 µM.

In Figure 7 (A) the total charge output for cells in pure YPD media and with 5% oil was illustrated. It is seen that in samples with 5% oil and 600 mV applied potential, the output is higher (±155,75 mC) than in pure YPD media (±108,4 mC), and the difference is statistically significant. There is also a difference between total charges obtained at 200 mV and 400 mV applied potential, where 200 mV demonstrated the least charge. Figure 7 (B) illustrates the percentage of SPE surface covered with yeast biofilm after 48-hour EF. In both pure and oil added YPD media, the most surface coverage was achieved at 400 mV. The least biofilm formation was observed at 600 mV within the groups with oil. There is no correlation between the total charge output and the amount of biofilm formed. There is a possibility that the applied potential changes the properties of the emulsion on the electrode, leading to high total charge output. As for most microorganisms, the high applied potential is toxic and leads to death. Therefore, 400 mV seems more suitable for EF of *Y. lipolytica*. Fluorescent images of SPEs are illustrated in Figure 8 and 9.

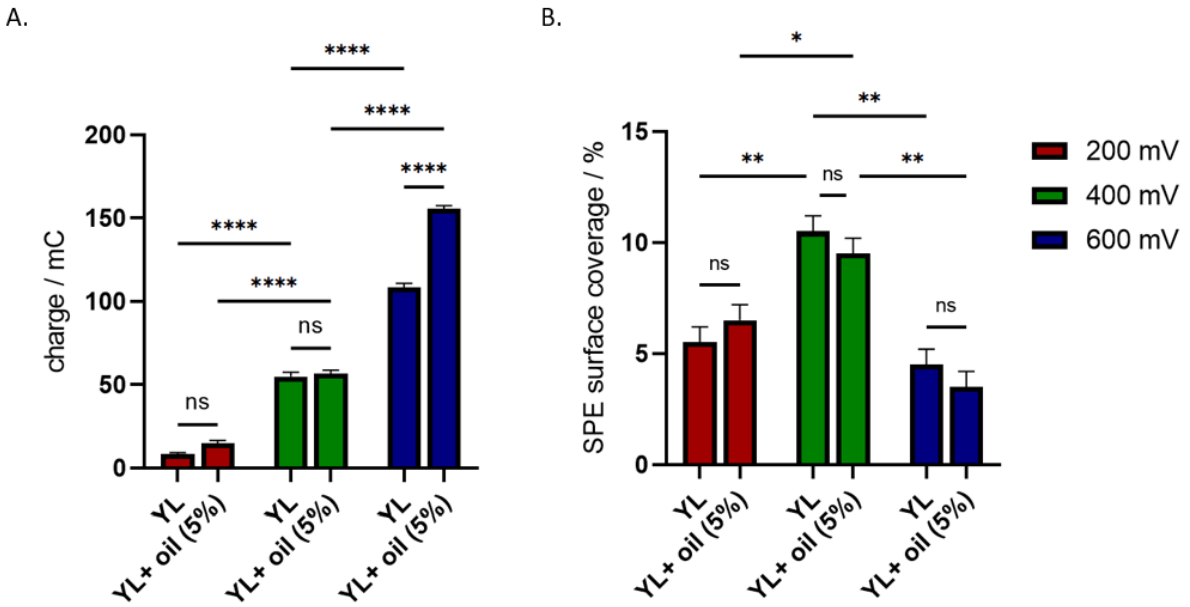

Figure 7. (A) shows the charge output of *Y. lipolytica* biofilm grown on SPEs within 48 hours with added $K_3[Fe(CN)_6]$ at 50 µM concentration, and different applied potential values. (B) shows the percentage of SPEs' surfaces covered with biofilm. 'YL' represents yeast cells grown in pure YPD media, while 'YL + oil (5%)' shows cells grown in media with sunflower oil.

