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Solid Electron Acceptor Effect on Biocatalyst Activity in Treating Azo dye Based Wastewater

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Abstract

Functional activity of anaerobic bacteria in concomitance with electrode as solid electron acceptor was evaluated during degradation of azo dye based wastewater. The experiments were performed using three different setups viz., bio-electrochemical treatment (BET; with electrode assembly and anaerobic biocatalyst), anaerobic treatment (AnT; with anaerobic biocatalyst and absence of electrode assembly) and abiotic reactor (control; with electrode assembly and absence of anaerobic biocatalyst) with azo dye wastewater of 50 mg/l dye concentration. Maximum dye removal was observed in BET (69.9%) followed by AnT (42%) and Control (2.4%). The bioelectrogenic performance was also observed to be higher in BET (92.1 mW/m^2) in comparison to abiotic-control (0.41 mW/m^2) which is attributed to the significant influence of bacteria as biocatalyst in concurrence with the electrode serving as solid electron acceptor in BET. The study also documented electron acceptor dependent respiration, exemplifying the influence of conjunction between electrodes and bacteria on dye degradation. Two possible electron transfer mechanisms viz., direct electron transfer (DET) through the membrane bound cytochromes to the solid electron acceptor and mediated electron transfer (MET) through the reduced dye intermediates as electron shuttles respectively were observed during BET operation. However, AnT and abiotic control operation resulted in less/no dye breakdown respectively due to the lack of conjunction between biocatalyst and electrode. The study provides a new insight into the electron acceptor dependent respiration where the electrode serving as solid electron acceptor enables the efficiency of anode respiring bacteria (ARB) in terms of electron flux towards dye degradation and electrogenesis.

Keywords: Bioelectricity; Electron flux; Redox mediators; Azo reductase; Dehydrogenase activity; Microbial Fuel Cell

1. Introduction

Azo compounds are characterized by its possession of one or more azo chemical moieties (-N=N-) and their linkages in a chemical compound could be flanked by alkyl or aryl groups. Presence of sulfo and azo groups in the dye structure, protects the dye molecule from attack of oxygenases making them resistant for oxidative biodegradation .[1] Hence these compounds are more difficult to degrade through conventional aerobic treatment process. [1-3] Azo dye molecule requires sequential redox conditions to cleave the chromophore (anaerobic) into corresponding aromatic amines and further degradation (aerobic) of toxic aromatics into simpler compounds.[4-6] The process of anaerobic degradation supports reductive breakdown of dye molecule by cleaving the chromophore into corresponding colourless aromatic amines.[7] This breakdown is supported by the utilization of redox powers obtained by oxidation of co-substrate in the surrounding microenvironment. Previous studies by Sreelatha et al. (2015) evaluated the dye based wastewater treatment in anaerobic systems and achieved considerable treatment efficiency.[7] Although anaerobic treatment has the potential to treat azo dyes to some extent, it cannot be used as standalone process and may be used in concurrence with current technologies to achieve better treatment.

Bioelectrochemical systems (BES) are typically a type of Microbial fuel cell (MFC), a promising technology for electricity production which can be advantageously combined with applications in wastewater treatment.[8, 9] When the major attention is on treatment rather than electricity production, BES can also be called as bioelectrochemical treatment systems (BET).[10, 11] BET use electrodes as solid electron acceptor for bacteria respiration and exploit microbial catabolic activities to generate electrons (e^-) and protons (H^+) by degrading organic molecules. The microbial metabolism is linked via electron donating and accepting conditions through the presence of artificially introduced electrodes (anode and cathode) that induces the development of potential difference which acts as a net driving force for bioelectrogenic activity and complex pollutant removal.[12, 13] BET has the potential to overcome the above limitations by converting chemical energy to electrical energy through cascade of redox reactions with simultaneous waste remediation.

BET performance is governed by various parameters that regulate the performance viz., physical, physico-chemical, chemical, biological, electrochemical, etc. One of the important factors is the biological factor where the microbes/bacteria acts as biocatalyst that will undergo diverse biochemical pathways and acquire various electron transfer mechanisms influencing the electron transfer rate and bioelectricity generation.[11] Bacteria as biocatalyst

will discharge reducing equivalents upon substrate degradation and will aid in the pollutant/wastewater degradation.

Besides, the important physical factor to be considered in BET operation is the presence of electrode assembly functioning as anode and cathode respectively. The electrode assembly in BET system acts as a solid electron acceptor and enables the bacterial respiration on its surface. Anode respiring bacteria (ARB) will respire on the electrode surface enabling electron transfer effectively towards dye/wastewater degradation. BET has the advantage of coupling both electrochemical and anaerobic biological processes which triggers the redox reactions for the degradation of complex pollutants with simultaneous power generation.[9]

Considering the specific functions of biological and physical factors of BET system, present study is designed to evaluate the influence of bacteria as biocatalyst and electrode assembly as solid electron acceptor for treatment of azo dye compounds. The experiment was evaluated using three different experimental operations principally with BET system comprising of both bacteria and electrode assembly in comparison to anaerobic treatment (AnT) and abiotic-control comprising of electrode assembly without biocatalyst. The specific influence of electrode assembly as a solid electron acceptor and the variation in biocatalyst behaviour with respect to electrode assembly was evaluated using mixed consortia as biocatalyst and non-catalyzed graphite electrodes as anode and cathode in BET system. All the three systems were simultaneously operated to assess the relative performance in conjunction with biocatalyst and solid electron acceptor during azo dye reduction.

2. Materials and Methods

2.1 Biocatalyst

An indigenous mixed anaerobic sludge acquired from effluent treatment plant (ETP-Hyderabad) was used as biocatalyst for the experimentation. The inoculum, prior to experimentation was washed twice with phosphate buffer saline and re-suspended in DSW (COD: 3 g/l (without dye) overnight at ambient room temperature. The grown culture was inoculated in to respective bioreactors by re-suspending through feed (VSS, 4660 mg/l) to facilitate the initial adaptation and stabilization of microorganisms in the respective bioreactors.

