

Development of a Cytosensor for the Detection of *Fusarium Oxysporum* - A Functional Approach Towards Bioanalytical Applications

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Abstract

The development of bio-analytical methods for monitoring microorganisms have created opportunities for applications in biosensors, bioprocess monitoring, assessment of cell signalling, analysis of drug responses, among several others. The voltammetric sensing system employed for studying the electrode behavior of the fungus *Fusarium oxysporum* comprised working (gold) electrode (0.2 cm²) platinum as counter electrode (0.2 cm²) and a saturated calomel as the reference, where the electrochemical response corresponded to the growth phases (lag, log, stationary and decline) of the fungus. The electrochemical method based on voltammetric response matched well with the response obtained through conventional methodology, where the dry weight of the fungus is estimated against time. The peak potential is a function of scan rate, which is one of the characteristic features of a totally irreversible electrode process. It is important to mention here that this dependence is true regardless of reversibility for any diffusing redox-active species. The proposed electrochemical method is less cumbersome and

more accurate. Furthermore, the proposed electrochemical method captures the decline phase of fungal growth, which is generally difficult using the conventional method of assessment of the growth curve. Further experiments confirm that the anodic peaks were not due to the biomass or the fungal spores and only due to the extracellular metabolites. However, at this stage it is difficult to exactly determine the metabolite or the group of metabolites that are responsible for the anodic peak. In conclusion this cytosensor is capable of accurately and rapidly quantifying fungi with *Fusarium oxysporum* as a model organism.

Keywords: *Fusarium oxysporum*; Cyclic voltammetry; Anodic peak; Cytosensor; Bio-electrochemistry

Introduction

Various activities of life and electron behavior have intimate relationship in living cells, which makes the study of physiological and biochemical characteristics an important area in analytical chemistry and bio-electrochemistry. (Okhi S 1985, Benjamin et al, 2018, Kizling & Bilewicz 2018). Bio-electrochemistry has increased our understanding of research areas such as electroporation (Hjouj et al. 2012; Zhan et al. 2012), direct electron transfer to enzymes and their applications in the areas of cloning (Mozzicafreddo et al. 2009, Cotter et al. 2011), drug discovery, (Hillard et al. 2008, Pauza et al. 2014) and biosensors (Tan et al., 1997, Subrahmanyam et al. 2001a, Shanmugam et al. 2001), and Enzymatic fuel cells (Bollella 2018). Potential applications of natural receptors in biosensors, drug discovery and bioassays (Subrahmanyam et al. 2002) have revealed novel opportunities for characterization of enzymes and antibodies. Advanced analytical methods have created opportunities such as ATP bioluminescence (Griffiths 1993), antibody-direct epifluorescent filter technique (Tortorello and Stewart 1994), enzyme immunoassays (Park et al. 1994, Watanabe & Hashida 2018), Polymerase Chain Reaction (PCR) based detection tools (Bej et al. 1994), Shi et al. 2018), microbial characterizations (Subrahmanyam et al. 1999, 2001d), and biosensors (Ding et al. 2011, Wang et al. 2012). Analysis of morphological changes in adherent cells using electric impedance sensing systems (Yang et al. 2003), and detection of viable microbes (Nakamura et al. 1991), Bajwa et al. 2013) have variety of applications. This is important because microbes when left undetected can cause a host of complications (Ba et al. 2011). Timely detection of microbes can be aided greatly by the use of appropriate sensors that can alleviate the progression of the disease (Balakrishnan et al 2006). Rapid real-time detection, high sensitivity, direct electron transfer and transfer of binding event into a signal are areas that need improvement for

the development of advanced analytical and detection tools. Cytosensors could address some of these issues and prove to be important analytical devices in future.

Several applications of cytosensors already exist. For example, cytosensors have had recent applications in oncology. Screening and recognition of two markers for breast cancer cells MCF-7 (Li et al. 2010) on the surface immobilizing an aptamer molecule on a gold surface have made it possible to conduct quick, sensitive and accurate detection and monitoring of cancer. Detection of overexpressing receptors have showcased interesting possibilities. Polyaniline-nanofiber (PANI-NF)-Gold nanoparticles (AuNPs), glutathione (GSH) and folic acid (FA) were sequentially self-assembled and immobilized for the detection of folate receptors (FR) overexpressed in cancerous cells using human cervical carcinoma HeLa cells as a testing system (Wang et al. 2012). Detection of bio-chemicals on cell surfaces are another interesting possibility. Overexpressing carbohydrate present on the cell surface was quantified using gold nanoparticles indicating metastasizing cancer cells. Leukemia cells (K562) were quantified using specific recognition of mannosyl on a cell surface to concanavalin-A (Con-A) and the signal amplification of gold nanoparticles (NPs) (Ding et al. 2011).