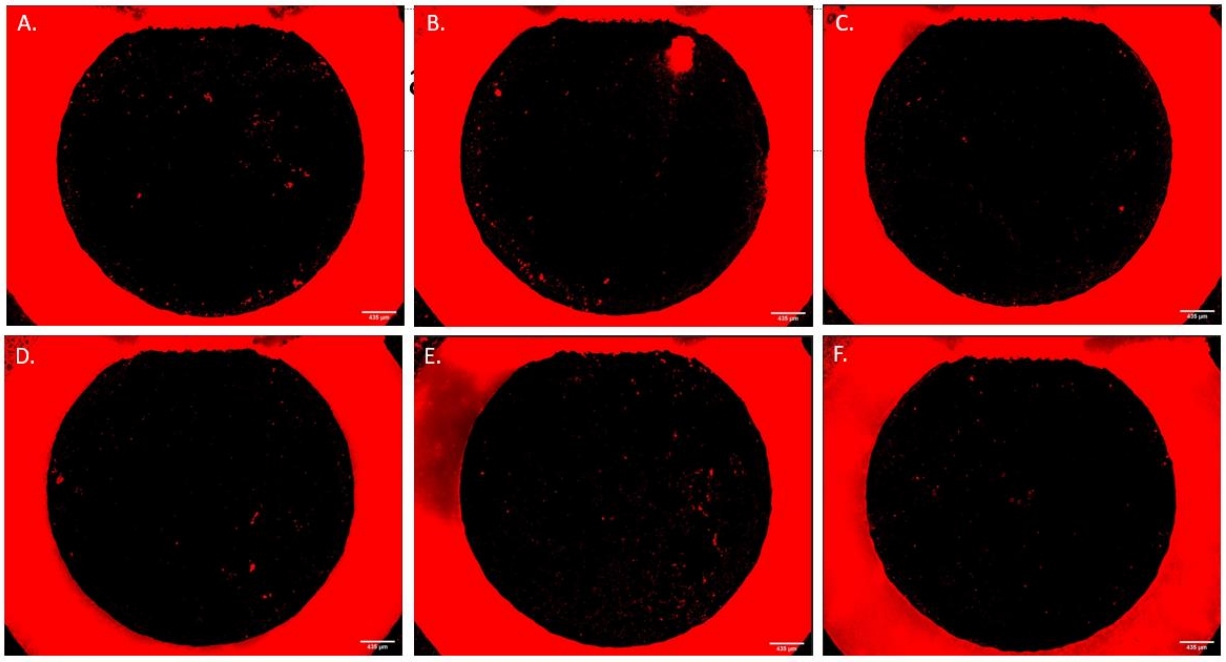

Figure 8. Fluorescent images of biofilm on SPEs collected from the electrochemical cells after 48-hour EF. Images (A), (B), and (C) show the biofilm of *Y. lipolytica* cells which were cultivated in YPD media with 50 µM $K_3[Fe(CN)_6]$. (A) shows biofilm formation with 200 mV applied potential, while (B) and (C) refer to 400 mV and 600 mV respectively. (D), (E),

and (F) images show biofilm of *Y. lipolytica* cells which were cultivated in YPD media with 50 µM $K_3[Fe(CN)_6]$ and 5% oil. (D) shows biofilm formation with 200 mV applied potential, while (E) and (F) refer to 400 mV and 600 mV respectively.