2.2 Experimental Methodology

The present study evaluates the influence of biocatalyst to decolorize azo dye based wastewater using three different experimental setups viz., bioelectrochemical treatment

(BET; electrode assembly and biocatalyst), anaerobic treatment (AnT; without electrode assembly and with biocatalyst) and Control (abiotic; with electrode assembly and without biocatalyst). AnT configuration described in the present study is adopted from Sreelatha et al. 2015. BET and control systems were operated using non-catalyzed graphite plates as electrodes (5 x 5 cm; 10 mm thick; 70 cm^2 surface area) in a single chamber open air cathode system. All the three reactors have a total/working volume of 0.50/0.45 l and were operated in fed-batch mode for 15 cycles (720 h) with a hydraulic retention time (HRT) of 48 h for each cycle. Comparison was made between BET and AnT to understand the influence of electrode assembly in conjunction with biocatalyst during the operation.

C.I. Acid Black 10B [(4-amino-5-hydroxy-3-[(4-nitrophenyl) azo]-6-(phenyl azo)-2,7-naphthalene disulfonic acid disodium salt ($C_{22}H_{14}N_6O_9S_2Na_2$), an azo dye belonging to acid applications class was used as a test dye. The simulated dye wastewater (SDW) was prepared by dissolving 50 mg/l of dye in designed synthetic wastewater [DSW (g/l): glucose-3.0, NH_4Cl-0.5, KH_2PO_4-0.25, K_2HPO_4-0.25, $MgCl_2$-0.3, $CoCl_2$-0.025, $FeCl_3$- 0.025, $ZnCl_2$-0.0115, $NiSO_4$-0.050, $CuCl_2$-0.0105, $CaCl_2$-0.005 and $MnCl_2$-0.015].[10] Prior to feeding, the pH of the SDW was adjusted to 7.1 ± 0.1 using 1N HCl/1 N NaOH.

2.3 Enzymes activity

2.3.1 Azo Reductase activity

Azo reductase activity was monitored to understand the reductive cleavage of azo bond in dye molecule. Colorimetric method was employed to estimate the extracellular azo reductase enzyme activity using co-substrate NADH [14.] To estimate enzyme activity, the reaction mixture was prepared with 200 µl sample, 400 µl of potassium phosphate buffer and 200 µl of C.I. Acid Black 10 B. The reaction was initiated by adding 200 µl of NADH (7 mg/ml) and absorbance was monitored at 618 nm (Venkata Mohan et al., 2012, 2013a). The linear decrease of absorption was used to calculate the azo reductase activity. One unit of azo reductase can be defined as the amount of enzyme required to decolorize 1 µmol of dye per minute. Well plate detection assay was performed by dissolving agar-agar (2%) in 100 ml milli-q water along with 50 mg of azo dye followed by boiling at temperature (60º C). After solidification, small and equal sized holes were made in the petri dish. First well was loaded with 80 µl of partially purified protein sample and 20 µl of NADH (7 mg/ml). The second well was loaded with crude protein extract (80 µl) followed by NADH. Well loaded only with milli-q water and NADH served as control.[14]

2.3.2 Dehydrogenase activity

Dehydrogenase (DH) activity (substrate linked) of the anaerobic biocatalyst was estimated using redox sensitive 2,3,5-triphenyltetrazolium chloride (TTC) based on the reduction to insoluble formazan[15.] DH was analyzed by adding 5 ml of TTC (5 g/l) and 2 ml of glucose solution (0.1 mol/l) to 5 ml of the bacterial culture and the resulting solution was stirred continuously (20 min; 200 rpm) followed by incubation (37 _C; 12 h). Subsequently, 1 ml of concentrated sulphuric acid was added to the reaction mixture to stop the deoxidization followed by addition of 5 ml of toluene to extract triphenyl formazan (TF) formed in the reaction mixture. The sample was agitated at 200 rpm (30 min). After keeping the reaction mixture idle for 3 min, the sample was centrifuged at 4000 rpm (5 min) and the supernatant was collected and the absorbance was measured at 492 nm using spectrophotometer (TF forms a colored complex with toluene).

2.4 Analysis

The electrogenic performance of BET and abiotic-control systems having electrode assembly was evaluated in terms of open circuit voltage (OCV) and current generation patterns. The electrochemical behavior was assessed by performing polarization with the function of current density against potential and power density measured at different resistances (30–0.05 kΩ). Anode potentials were also measured at variable external resistances to find the sustainable power generation. Samples were analyzed for the change in dye and COD concentrations and enzymes activities. Dye concentration was monitored colorimetrically at λ_{max} 618 nm using UV-Vis spectrophotometer. Reactor performance was assessed by monitoring COD.[8] Cyclic voltammetry technique was employed to observe redox variations in bio-electrochemical behaviour of the biocatalyst under three different experimental setups using potentiostat-galvanostat system. A potential ramp of +0.5 to −0.5 V was applied at a scan rate of 30mV/s to record the voltammograms. All the electrochemical assays viz., derivative cyclic Voltammogram (DCV), Tafel plots, slopes (β_a and β_c) and polarization resistance (R_p) were deduced from the Voltammetric studies.[16] All the analyses were performed in triplicates and the mean values were plotted in the graphs.

3. Results and Discussions

3.1 Colour Removal

Initially, all the three bioreactors (BET, AnT and control) were optimized with DSW at an organic loading rate (OLR) of 1.36 kg COD/m^3-day (without dye) for 10 cycles (HRT, 48 h) to facilitate the biomass growth. After stabilization with respect to substrate removal, the bioreactors were subsequently fed with SDW (azo dye load, 50 mg /l) and operated continuously for 15 cycles. Azo dye removal efficiency was evaluated for all the three reactors, wherein BET documented decolourization of 52% followed by AnT (32%) and control (3%) during initial period of operation (3^{rd} cycle) (Fig.1). With increase in number of cycles, the decolourization efficiency was observed to increase gradually (BET and AnT) and maximum performance was observed at 15^{th} cycle of operation which is attributed to the acclimatization of biocatalyst. BET documented higher colour removal efficiency (70 %) accounting to a dye removal of 35 mg dye/l, which is relatively higher than the AnT (42%; 21 mg dye/l removal) and control (2.44%; 1.22 mg dye/l removal) operations.