Cytosensors have been used to identify receptor ligands in tissue extracts and for examining signal transduction of neurohormones (Lenkei et al. 2000). Other applications of cytosensors include detection of fungi (Subrahmanyam et al. 2000b) & eukaryotic cells (Eldefrawi et al. 1998), assessment of compound toxicities, (Cooke & O'Kennedy 1999, Liu et al. 2013), sensing of glomerular inflammation and subsequent control of transgenic activity (Kitamura 1999), and cancer cells (Jiang et al. 2018, Zhang et al. 2018, Tang et al. 2018, Dervisevic et al. 2017). While the above examples are useful, development of cytosensor for detection of whole cells will have better applications, including detection of contamination, identification of cell cultures and understanding competitive inhibition in mixed cultures.

While previous cytosensor work have largely targeted yeast cells (*Saccharomyces cerevisiae*) and fresh water ciliate (*Tetrahymena shanghaiensis*), there has been no work reported on development of cytosensors for pathogenic fungi. The aim of this work therefore, is to demonstrate a novel cytosensor based on cyclic voltammetric response for the fungus *Fusarium oxysporum*.

Materials and Methods

Materials

Fungal strain

The fungus was isolated from soil collected from an industrial site where waste polyurethane (PU) scraps are disposed near Vellore, India. 2 mg of surface soil were collected. Serial dilution technique was employed for the isolation. Several rounds of sub-culturing and dilutions were done until a pure culture of *Fusarium oxysporum* was obtained. The fungus was identified and confirmed to be *Fusarium oxysporum* at the Mycology Division, Indian Agricultural Research Institute, India.

Culture media and buffer

Czepek-Dox media containing $\text{KH}_2(\text{PO}_4)_2$ (1g), NaNO_3 (2g), MgSO_4 (0.5g), KCl (0.5g) and glucose (30g) dissolved in a liter of distilled water with pH adjusted to 7.2 were used for the culture. Phosphate buffer (pH 7.2), 100mM was used for washing the fungal media.

Fungal culture

The fungal media was autoclaved at 121°C at 15 psi for 15 minutes and cultured in 500ml flasks with 100 ml of culture medium. After inoculation the culture was maintained at 27°C in an aerated condition. The fungus was isolated from the broth by centrifuging the broth at 3400 rpm for 45 minutes, at 4°C.

Methods

The voltammetric sensing system

The voltammetric sensing system employed for studying the electrode behavior of the fungus comprised gold as working electrode (0.2 cm^2) platinum as counter electrode (0.2 cm^2) and a saturated calomel as the reference. The working electrode was polished well before dipping into the fungal broth. All measurements were done at room temperature (27°C \pm 2°C). The electrode was cleaned well and was cyclically scanned several times from 0.0 to 1.0V (vs Saturated Calomel Electrode [SCE]) for baseline qualification. The fungus was washed well with buffer before experimentation.

Apparatus

The measurements were performed using a Wenking potentiostat, (Tokyo, Japan) model POS 88 with a Rikadenki (Tokyo, Japan) X–Y–t recorder (RW–201 T).

Measurement of growth using voltammetric sensing system

The working electrode was thoroughly polished and was cleaned before it was put into the sensing system. It was treated by cyclic scan several times from 0.0 to 1.0 V vs SCE before immersing in the broth containing the fungus.

Measurement of the fungal growth in the media

For measurement of fungal growth using the ‘conventional method’, the fungus was harvested from the culture medium after thorough filtration on tarred filter paper after washing it at least three times with petroleum ether, hexane and methanol Ci et al. (1997). After harvesting, the biomass was dried to constant weight at 95°C and replicate tests were conducted to obtain average values.

Results

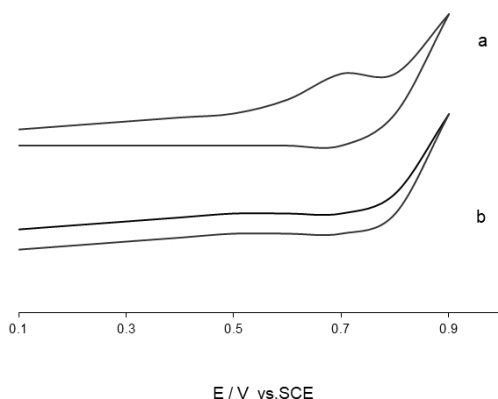


Figure 1: Cyclic voltammogram of *Fusarium oxysporum*, (Scan rate of 50 mV/s)

