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Thionine Increases Electricity Generation from Microbial Fuel Cell Using *Saccharomyces cerevisiae* and Exoelectrogenic Mixed Culture

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Microbial fuel cells (MFCs) have been shown to be capable of clean energy production through the oxidation of biodegradable organic waste using various bacterial species as biocatalysts. In this study we found *Saccharomyces cerevisiae*, previously known electrochemcially inactive or less active species, can be acclimated with an electron mediator thionine for electrogenic biofilm formation in MFC, and electricity production is improved with facilitation of electron transfer. Power generation of MFC was also significantly increased by thionine with both aerated and non-aerated cathode. With electrochemically active biofilm enriched with swine wastewater, MFC power increased more significantly by addition of thionine. The optimum mediator concentration was 500 mM of thionine with *S. cerevisae* in MFC with the maximum voltage and current generation in the microbial fuel cell were 420 mV and 700 mA/m^2, respectively. Cyclic voltametry shows that thionine improves oxidizing and reducing capability in both pure culture and acclimated biofilm as compared to non-mediated cell. The results obtained indicated that thionine has great potential to enhance power generation from unmediated yeast or electrochemically active biofilm in MFC.

Keywords: microbial fuel cell, mediator, electron shuttle, electricity generation, *Saccharomyces cerevisiae*

Introduction

Production of energy from renewable resources is now a widely accepted and utilized sustainable concept, which reduces global carbon dioxide emissions and thus decrease the environmental burden derived from fossil fuel. The microbial fuel cell (MFC) is a novel technology that can recover bioenergy in the form of hydrogen and/or electricity directly from organic matter, while simultaneously treating biodegradable contaminants in wastewaters (Oh and Logan, 2005; Oh *et al.*, 2010). It has been extensively investigated and shown that the MFC performance mainly depend on operational and design factors, such as system architecture, electrode material and surface area, catalytic bacterial species on the electrode, types of substrate, and operating conditions (pH, conductivity and flow rate) in the anode chamber, as well as cathode catalyst and electrolyte (Oh *et al.*, 2004, 2009, 2010; Kim *et al.*, 2005, 2007a; Liu *et al.*, 2005; Oh and Logan, 2006).

Bacteria have been presented as key catalysts in MFCs and therefore the improvement of biocatalyst on the electrode has been widely investigated from this perspective in relation to other features; increase electrode surface area by brush carbon electrode (Logan *et al.*, 2007), activation of electrode surface by chemical treatment (e.g. ammonia) (Kim *et al.*, 2007b); active selection of electrogenic species (Kim *et al.*, 2005). The combined electron transport mechanisms between bacterial cell membrane and electrode surfaces is believed to be a rate limiting factor which determines the whole MFC system performance. The bacterial electron transfer mechanisms reported so far are; direct electron transfer from outer cell membrane to electrode; electrically conductive nanowire (Beveridge, 2004; Reguera *et al.*, 2005); electron shuttles using externally added or self produced chemicals (e.g. pyocyanin from *Pseudomonas aeruginosa*) (Rabaey *et al.*, 2004, 2005).

A significant improvement in cell current has been observed with the addition of electrochemical mediators that facilitate the electron transfer between bacteria and electrode (Allen and Bennetto, 1993; Park and Zeikus, 2000; Rabaey *et al.*, 2005). Typically, MFCs performances have been known to be enhanced by the addition of electron shuttles with e.g. *Shewanella*, *Pseudomonas*, and *Escherichia coli*; particularly also in Gram-positive bacteria, *Bacillus*, which were otherwise inefficient to transfer electrons from their internal electron transport chain to outer electron acceptor. Electron mediator which has a redox potential close to that of NADH/NAD$^+$ can facilitate electron shuttling between the reaction center inside of the cell and terminal electron acceptor (anode electrode). Several exogeneous electron mediators such as methyl viologen, methylene blue, neutral red, and thionine have been used in MFCs (Table 1).