Fig. 1

3.1.1 UV-Vis Spectral Scan

Decolourization pattern of azo dye was observed under static condition with multi-scan spectrum analysis (200-800 nm) using UV-visible spectroscopy at regular time intervals for all the three reactors (Fig. 2a). Two peaks were observed in UV region near the spectral ranges of 500 to 700 nm and 320 to 360 nm. The intense peak at 618 nm corresponding to chromophore (-N=N-) and peak at 340 nm is associated with the presence of oxidized aromatics such as phenolic and naphthoquinone compounds.[14,15] The peaks at 340 nm increased with operation time indicating the accumulation of amines under reductive microenvironment which was observed to be similar in all the three reactors. Among three reactors, UV-Vis spectrum of BET reactor at various time intervals clearly portrayed the decrement in peak height at 618 nm depicting the degradation of dye into corresponding intermediates.[4] Less absorbance was observed at 48 h, illustrating the reduction of chromophores by the electrons liberated in the reactor. The self-induced bio-potential developed in BET system was able to cleave the azo dye compounds towards enhanced dye degradation. Subsequently, AnT also showed decrement in the peak height indicating effective reductive behaviour in anaerobic conditions, which might have resulted due to the

increase in the amount of aromatic amines.[7] The shift in wave length in the UV-scan indicates the biochemical interface of dye which is consistent with azo bond cleavage and transformational changes to the aromatic structure. In control operation, the decrement in peak height was not observed indicating that the chromophores were not cleaved due to the absence of biocatalyst. In BET operation, the peaks corresponding to 6h and 12h at 340nm increased with time depicting the accumulation of aromatic amines and decreased at 24h to 48h depicting the degradation of aromatic amines to lower molecular weight aliphatic hydrocarbons. BET can be considered more favourable than AnT for the treatment of azo based wastewater due to the development of high density electroactive species on the anode with faster electron transfer rate which might be used for the electrochemical substitution and electrolytic dissociation of complex compounds.

The disparity in treatment efficiency is attributed to the capability of biocatalyst to undergo metabolic shift with regard to the experimental conditions. The presence of electrode assembly as well influenced the performance in BET compared to the AnT system. In the control operation, removal efficiency was almost negligible and the dye concentration remained almost similar until the end of operation. In AnT system, azo dye is degraded by bacteria through reductive enzymatic cleavage of azo bond which acts as an electron acceptor under anaerobic condition.[7] However, in BET system, the presence of solid electron acceptor influenced the effective degradation of the complex dye molecules faster than the conventional anaerobic treatment process due to the enrichment of electrochemically active bacteria (EAB).

Fig. 2a

3.2 Substrate Degradation

Initially, the bioreactors were fed with DSW (without dye) at an OLR of 1.36 kg COD/m^3-day and operated for 6 cycles with a HRT of 48 h. Subsequently, the reactor was fed with synthetic azo dye bearing wastewater (50 mg dye/l) and operated for 15 cycles. Higher substrate degradation was observed in BET (85%; SDR: 1.15 kg COD/m^3-day) followed by AnT (75%; 0.78 kg COD/m^3- day) and Control (2.9%; 0.04 kg COD/m^3- day) without dye addition, which indicates the rapid metabolic capabilities of bacteria in utilizing glucose as co-substrate and abiotic-control resulted in negligible performance (Fig. 2b). After subsequent dye addition, lower substrate degradation was observed in both BET (3^{rd} cycle: 55%) and AnT reactors (3^{rd} cycle: 35%) and observed to increase with increase in cycle

operation which is attributed to the non adaptability of bacteria to dye environment. However, increased COD removal efficiency with additional cycles is due to the adaptable nature of biocatalyst in utilizing dye molecule rapidly. Maximum substrate removal was observed at 15th cycle in BET (79.5%) followed by AnT (48%) and Control (2.33%). Carbon sources have an important influence on dye degradation because azo dyes are deficient in carbon and require co-substrate for the breakage of the azo bond, energy for the growth, survival of the microorganisms and as electron donors.[17]

The requirement of simple carbon source is inevitable for dye degradation.[10] Apart from the sole substrates of recalcitrant dyes in anode chamber, co-metabolism using simple substrate (glucose) provides a good candidate for bioremediation of these recalcitrant azo compounds.[18-20] The mechanism of co-metabolism involves the liberation of reducing powers which can be utilized for the degradation of dye molecules as terminal electron acceptors with the help of *in situ* mediators produced during the process. The dye molecules and its reduced intermediates also act as mediators during the operation and enhance the electricity generation and substrate removal in BET system. The substrate removal is expressed in terms of SDR and the removal efficiency varies with respect to reactor operation in the experiment. The substrate degradation rate (SDR) was observed to be higher in BET (1.0789 kg COD/m^3-day) followed by AnT (0.653 kg COD/m^3-day) and Control (0.032 kg COD/m^3-day). It is clear evidence that BET operation depicted 1.7 folds higher substrate degradation efficiency than AnT due to the advantage of self-induced bio-electro catalytic microenvironment and enabled electron acceptor dependent respiration. The anodic electron flux reactions might have also played a role in enhancing the substrate degradation in BET operation.[21] The use of biocatalyst, presence of *in situ* mediators and the type of metabolism (co-metabolism) may potentially affect the degradation pathways of azo dye substances.[18,22]