Figure - 1a) *Fusarium oxysporum* in broth, 38 mg/250 ml

Figure - 1b) Background electrolyte only

Cyclic voltammogram generated for the broth containing the fungi is presented in Figure—1a and b (The scan rate of 50 mV/s vs SCE was employed). At 0.7 V vs SCE, anodic wave was obtained. The current on the anodic peak increased with increase in time. The anodic peak seen in Fig—1a, could be attributed either due to fungal mycelia, or extracellular metabolite secreted by the fungus. A control experiment was performed to ascertain the reason for the anodic peak. The fungus was isolated from the broth by centrifugation and the clear supernatant was collected. The conditions for centrifugation including rate and time are presented in section 2.1.3. The fungus was then filtered in a Whatman 40 filter paper and washed thoroughly with phosphate buffer (pH 7.2, 100mM) to remove any metabolite that

adhered to the fungus. Centrifugation was done again to confirm that the metabolite was completely devoid of any fungal biomass that can possibly be found in the supernatant. The isolated fungus was suspended in phosphate buffer and the cyclic voltammograms were generated (See Fig—1b).

Discussion

Reasons for the anodic peak

As can be seen from Fig—1b, CV peak characteristics are similar to that of the background electrolyte confirming that the fungal mycelia are not responsible for the anodic wave. Similarly we also recorded the CV of the supernatant using the same experimental conditions. The solution containing the metabolites provided the anodic wave. The results confirm that the anodic peaks were not due to the biomass or the fungal spores and only due to the extracellular metabolites. However, at this stage it is difficult to exactly determine the metabolite or the group of metabolites that are responsible for the anodic peak. Unfortunately, the metabolite or the mixture of metabolites that is/are responsible for the anodic peak could not be identified. While this is most certainly a limitation of this research, the fact that we are able to characterize an anodic peak and deduce scientific explanation for it, has tremendous potential in the near future.

Correlation between peak current and the fungal growth

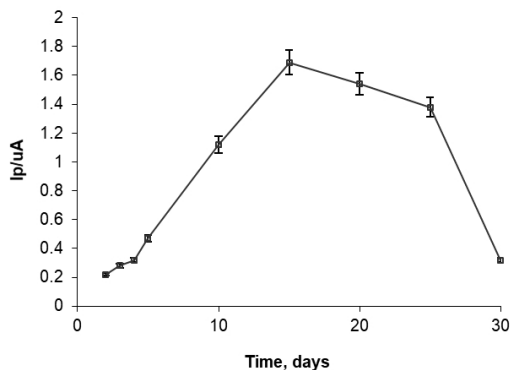


Figure 2: Growth of *Fusarium oxysporum* in culture media characterised by peak current

Peak current values were calculated for several days during the growth phase of the fungus. A graph was plotted with the peak current values against the growth period (See Fig—2). The cytosensor was able to evaluate all the three metabolically active phases of the growth curves, namely the lag, log, stationary phases, and one metabolically inactive

decline phase. Earlier authors have reported a similar trend for the microbial species of *Saccharomyces cerevisiae* Feng et al. (1997), and *Tetrahymena shanghaiensis* Cofone et al. (1973). In our earlier study, our group demonstrated similar responses for *Aspergillus niger* Subrahmanyam et al. (2001c); *Fusarium solani*. Subrahmanyam et al (2000a) and *Aspergillus terreus* Subrahmanyam et al (2001b). The results have clear advantages in terms of the bioanalytical application. Conventional growth curve can be estimated easily for bacteria, whereas it is difficult, cumbersome and unreliable for fungi, because colony counting cannot be used as in the case of bacteria. Estimation of dry weight for fungi and colony counting for bacteria make it especially difficult to capture the decline phase. These limitations of the ‘conventional’ colony counting methods are exactly the ones that the electrochemical technique proposed here aims to overcome. In addition to the electrochemical estimation, another batch of experiments were performed in parallel to obtain the growth characteristics of the fungus using the “conventional technique”.

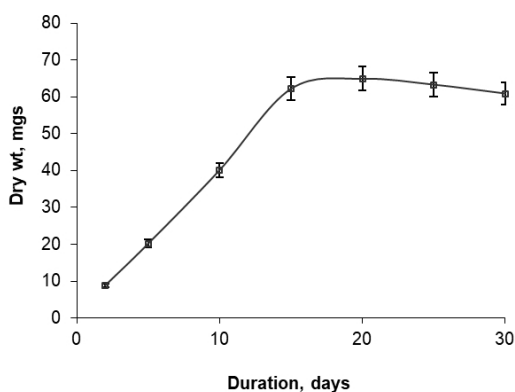


Figure 3: Growth of *Fusarium oxysporum*, Conventional methodology of measurement of dry weight against time

Cytosensor approach

It is well established that in the conventional method of determining growth curve, the decline phase is not detected clearly for the fungus, as the increase in dry weight is monitored during fungal growth (see Fig—3). We can also observe a marginal decrease in weight after 24 days of incubation. A comparison of Figures—2 and 3 confirms that the first three phases of the growth (lag, log and stationary growth phases) match well with the peak current values, confirming the fact that the electrochemical technique has potential applications as cytosensors. As has been shown the decline phase

is clearly defined only using the electrochemical cytosensor approach (see Fig—2). This is important for determining the growth characteristics of fungal species for the following reasons. Firstly, this approach is useful for capturing growth phases of microorganisms, whose growth phases run for several days rather than hours. The four growth phases for fungi run for a couple of weeks, when compared to that of bacteria, which require only about a few hours for their entire growth cycle. Secondly, using conventional techniques decline phases cannot be estimated accurately. This is because, the decay of the dead cells is not immediate, making it impossible for the conventional method to provide us with decrease in activity of the living cells.