Choi *et al.* (2003) suggested that the desirable characteristics of mediators are facilitation of reversible electron transfer and minimal mediator accumulation inside of the

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Table 1. Microorganisms, substrates, and mediators reported in MFCs implementations for the enhancements of power generation

Microorganism	Substrate	Mediators	Reference
Actinobacillus succinogenes	Glucose	Neutral Red, Thionine	Park and Zeikus (1999, 2003)
Erwinia dissolven	Glucose	Fe (III) EDTA	Bennetto *et al.* (1983)
Gluconobacter oxydans	Glucose	HNQ, resazurin, Thionine	Lee *et al.* (2002)
Shewanella putrefaciens	Lactate, pyruvate, Acetate	Neutral Red	Park and Zeikus (2002)
Streptococcus lactis	Glucose	Ferric chelate complex	Grzebyk and Pozniak (2005)
Escherichia coli	Glucose, acetate	Neutral Red, 2-Hydroxy-1,4-Naphthoquinone, Methylene blue	Bennetto (1990), Park and Zeikus (2000), Schroder *et al.* (2003), Grzebyk and Pozniak (2005), Ieropoulos *et al.* (2005)
Proteus vulgaris	Glucose, Sucrose	Thionine	Bennetto *et al.* (1985), Thurston *et al.* (1985), Park *et al.* (2000)
Enterococcus faecium	Glucose	Pyocyanin	Rabaey *et al.* (2005)
Micrococcus luteus	Glucose	Thionine	Yoon *et al.* (2007)
Saccharomyces cerevisiae	Hydrolyzed Lactose	Methylene blue, Neutral Red	Najafpour *et al.* (2010a)

cell membrane. They reported that the thionine can easily penetrate through the phosphatidylcholine (PC) layer in the cell membrane during the process, compared to other mediators, namely HQN, phenothiazine, quinone, and azo family mediators. It is also reported that thionine is not involved in any assimilatory biochemical reaction thus it cannot be accumulated in the cell membrane, and can therefore facilitate reversible electron transfer between cell and terminal electron acceptor. The increased electron transfer rate with thionine should result in higher current generation and therefore could be used in MFCs for sustaining improved performance.

Park and Zeikus (2000) have also shown the interactions between bacterial cultures and electron mediators. Incorporation of mediators onto the graphite electrode increased power output 10-fold because of the facilitated interaction of these mediators with NAD^+ (Park and Zeikus, 2003; Ringeisen *et al.*, 2006; Yoon *et al.*, 2007).

The soluble redox mediators used in MFCs for the improvement of electron transfer have been summarized in Table 1.

In this paper, we demonstrate the yeast, *Saccharomyces cerevisiae*, which has previously been known to be exoelectrogenically inactive, can produce electricity in an MFC and power can be increased by using thionine as mediator. *S. cerevisae* has been used as biocatalyst for biofuel cells with using methylene blue as mediator in the previous studies (Gunawardena *et al.*, 2008; Najafpour *et al.*, 2010b). However its electrogenic activity was considered too low to be implemented in a system when chemical mediator was not added. However, its metabolic and genetic information has been widely investigated, which would be helpful in studying its performance in MFCs. The main objectives of the study were to determine an optimum concentration of thionine as electron mediator in the anode chamber and its electrochemical characteristics, determined by adding electron shuttle and considering sensitivity to operational parameter such as aeration in the cathode chamber.