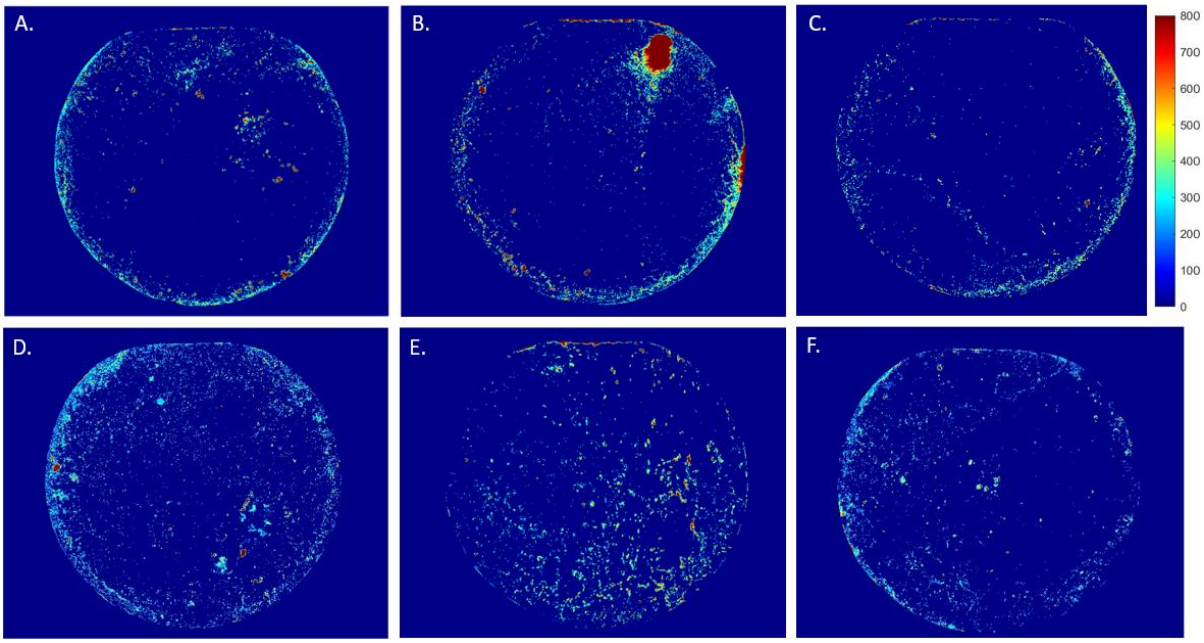

Figure 9. Heatmaps of biofilm on SPEs collected from the electrochemical cells after 48-hour EF. Images (A), (B), and (C) show the biofilm of *Y. lipolytica* cells which were cultivated in YPD media with 50 µM $K_3[Fe(CN)_6]$. (A) shows biofilm formation with 200 mV applied potential, while (B) and (C) refer to 400 mV and 600 mV respectively. (D), (E), and (F) images show biofilm of *Y. lipolytica* cells which were cultivated in YPD media with 50 µM $K_3[Fe(CN)_6]$ and 5% oil. (D) shows biofilm formation with 200 mV applied potential, while (E) and (F) refer to 400 mV and 600 mV respectively.

Chapter – 5 Discussion

First step in the experimental part of this study was to test the toxicity of two redox mediators on *Y. lipolytica*. Both planktonic growth and biofilm formation experiments demonstrated that 2-HNQ has a toxic effect on yeast at concentrations > 200 µM and made no significant change at lower concentrations. 2-HNQ was previously reported to have antibacterial and antifungal properties, that are achieved through various mechanisms including inhibition of protein synthesis, activating a mechanism of apoptosis in cells, affecting redox balance, and disruption of cell membrane synthesis (Wang et al., 2022). Unlike 2-HNQ, $K_3[Fe(CN)_6]$ has demonstrated an impressive effect on fungus by increasing biofilm formation at 50 and 100 µM concentrations, while not influencing the planktonic growth. $K_3[Fe(CN)_6]$ is widely used as a redox mediator for different microorganisms, including both yeast and bacteria (Dmitrieva et al., 2022).

Electrochemical experiments demonstrated the absence of a correlation between biofilm formation and total charge production. Even though the highest electrical conductivity was obtained at E = 600 mV, the biofilm-forming ability of the cells significantly decreased. Contrary to this, the most suitable applied potential value for *Y. lipolytica* seems to be E = 400 mV, which is proved by the biofilm formation percentage on SPEs.

The yeast Y. lipolytica is a particularly desirable host for the utilization of waste oils and lipid-based feedstock due to its capacity to digest lipids and withstand various undesirable conditions (Worland et al., 2020). Sunflower oil was added as an additional carbon source to the YPD medium to test the ability of the fungus to break down the lipids and uptake them as a carbon source. Biofilm formation and planktonic growth experiments showed that *Y. lipolytica* is capable to do so with 5% sunflower oil added to the media. This alteration had a significant effect on biofilm formation and electrochemical system output. The next step that should be done after performing experiments is a quantification of lipase from oil containing *Y. lipolytica* broth under an electrochemical system. Studies showed that olive and corn oil as a carbon source, as well as urea as a nitrogen source, resulted in the highest lipase production (Corzo & Revah, 1999). High levels of lipase production were previously reported to have been attained using high-density fed-batch fermentation (Pan et al., 2023); this achievement must serve as a standard for lipase production with the EF method.

A possible decrease in surface adhesion caused by the *Y. lipolytica* growing at the water/liquid interface due to the formation of the emulsion with TWEEN80 may serve as a limitation of this work, causing wide variation in optical density values obtained from biofilm formation in oil-added media.

Chapter – 6 Conclusion

Y. lipolytica is an unconventional yeast that gained attention from both researchers and industry professionals due to its ability to efficiently produce high-yield products, utilize a wide variety of substrates and be easily genetically modified. There has previously been no published research on the EF of *Y. lipolytica*, making this work unique. This study determined $K_3[Fe(CN)_6]$ as a suitable redox mediator at 50 µM concentration, due to its minimal effect on planktonic cell growth, as well as a significant boost in biofilm formation. As a result of electrochemical experiments, the highest percentage of SPE surface coverage was achieved at E = 400 mV, reaching 11%. In contrast, the best charge output (108,4 mC) was received at E = 600 mV applied potential. By adding 5% sunflower oil to the nutrient medium, the total charge output was increased (155,75 mC). Biofilm formation experiments with oil demonstrated that *Y. lipolytica* tends to form thicker biofilm in presence of 5% oil in a culture medium, which has to be studied further in regard to the quantification of a lipase produced by yeast in an electrochemical system.