Fig. 2b

3.3 Enzymes Activity

3.3.1Azo reductase Activity

The cleavage of azo bonds catalyzed by azo reductase enzyme with the aid of an electron donor (NADH and/or NADPH) was monitored with respect to time (BET, AnT and control reactors) (Fig.3a). At initial cycles of operation, lower azo reductase activity was observed for all the three reactors and gradual improvement in the enzyme activity was observed during the due course of operation. Maximum enzyme activity was observed at 15th cycle of

operation in BET (26.2 ± 1.2 U) followed by AnT (18.9 ± 0.9 U) and abiotic control (0.15 ± 0.05 U). Azo reductase enzyme plays a major role to cleave -N=N- bond of azo dyes into colorless metabolites analogous to the aromatic amines. The azo reductase enzyme consumes NADH and/or NADPH as an electron donor and dye as an artificial electron acceptor to cleave the bond. The conversion of NADH to NAD liberates the reducing powers that are used for the reduction of azo dyes to aromatic amines via hydrazines formation.[23] The dye degradation evidenced in this study is attributed through two main routes viz., symmetrical and asymmetrical cleavage of azo bond, where the typical microbial metabolism with respect to the available carbon source (glucose and dye) might have contributed for the generation of reducing powers.[24,25] In AnT system, presence of redox mediators such as NAD/NADH accelerate the decolorization rate of azo dyes by shuttling electrons between biological oxidation of primary electron donor to electron acceptor such as azo dye.[26] Besides, NAD/NADH in BET system, the presence of electrode assembly develops the self driven redox mediators to enhance the dye degradation. The dye removal pattern correlates well with the azo reductase enzyme indicating a major role of enzyme playing in the cleavage of azo compounds. Stable enzyme activity with each feed event in both the bioreactors supports the robustness of systems in dye reduction.[4, 10]

Fig. 3a

3.3.2 Dehydrogenase Activity

Dehydrogenase (DH) belongs to oxido-reductase group of enzymes catalyzing the redox reactions for the inter-conversion of metabolites and also helps in shuttling the protons (H^+) between metabolites with the help of electron carriers (NAD^+, FAD^+, etc).[27] They play a crucial role in dye degradation to its intermediates by catalyzing the proton transfer from the substrate or intermediates to the dye molecules. Higher DH activity was observed with BET operation (2.6 μg/ml), followed by AnT (1.4 μg/ml) and control (0.07 μg/ml) (Fig.3b). DH enzyme activity represents metabolic activities of the microorganism and can be considered as a good measure of microbial oxidative activities.[28] BET showed relatively higher enzyme activity when compared to other operations because the electroactive consortia developed in BET is robust to oxidize complex substrates indicating higher metabolic activity of self-immobilized bacteria on the working electrode. It can also be hypothesized that high DH activity in the BET reactor was due to close proximity of electron donors and acceptors thus facilitating rapid electron transfer compared to AnT operation. Proton shuttling between the

intermediates during metabolic activity might also be involved in the dye reduction. It is clear that the increased enzyme activities are proportional to dye degradation and electrogenic activity which might have stimulated the metabolic and enzyme activities of the biocatalyst robust to the subjected microenvironment.

Fig. 3b

3.4 Self Induced Electrogenesis

BET depicted biogenic electricity production along with concomitant azo dye removal. Initially the reactors were fed with DSW (with no dye addition) at an OLR of 1.36 kg COD/m^3-day and operated for 10 cycles with a HRT of 48 h. Maximum OCV of 365 mV and PD of 100 mW/m^2 were observed at 10^{th} cycle in BET system, while the control reactor documented OCV of 58 mV (abiotic) (Fig.4a). The results clearly indicate the influence of biocatalyst in BET system, which facilitated higher biomass growth, documenting higher bioelectrogenic performance than control. The reactors were further fed with synthetic azo dye bearing wastewater (SDW-50 mg/l) and operated continuously accounting for total time of 720 h with an OLR of 1.36 kg COD/m^3-day. Both the systems showed a drop in performance during initial cycles (Cycle 1-BET-OCV: 130 mV and PD: 12 mW/m^2; Control: OCV:20 mV and PD: 0.12 mW/m^2) which might be due to the inhibitory effect of toxic dye. With increase in cycles, a gradual increment in performance was observed in BET system. During 8^{th} cycle, BET documented OCV of 200 mV and PD of 40 mW/m^2 and control reactor documented OCV of 48 mV (0.41 mW/m^2). The performance of control reactor was almost similar to that of without dye operation, since reducing equivalents that degrade dye molecules are not liberated due to the absence of biocatalyst. However, the electrogenesis observed in the control operation might be because of the potential produced by electrode assembly. The reactors were operated continuously for 15 cycles and a stabilized performance was observed from 12th cycle and depicted maximum electrogenesis. In BET system, a stable voltage of 350 mV and PD of 85 mW/m^2 was observed at 15 cycle of operation indicating the acclimatization of the biocatalyst. The organic and inorganic substrates present in the azo dye based wastewaters were oxidized by the bacteria producing excess of electrons (e^-) and protons (H^+) in the anode chamber. The reducing equivalents generated in the system were partially used to harness electricity and partially used to cleave the azo bonds of the dye that acts as alternate electron acceptor (dye molecules) present in wastewater which favoured the breakage of chromophores in the anode chamber. Besides the

benefits of power generation, the reduction of azo dyes is accomplished through bioelectrochemical reduction without the application of external power source.[16, 29] On the contrary, abiotic-control depicted no increase in power generation due to the absence of biocatalyst (OCV- 50 mV; PD - 0.5 mW/m^2).

Fig. 4a

3.4.1 Anode potential

Variations in anode potential against external resistance (30kΩ to 50Ω) were recorded against saturated Ag/AgCl (S) electrode during both BET and control operations (Fig.4b). Maximum open circuit anodic potential was -350 mV for BET system and -50 mV for control system with the absence of resistor. Higher anode potential indicates transfer of more energy for microbial growth and cell maintenance due to the availability of substrate in BET system.[30] Although substrate availability was same in the control system the degradation of substrate was not observed due to the absence of biocatalyst. Operation for longer period (15 cycles) in BET system favours effective growth of electrochemically active bacteria on the anodic electrode. The formation of biofilm on the electrode and the absence of membrane reduce internal losses documenting higher anode potential in BET system. In BET system, anode potential varied between -312 to -175 mV with varying resistor of 30 and 0.05 kΩ. The potential was observed to drop from 5 kΩ in BET reactor and 0.5 kΩ in control reactor, suggesting the possibility of effective electron discharge at the respective resistors. The electrochemically active consortia will have higher membrane potential, which helps in delivering electrons against the anode potential. The induced electrochemical oxidation during electrogenesis as well as the simultaneous bio-electrochemical reactions might have helped in transferring the electrons to dye molecules, resulting in the decolorization of wastewater.