Materials and Methods

Microorganism and cultivation

Saccharomyces cerevisiae PTCC 5269 supplied by the Iranian Research Organization for Science and Technology (Tehran, Iran) was used as active biocatalysts in this study. The microorganism was grown anaerobically in a jar vessel. The medium for the pure culture consisted of glucose, yeast extract, NH_4Cl, NaH_2PO_4, $MgSO_4$, and $MnSO_4$: 10, 3, 0.2, 0.6, 0.2, and 0.05 g/L, respectively. The medium was autoclaved at 121°C and 15 psi for 20 min. The pH was initially adjusted to 6.5. The *S. cerevisiae* were inoculated into sterilized media at ambient temperature and incubated at 30°C. The pure culture was fully grown in a 100 ml flask without any agitation for the experiment duration of 24 h. In a MFC operation 1 g/L of glucose was used in an anode chamber. For a mixed culture inoculum, swine wastewater (0.3 ml) in Chuncheon city, Korea was used. After that MFCs were inoculated with anode medium of a working MFC initially inoculated with swine wastewater. Glucose (20 mM) was used as an energy source in a nutrient solution (pH=7.0) containing (per L of deionized water): $NaHCO_3$ (3.13 g/L), NH_4Cl (0.31 g/L), KCl (0.13 g/L), NaH_2PO_4 (4.22 g/L), Na_2HPO_4 (2.75 g/L), and trace metal (12.5 ml) and vitamin (12.5 ml) solutions. (Kim *et al.*, 2005)

Chemicals and analyses

All chemicals and reagents used for the experiments were analytical grades and supplied by Merck (Darmstadt, Germany). The pH meter (Model HANA 211, Romania) was employed to measure pH values of the aqueous phase. The initial pH of the working solution was adjusted by addition of diluted HNO_3 or 0.1 M NaOH solutions. The surface images of the graphite plate electrode were obtained by a Scanning Electronic Microscope (SEM) (Phillips XL30, Netherland). The image from the surface of the graphite electrode was taken before and after the experimental run. The sample specimen size was 1 cm×1 cm for SEM analysis. Finally, images of the samples were taken under SEM at magnifications of 500 and 5000. SEM images were used to demonstrate the physical characteristics of the electrode surface and to examine the morphology of the microorganism on the anode surface.

MFC construction and operation

The fabricated cells made of glass (Pyrex) material at laboratory scale were used for the MFC. The volume of each chamber (anode and cathode) was 850 ml with a working volume of 760 ml. A sampling port, wire point inputs and inlet port were provided for the anode chamber. The se-

lected electrodes in MFC were 40×70×2 mm graphite plates. When calculating power density, surface area of electrode of 0.0056 m^2 was used. Proton exchange membrane (PEM; NAFION 117, Sigma-Aldrich) was used to separate the two compartments (cross-sectional area: 3.79 cm^2). Nafion was pretreated to remove any impurities by boiling the film for 1 h in 3% H_2O_2, followed by treating with deionized water, 0.5 M H_2SO_4, and finally washing with deionized water. Thionine at different concentrations (100 to 600 mmol/L), was used as an electron mediator in the anode chamber of the MFCs.

Electrochemical measurements and calculations

The cell voltage across the external load was continuously measured by a multimeter with a data acquisition system (Model 2700, Keithly, USA). Power and current were calculated according to $P=IV$ and $I=(V/R_{ext})$, where P (W) is the generated power, V (V) is the measured cell voltage, R_{ext} (Ω) is the external load, and I (A) is the current. The current and power produced were normalized to the surface area of the electrode. Polarization curves were obtained by using varying external resistances over a range of 65,535-1 Ω.

Cyclic voltammetry (Ivium CompactStat, Ivium Technology, Netherland) was carried out to characterize the oxidation and reduction of thionine on the electrode. A conventional three-electrode system was employed with the anode as the working electrode, cathode or platinum mesh (Platinum, gauze 100 mesh, Sigma Aldrich) as the counter electrode, and an Ag/AgCl reference electrode (0.195 V corrected to a normal hydrogen electrode; NHE). The potentials were shifted from -400 mV to 1000 mV at a scan rate of 50 mV/sec for comparison of redox property except where stated otherwise.