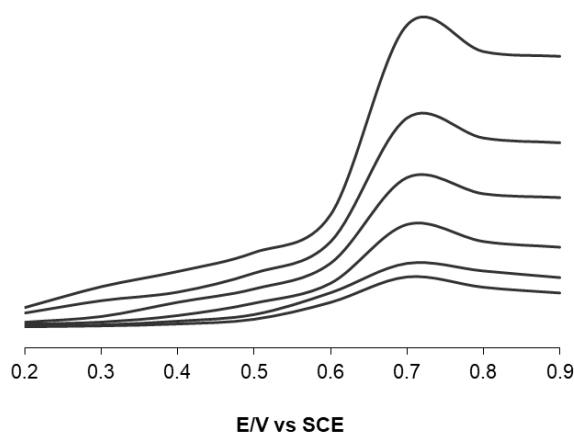


Figure 4: Peak potential of the fungus against different scan rates (Scale 1cm = 2uA). Dry weight (*Fusarium oxysporum*) of 32mg/250 ml of media; Scan rates of 5 mV/s, 10 mV/s, 20 mV/s, 50 mV/s, 100 mV/s, and 200 mV/s

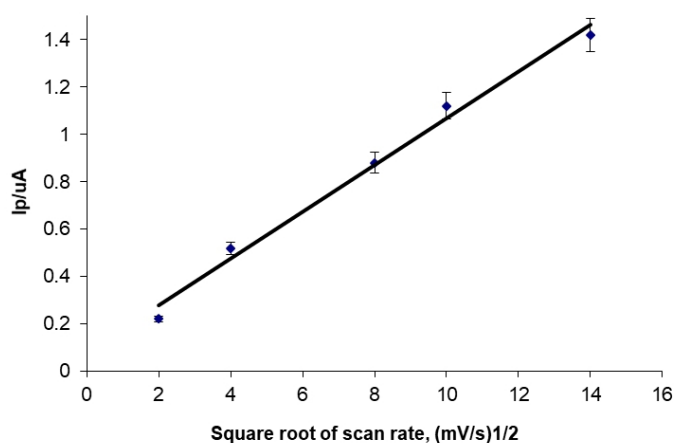


Figure 5: Relation between peak current and square root of scan rate, *Fusarium oxysporum*, 40 mg dry weight in 250 ml of media

The electrode process and behavior

The fungus (*Fusarium oxysporum*) was scanned for the peak potential against different scan rates. As has been shown in Fig—4 the peak potential is a function of scan rate, which is one of the characteristic features of a totally irreversible electrode process. It is important to mention here that this dependence is true regardless of reversibility for any diffusing redox-active species. This was also confirmed by studying the linearity between the square root of the scan rate and peak current (see Fig—5). The response is not due to the fungal biomass but due to the electroactive metabolites.

Conclusion

This work describes the development of a cytosensor capable of accurately and rapidly quantifying fungi with *Fusarium oxysporum* as a model organism. It is also clear that the anodic peak is a response exclusively due to the extracellular metabolites and not during the fungal mycelia. We believe that the electrochemical technique described here potentially has applications in a variety of areas such as biosensors, detection of extracellular metabolites, estimation of fungal contamination, bioprocess and pharmaceutical industries.

Firstly, one of the immediate applications is in cell biology, where estimation of cells using conventional methods could be readily replaced saving both time and labour.

Secondly, this technique could find applications in food industry. Food-borne illness typically arise due to improper handling, preparation and food storage Mead et al. (1999); McCabe-Sellers & Beattie, (2004); Scallan et al. (2011). Standardizing the detection of microbes could detect microbial toxins.

Thirdly, the technique could be applied within bioprocess industry including in the production of antibiotics such as insulin, microbial enzymes, vitamins, vaccines, growth factors and steroids Madigan et al. (1997); Samaha et al. (2004). In order to estimate growth of fermenting microbes, samples are drawn at regular intervals. Incorporating a cytosensor within a bioreactor, would enable continuous estimation of the product with less probability of contamination.

Lastly, drugs and their effectiveness on microorganisms could be easily researched using the proposed cytosensor, by analyzing the cells' viable state in a culture media.

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