3.4.2 Cell electromotive force

Polarization behaviour of the electrodes was observed by varying the resistances (30 to 0.05 kΩ) at 15th cycle of operation for BET and control reactors (Fig.4b). In BET operation, the potential at 30 kΩ was 380 mV and observed to decrease with decrease in resistance (0.05 kΩ - 192 mV). The voltage profile indicates that, until 5 kΩ the flow in potential was restricted and observed to drop gradually until 1 kΩ and dropped drastically from 0.5 kΩ. Correspondingly, current density (450 mW/m^2) increased with decrease in resistors indicting

the flow of electrons. The self-immobilized biofilm developed on the anode surface of the BET system showed marked influence on both bioelectricity production and substrate degradation efficiency. In the case of abiotic control operation, the performance was very low due to the absence of biocatalyst. Generally, the microbes that acts as the biocatalyst have high electron discharge capability and are considered to be electrochemically active and are crucial in the BET operation. Maximum power density of 80 mW/m^2 was observed at cell design point (CDP) of 100 Ω with corresponding current density of 381 mA/m^2 in BET performance.

Fig. 4b

3.5 Bioelectrochemical Behaviour

3.5.1 Cyclic Voltammetry

The bioelectrochemical redox transition coupled to catalytic oxidation and reduction behaviour was analyzed for all the three reactors for every 6 hr interval (Fig. 5a). Maximum redox catalytic currents were observed in BET system (oxidative current, OC: 39.6 mA and reductive current, RC:-19.5 mA) followed by AnT (OC: 6.2 ± 0.2 mA; RC: -7.1 ± 0.15 mA) and control (OC: 0.326 ± 0.1 mA; RC: -0.586 ± 0.2 mA). OC was higher than RC in BET system indicating that the redox behaviour is more favourable towards oxidation due to the development of electrogenic microenvironment that develops a continuous stress on the selectively enriched electrochemically active consortia facilitating on the anode surface by mediating higher electron transfer rate.[29] However, BET operation resulted higher redox currents than AnT and control operation due to the effective contact of biofilm for efficient electron transfer, minimization of mass transfer losses as well as for the involvement of dye molecule itself as a mediator. In AnT system, the increment in reduction current is ascribed to the increased reduction reactions towards the breakdown of dye into reduced intermediates and its persistent accumulation.[7] Control operation showed a very less catalytic current due to the absence of biocatalyst. The redox catalytic currents observed in BET system was well correlated with the higher COD and dye removal, enzyme activities during the reactor operation.

Voltammogram also depicts the redox mediators involved to alleviate the shuttling reactions between the electron donor and acceptor. When the redox potential of a mediator equals the applied potential, the peaks were deducted indicating the presence of mediators for electron

transfer.[31, 32] Distinct peaks were observed in BET operation, with respect to time different interval. The peak potentials detected are -0.164 V (6 h), 0.32 V and 0.258 V (12 h), 0.27 V (24 h), -0.235 V (36 h) and 0.431 V (48 h) which might correspond to the bacterial membrane bound proteins viz., Fe-S proteins, NAD/NADH, Cytochrome-bc1, Cytochrome-C, Flavoproteins and NO_3/NO_2 respectively.[31] The presence of electrode assembly in BET system enables the enrichment of ARB on electrode surface that transfer electrons via the observed membrane bound proteins. The cytochrome-C complex present in almost all the bacterial species acts as an electron carrier which helps in the efficient direct electron transfer and the NO_3/NO_2 detected is the dye intermediate formed at the end of cycle operation depicting the breakdown of dyes into simple non toxic compounds. The electrode assembly is the integral part of the BET system that acts as a solid electron acceptor. The involvement of these mediators and membrane bound proteins is in correlation to the higher redox currents attributing its role in dynamic electron flux in BET operation.

3.5.2 Derivative Cyclic Voltammetry (DCV)

The derivative Voltammogram represents the rate of change of voltammetric current with respect to time and electrode potential E (di/dt). The numbers of peaks were comparatively higher in DCV than the corresponding CV analysis. The derivative of CV helps to find the EET site of the redox mediator involved in the process in the form of a peak in both BET and AnT operations (Fig.5b). However, numbers of peaks were comparatively higher in BET system than the corresponding AnT operation. In BET system, two reversible peaks were detected at -0.165 V and 0.188 V corresponding to the involvement of Cytochrome-bC1 and Fe-S proteins respectively. In addition, five quasi reversible peaks were detected at -0.188 V, 0.035 V, -0.141 V, 0.023 V and -0.094 V which correspond to the involvement of Fe-S proteins, Quinones and Cytochrome bC1 respectively. In the case of AnT operation, six quasi reversible peaks were detected, each with a potential of 0.141 V, 0.094 V, -0.129 V, 0.177 V, 0.011 V and -0.117 V corresponding to the involvement of Cytochrome-bC1 and Fe-S proteins respectively. All the redox mediators detected during DCV analysis are the membrane bound proteins of bacteria that act as electron carriers during the process. However, detection of quinones as redox shuttlers in BET system implies the direct evidence of dye degradation mechanism which forms the aromatic derivatives. Quinones are the secondary intermediates formed during the degradation of aromatic compounds such as dye molecules which serves as electron carriers/ acceptors during the process. This additional advantage of quinone as redox mediator in BET system proves the effective dye degradation

421 compared to other two reactors. On the contrary, control operation did not show any peaks
422 which might be attributed to the absence of biocatalyst activity.