Results and Discussion

The effect on power of electron shuttle in the anode and aeration in the cathode chamber are shown in Fig. 1. Very low maximum power density was obtained with *S. cerevisiae* PTCC 5269 in a control MFC in which an exogeneous electron mediator was not used (3 mW/m^2) (Fig. 1A). However, the addition of thionine (200 μM) in the anode chamber significantly increases the power density (16 mW/m^2), as compared to the control (Fig. 1B). In order to see the impact of aeration in the cathode chamber, the cathode was aerated using an air pump with 50 ml/min (dissolved oxygen: 6.5 mg/L). Aeration into the cathode further increased the maximum power up to 29 mW/m^2 (at 280 mA/m^2), due to improved cathode potential (Fig. 1C) as shown in the previous report (Oh *et al*., 2004). Some microorganisms (e.g. *Geobacter* sp.) produce nanowires to transmit electrons directly without using any mediator but yeast like *S. cerevisiae* is not known to produce conductive nanowire. Therefore *S. cerevisiae* use electron shuttle (thinoine) for respiration rather than direct contact with external electron acceptor. We found small power generation was obtained even without adding mediator, probably through naturally produced self-mediator or direct contact of living cell on the electrode, possibly through membrane bound cytochromes. However, the maximum power density can be significantly increased

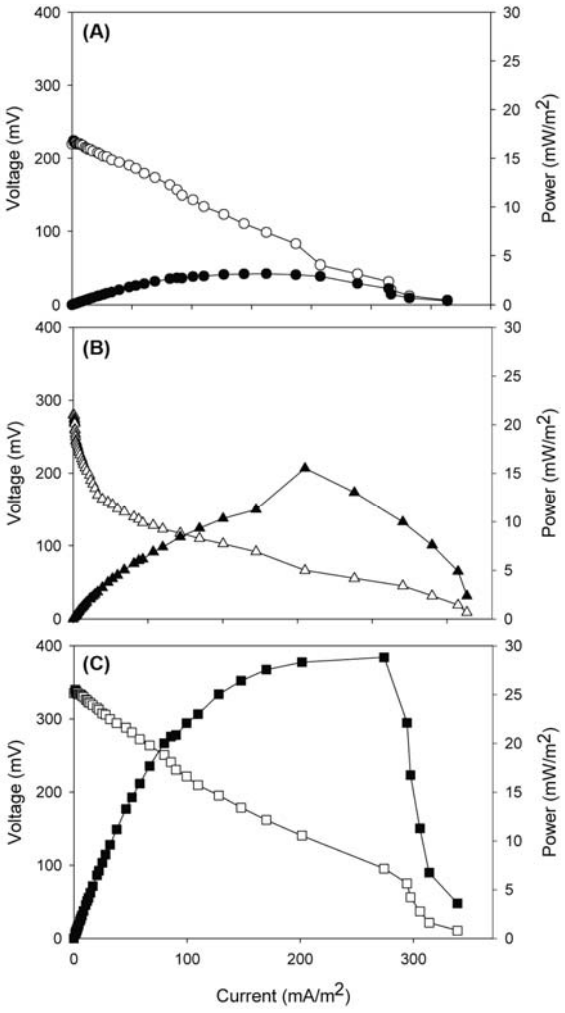

Fig. 1. Effect of mediator on polarization curve of the MFC with pure culture of *S. cerevisiae*. (A) Without thionine and aeration at the cathode. (B) Using 200 mmol/L thionine in the anode chamber and without aeration at the cathode. (C) 200 mmol/L thionine in the anode and aeration at the cathode. In all cases, graphite was used as the anode and cathode.

by exogeneous mediator, thionine.

The optimal thionine concentration in the fabricated H-type MFC was investigated using various concentrations of thionine (100 to 600 mM with an increment of 100 mM) analysed through polarization curves (Fig. 2). The maximum power and current were obtained at 500 mM of thionine concentration (60 mW/m^2 and 400 mA/m^2) although the optimized concentration is system specific value for two-chamber MFC tested. The further increase of thionine concentration (600 mM) results in similar current and power generation (Fig. 2). Kim *et al*. (2000) reported that voltage and coulombic efficiency increased when thionine (0.33 mM) was added in the MFC containing *Proteus vulgaris*. Thurston *et al*. (1985) also obtained an increased coulobmic yield of 50% and respiration rate in thionine-mediated biofuel cell using *P. vulgaris*. These results implicate that increased concentration of electron mediator can facilitate power production, but it is limited by capability of microbial exoelec-