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Appendices

Appendix A

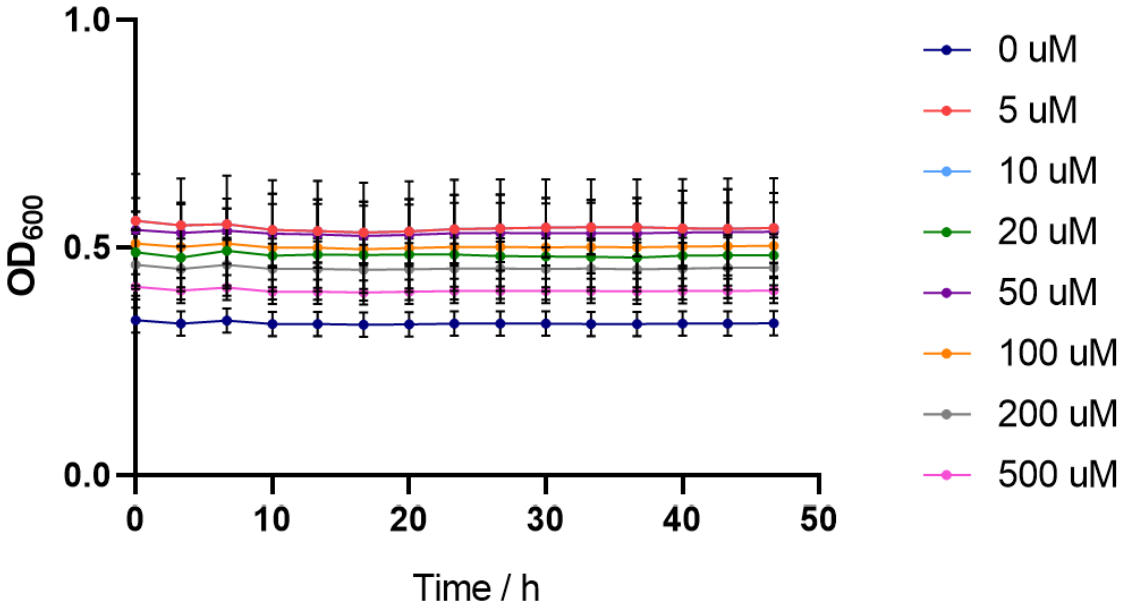

Figure A1. Planktonic growth curve of Y. lipolytica with 10% of sunflower oil. The culture medium is YPD.

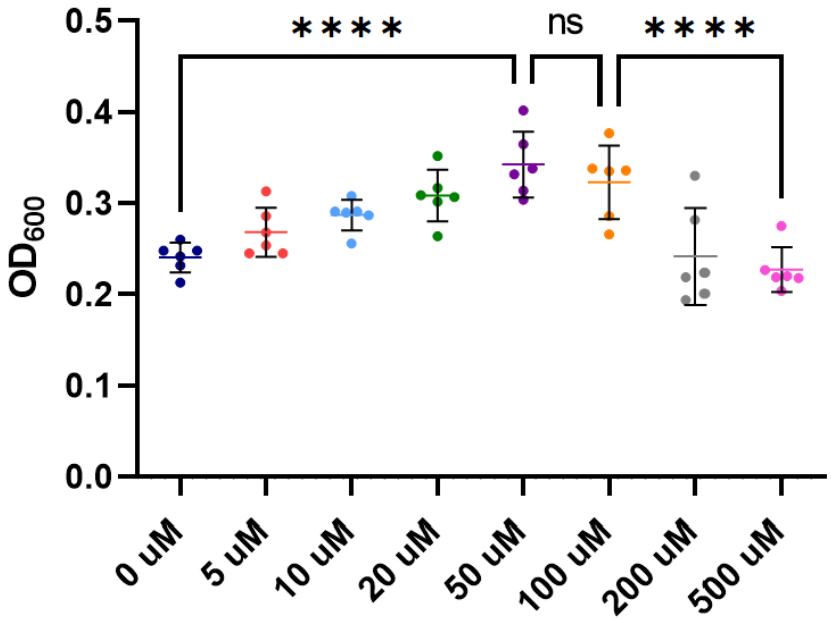

Figure A2. Biofilm growth curve of Y. lipolytica with 10% of sunflower oil. The culture medium is YPD.