423 **Fig. 5**

424 **3.5.3 Charge**

425 Charge distribution was observed to vary during the anodic oxidation and cathodic reduction
426 with the function of biocatalyst and electrode assembly. Charge distribution obtained from
427 the voltammetric profiles was observed to be higher for BET than AnT and control operations
428 (Sfig1). At initial period of operation (0 h) the charge was observed to be 0.64 C which
429 increased with time (6 h; 0.89 C) and gradually decreased to 0.804 C until 36^{th} h and random
430 drop was observed at 48^{th} h (0.671C). This depicts the rapid oxidation of substrate and co-
431 substrate at the initial hours of cycle operation and hence the electron availability on the
432 working electrode is higher which yields the elevated power in BET operation. In case of
433 AnT operation, initial operation (0 h) documented 0.167 C which changed marginally with
434 respect to cycle operation (6 h, 0.173 C; 12 h, 0.187 C; 24 h, 0.176 C) with a relative rapid
435 drop at 36 h (0.158 C). The absence of electrode assembly in AnT system indicates the non-
436 adaptation of electrochemically active biomass in the reactor resulting in low performance
437 than BET system. In the control operation, negligible charge of $\sim$0.003 C was observed due
438 to the absence of biocatalyst as well as electrode assembly. Almost eight fold higher charge
439 distribution was observed with BET when compared to AnT which might be attributed to the
440 variation in the relevant energy levels of the biocatalyst, participation of the soluble redox
441 species and electron transfer efficiencies. Charge separation can also be expressed in terms of
442 capacitance with respect to the voltage applied. Higher capacitance was observed with BET
443 (1.76 F; 6 h) followed by AnT (0.37 F; 12 h) and Control operation (0.03 F; 48 h) which
444 correlates with the distribution of charge, indicating that the electron holding capability of
445 biocatalyst was higher with the conjunction of biocatalyst and electrode assembly in BET.
446 As charge is directly proportional to capacitance, increase in charge also concurrently
447 increases the capacitance that indicates higher availability of electrons due to the inter-
448 conversion of metabolites at higher substrate load. The energy (J) generated and number of
449 electrons (n) during potential sweep also followed a similar pattern as charge and capacitance
450 with respect to time intervals. The energy stored and number of electrons is higher with BET
451 operation at 6 h (0.45 J; 5.6 x 10^{18}) compared to AnT (12 h, 0.094 J; 1.179.18 $\times 10^{18}$). This
452 higher energy stored and number of electrons generated depicts the optimization of
453 bioelectrogenic biomass for the higher electrogenic activity and dye removal in BET than

AnT depicting the robustness of bioelectrogenic bacteria in dye removal and electricity generation in BET system.

SFig. 1

3.5.4 Tafel Slopes

Tafel plots provide a visual understanding of the electron losses present in the system and help to interpret the biocatalytic activity based on the derived kinetic parameters viz., oxidative Tafel slope (β_a), reductive Tafel slope (β_c) and polarization resistance (R_p) (Fig.6). Tafel analysis depicted a marked variation in all the three systems viz., BET, AnT and abiotic control operations during oxidation and reduction reactions.

Reduction slopes (β_c) were comparatively lower than the oxidation slopes (β_a) in all the three systems which indicate the feasibility of effective neutralization/reduction of dye molecules in the system. In BET operation, the reductive slope of 0.165 V/dec (0 h) decreased to 0.1 V/dec till 24 h and slightly increased to 0.12 V/dec and 0.14 V/dec at 36 and 48 h respectively. The decrement in reduction slope till 24 h depicts the scope of reduction reactions in the bioreactor and further enhances the breakage of chromophore groups, whereas the oxidative slope of 0.53 V/dec (0 h) decreased slightly to 0.5 V/dec (12 h) and again showed slight increment till the end of cycle operation and visualized 0.62 V/dec (48 h). This is attributed to the influence of electrode assembly coupled with biocatalyst activity that induces the bioelectrogenic micro environment in the system to carry out the oxidation reactions at a higher rate. Degradation of dye molecule was found to be higher in BET system which is in accordance with the observed low oxidation slope and COD removal. Higher oxidation indicates the liberation of more number of redox equivalents to perform effective dye degradation process. Degradation of dye molecule leads to the liberation of aromatic amines (intermediates) which further acts as redox shuttles in the electron transfer. But in the case of AnT operation, the reduction slopes were little lower when compared to BET operation depicting the higher reduction activity of dye molecules. The reduction slopes which were little lower till 6 h (0.6 V/dec) showed nominal increment till 36 h (0.1 V/dec) and visible shift was observed at 48 h (0.18 V/dec) depicting the dye reduction was higher during the initial hours of cycle operation in AnT, whereas the oxidative slopes showed nominal decrement from 1.12 V/dec (0 h) to 0.7 V/dec (48 h) till the end of cycle operation with respect to the time intervals illustrating the utilization of carbon and dye as electron donors until the end of cycle operation. Abiotic-control illustrated higher redox slopes as there is no biocatalyst to perform redox reactions.

Polarization resistance (R_p), derived from Tafel analysis, refers to the resistance offered towards electron transfer from the biocatalyst to the electrode. R_p was found to be lesser in BET operation when compared to AnT and abiotic-control. Highest resistance was offered by control operation (7000Ω; 0 h) followed by AnT (97Ω; 48 h) and BET (30 Ω; 0 h). This lower resistance in BET could be the probable reason for higher dye degradation and more enzymatic activity. Low resistance present in the BET and AnT systems minimizes losses and provides effective electron transfer between substrate and the dye molecule and thus enhances the dye removal.