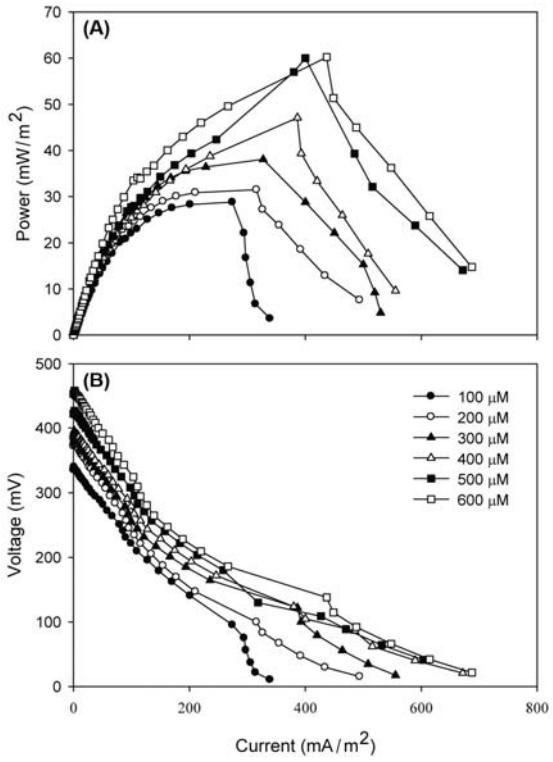

Fig. 2. Effect of different thionine concentrations (from 100 to 600 µM) in the anode chamber with pure culture of *S. cerevisiae*. (A) on power density and (B) cell potential in the MFC. Cathode electrode was aerated with air.

trogenesis and cell density. Further investigation for optimum concentration should be conducted in more advanced system.

The microbial electron transfer characteristics were analyzed with cyclic voltammetry (CV). Three different conditions (no microbe, microbe without thionine, and microbe with 500 µM thionine as mediators) were compared in anaerobic (Fig. 3A) and aerobic condition (Fig. 3B), respectively. Oxidation and reduction peaks were not observed before adding *S. cerevisiae* in the anode compartment. Inoculation of *S. cerevisiae* in the anode chamber also does not show redox capability greater than that of a control (no microbe). However, thionine significantly improves the oxidation (0.26 mA at -45 mV vs Ag/AgCl) and reduction peak (-0.61 mA at -70 mV). It should be noted that the CV analysis was conducted immediately after inoculation; therefore no biofilm was formed on the electrode. The difference between oxidation and reduction peaks from the result using microbe without thionine, was most probably due to the facilitation of electron transference between suspended microbe and electrode. The redox capability of *S. cerevisiae* decreases in aerobic condition as compared to anaerobic condition (Fig. 3B) as oxygen can oxidize a reduced mediator molecule or intercept microbial respiration directly. However, clear oxidation and reduction peaks are obtained with indicating that the electrochemical property by using thionine can be maintained even in aerobic condition.

In order to compare the electrochemical performance of *S. cerevisiae* with natural biofilm, an anode electrode with a developed mixed culture biofilm (after cultivation of 2 weeks in MFC reactor) was analyzed with CV, under the same conditions. Before formation of biofilm on anode surface, oxidation and reduction peaks were not observed in CV, as with the control in Fig. 3. However, after biofilm formation on the electrode, after two weeks, clear redox capability was observable in the current-potential curves (Fig. 4B), with typical two oxidation peaks (-0.199 mA at 3 mV and 0.435 mA at 48 mV). Similar CV results were reported by Kim *et al.* (2007b) using ethanol as the electron donors in an MFC. This result indicates that the redox reaction on the electrode with acclimated biofilm might be irreversible since the attached microbe should be continuously oxidized in a one-way process. SEM image shows that the *S. cerevisiae* attach to the carbon electrode surface (Fig. 4).

The addition of thionine to the acclimated anode electrode with a formed biofilm significantly changes the CV response in MFC, increasing the oxidation peak to 0.56 mA at 42 mV and appearance of a reduction peak (-0.64 mA at -105 mV) on the reverse scan (Fig. 5C). The larger difference of forward (oxidation) and reverse (reduction) scan shows that the practical capacitance of mediating system increased as compared to the non-biofilm result (Fig. 3A). The CV analyses imply that thionine can be captivated within the biofilm matrix on the anode electrode. This result also indicates

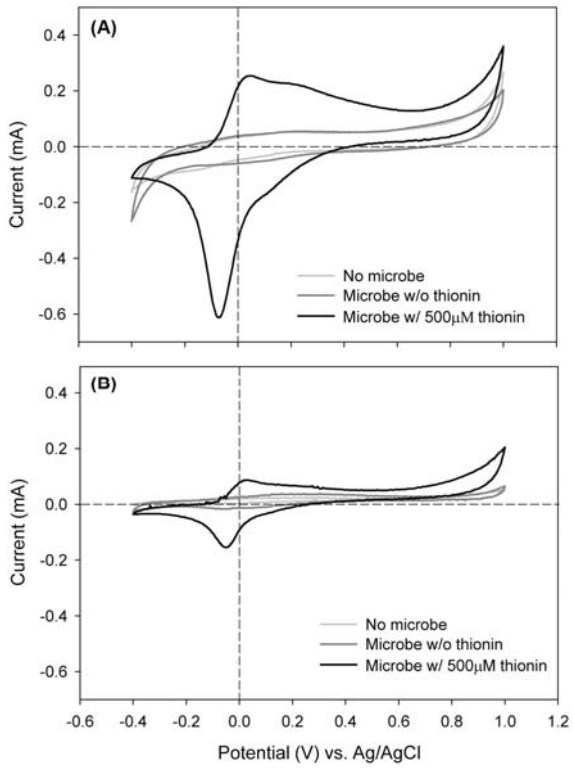

Fig. 3. Cyclic voltammograms of the anode with pure culture of *S. cerevisiae* under (A) anaerobic and (B) aerobic conditions in three different conditions (Without inoculums and no thionine, with inoculum no thionine, and with inoculum and 500 µM thionine).

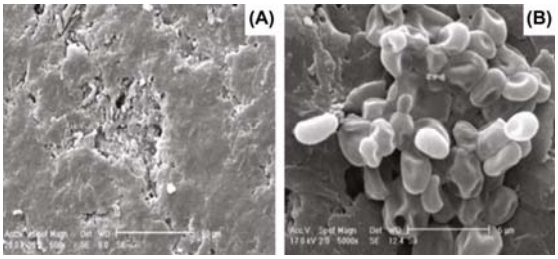

Fig. 4. SEM images from anode electrode surface, (A) before and (B) after using in MFC reactor inoculated with *S. cerevisiae*.

that thionine affects the electron transfer behavior in the biofilm, with increasing redox capability augmenting that of the suspended microbe.

The effect of thionine on power production in the operating MFC are more clearly presented in Fig. 6. The voltage generation with 1,000 Ohm external resistance was stabilized at 0.052 V when the system operated without electron mediators. The voltage was immediately increased to 0.095 V when thionine (500 mM) was dosed to anode chamber at 110 h.

The present research demonstrates that Gram-positive microorganism, *S. cerevisiae* can produce electricity in the MFC, but the power production was limited by a poor electron transfer rate. However, this can be overcome by using a proper mediator such as thionine at an appropriate concentration. To the author's knowledge, this study is the first investigation into the effects of thionine with *S. cerevisiae* on electrochemical performance in MFC. Also even for exoelectrogenic mixed culture enriched from swine wastewater, electron transfer rate increased by adding thionine. Thionine significantly increases redox capability of both suspended *S. cerevisiae* and an exoelectrogenic biofilm on the electrode. Aeration in the cathode can further improve the system performance when the optimum mediator concentration is used. When initial thionine concentration was 500 mM, the maximum power generation and current were 60 mW/m^2 and 436 mA/m^2, respectively. For the mixed culture MFC stable power at 1,000 Ohm was increased from 0.48 mW/m^2 to 1.6 mW/m^2 by adding thionine to the anode (Fig. 6). These results indicate that *S. cerevisiae*, Gram-positive microbe which has relatively rigid cell wall containing teichoic acids, can be used as active biocatalyst in MFC by using electron mediator. It is expected that further investigation for electron shuttle and its optimum concentration could expand electrochemically active species, thus increase applicability of Gram-positive microbes for MFC. It is clearly shown that CV results improve redox capability by adding thionine on both pure and mixed culture. We also found that electrogenic biofilm matrix can have advantage of holding of mediator molecule, therefore provide beneficial effect of operation in continuous and high throughput system.