Fig. 6

3.6 Electron Acceptor-Dependent Respiration

In BET, electron acceptors are of crucial importance as the terminal reduction reactions are dependent upon the availability of terminal electron acceptors for the degradation of wastewater/pollutants. The electron acceptors respired by bacteria often have solid and soluble forms that typically coexist in the bioreactor environment.[33] In the present study, BET system functioned with coupled action of electrode assembly as solid electron acceptor as well as bacteria as biocatalyst documented significant dye remediation with simultaneous power generation in comparison to the corresponding AnT and abiotic-control operations (Table 1). ARB present in BET system would respire on the electrode surface by utilizing the solid electrode as electron acceptor, thereby enhancing the dye degradation and power generation efficiency of the system. The present study elucidates the significant role of solid electron acceptor in conjunction with biocatalyst for dual benefits of power generation with simultaneous dye reduction. Direct electron flux between a microbe and a solid phase is a widespread and environmentally significant process.[33,34] Using solid electrodes as electron acceptors enables efficient electron transfer between biocatalyst and anode during dye degradation process in BET. During BET operation, two overlapping mechanisms might occur for coordinating extracellular electron transfer to solid phase electron acceptors viz., direct cell-electrode contact with bacterial outer membrane bound cytochromes serving as reductases as well as dye molecules functioning as electron shuttles. BET system visualized dual electron transfer mechanisms viz., direct and mediated. Voltammetric analysis depicted the involvement of outer membrane bound cytochromes, flavoproteins, Fe-S proteins and quinones that are responsible for DET to the solid electron acceptor (electrode) in BET system. DET mechanism is possibly done by the ARB present on the electrode surface. The organic fraction of wastewater will be utilized by the bacteria present in suspension as well as

in biofilm and will liberate the reducing equivalents towards the electrode. During the BET operation, electrode assembly induces the development of potential difference by the biocatalytic action of electrochemically active bacteria enriched around the electrodes in carrying out the simultaneous redox reactions towards the breakdown of dye molecules. Besides, the breakdown of complex dye molecule results in the generation of reduced dye intermediates that in turn acts as redox mediators/electron shuttles during the transfer of electrons to the solid electron acceptor/ for the other dye molecule reduction enabling the mediated electron acceptor.[4,34,35] Bio-electrogenic activity enhanced when bacterial respiration was coupled with the electrode assembly in treating/utilizing the azo dye based wastewater which can be attributed to the electron acceptor-dependent respiration.[36] Comparative analysis between three reactors during the study depicts the significant influence of biocatalyst and electrode assembly in BET system towards higher color removal (79.5 %), power generation (85 mW/m^2), electron transfer mechanism, redox catalytic currents (39.6 mV), azo reductase (26.2 U) and DH activity (2.6 μg/ml of Toluene) in comparison to AnT and abiotic control systems. On the contrary, abiotic control system lacking biocatalyst resulted in poor/no dye degradation in comparison to respective systems operated. AnT operation (with biocatalyst and without electrode assembly) resulted in poor azo dye degradation, enzymatic activities as well as redox catalytic currents. In general, the terminal reduction reactions are dependent upon the hierarchy of the most electronegative species/solid electron acceptor existing in the surrounding microenvironment. Absence of an electron acceptor results in inter species electron transfer, where the electrons liberated by one microbe will get utilized by another bacteria towards its growth and metabolic activities.[37] However, voltammetric analysis during AnT operation documented the involvement of membrane bound cytochromes and flavo proteins during the electron transfer, where the mixed bacterial population would have utilized these in transferring the electrons among the inter species. The results obtained during the study provide new insights into the bacterial respiration during BET operation utilizing various electron acceptors viz., electrodes as well as dye intermediates that enhanced the dye degradation efficiency with simultaneous power generation.

Table 1

4. Conclusion

- The effect of electrode as solid electron acceptor and bacteria as biocatalyst documented significant and specific process efficiency towards dye degradation with simultaneous power generation in BET system compared to the corresponding AnT and abiotic-control systems.
- The self induced bio-potential developed in BET system as a result of electrode assembly stimulated enrichment of electrochemically active bacteria that discharge higher number of reducing equivalents towards dye degradation and power generation.
- Two overlapping mechanisms for electron transfer were observed during BET operation, where ARB contributed for DET via cytochromes, flavo proteins towards the electrode (anode) and the reduced dye intermediates functioned as electron shuttle/mediator for other dye molecule reduction/electrode surface.
- Absence of electrode assembly in AnT and bacteria in abiotic-control system respectively, resulted in poor system performance in terms of azo dye degradation, azo reductase activity, colour removal, DH activity as well as redox catalytic currents depicting the lack of concurrence between the biocatalyst and solid electron acceptor in both AnT and abiotic-control systems.
- The study clearly documented that the presence of electrode assembly and the anaerobic consortia as biocatalyst in a system procures the advantage of electron acceptor dependent respiration offering dual benefits of dye degradation and power generation.

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640 **Table. 1 Comparative performance of BET, AnT and Abiotic Control reactors**

S. No	BET	AnT	Abiotic
Color (%)	70	42	2.4
COD (%)	79.5	48	2.33
OCV (mV)	350	-	50
PD (mW/m^2)	85	-	0.5
Azoreductase Activity (U)	26	18.9	0.15
DH (µg/ml of Toluene)	2.6	1.4	0.07
Oxidation Currents mV	39.6	6.2	0.326
Reduction Currents - mV	-19.5	-7.1	-0.586
β_a (V/dec)	0.599 (36 h)	1.097 (0 h)	0.841 (24 h)
β_c (V/dec)	0.164 (0 h)	0.171 (48 h)	0.197 (0 h)
R_p (Ω)	31 (0 h)	112 (48 h)	7373 (0 h)
CV mediators	Fe-S proteins, NAD/NADH, Cyt bc1, Cyt C, Flavoproteins and NO_3/NO_2	Cyt C and Flavoproteins	-
DCV Mediators	Fe-S proteins, Quinones and Cyt bC1	Cyt-bC1 and Fe-S proteins	-
Charge (C)	0.89 (6 h)	0.19 (12 h)	0.0147 (48 h)
Capacitance (F)	1.76 (6 h)	0.37 (12 h)	2.91E-02 (48 h)
Energy Stored (J)	0.45 (6 h)	0.094 (12 h)	7.42E-03 (48 h)
No. of electrons (n)	5.5638E+18 (6 h)	1.1684E+18 (12 h)	9.18E+16 (48 h)
Reference	Present Study	Sreelatha et al., 2015	Present Study

641

Captions for Figures

Fig 1: (a) Variation in enhancement of overall color removal efficiency in BET, AnT and abiotic-control bioreactors with 15 cycles operations (b) Relative variation of color removal efficiency (15^{th} cycle) under BET, AnT and abiotic-control operations.