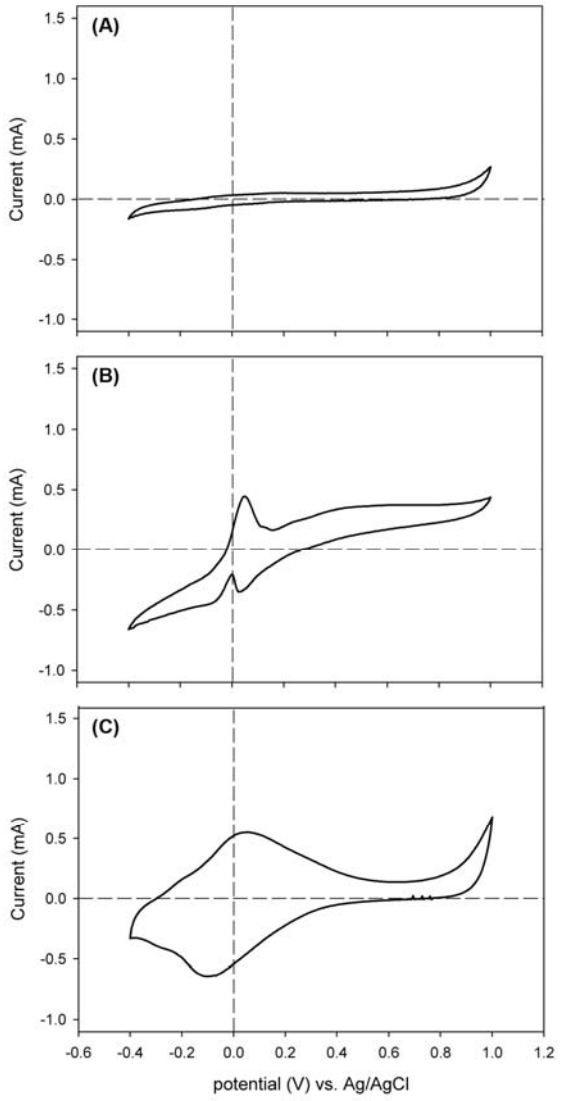

Fig. 5. Effect of electrochemically active biofilm of mixed culture on anode surface with CV analysis. (A) in the absence of biofilm (after addition of suspended mixed culture to the anode chamber), (B) after formation of biofilm without mediators and (C) after formation of biofilm with 500 μM thionine as electron mediators. Scan rate was 0.01 V/S.

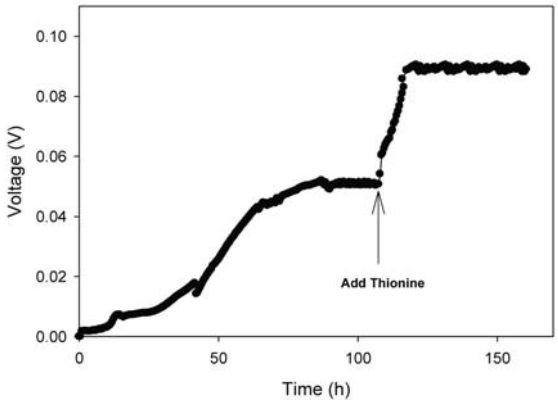

Fig. 6. Effect of thionine on MFC power generation. External load R_{ext}= 1000 Ω. Thionine concentration added to the anode chamber was 500 μM. The MFC was inoculated with 10 ml of anode medium of a working MFC originally inoculated with swine wastewater.

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