Fig 2: (a) UV-Vis Spectral profiles recorded at regular time intervals of different experimental variations and comparative spectrum of BET, AnT and Control operations. (b) Comparison of Substrate degradation rate and COD removal efficiency with respect to BET, AnT and abiotic-control operations

Fig 3: (a) Change in Azo reductase enzyme activity profiles of various experimental conditions studied with respect to regular time intervals. (b) Comparative evaluation of Dehydrogenase activity studied at regular time intervals.

Fig 4: (a) Bio-Electrogenic activity in terms of OCV and PD for BET and control operations. (b) Polarization Curve and anode potential measured at various resistances during stabilized performance for BET and abiotic-control operations.

Fig 5: (a) Electrochemical (Redox) behaviour of BET, AnT and abiotic-control operations recorded (scan rate; 30 mV/s) at regular time intervals and comparative evaluation of voltammograms recorded at 6 h under varying operating conditions (b) Timely varied first DCV profiles at varying operations of BET, AnT and control reactors.

Fig 6: Variation of Tafel plots along with redox Tafel slopes and polarization resistance variations with the function of BET, AnT and abiotic-control operations.

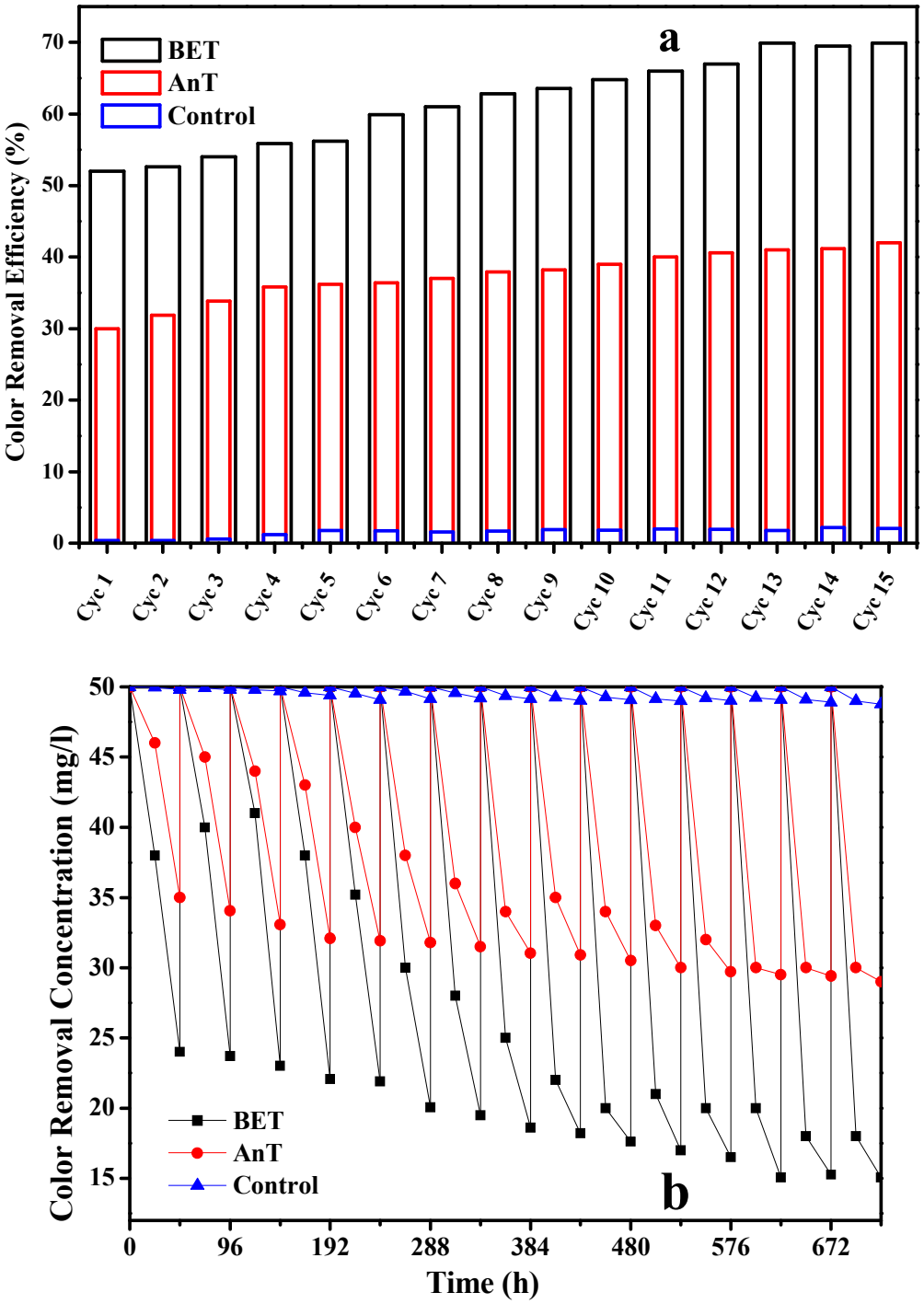

a
BET
AnT
Control
Color Removal Efficiency (%)
0
10
20
30
40
50
60
70
Cyc 1
Cyc 2
Cyc 3
Cyc 4
Cyc 5
Cyc 6
Cyc 7
Cyc 8
Cyc 9
Cyc 10
Cyc 11
Cyc 12
Cyc 13
Cyc 14
Cyc 15
b
BET
AnT
Control
Color Removal Concentration (mg/l)
15
20
25
30
35
40
45
50
0
96
192
288
384
480
576
672
Time (h)

Fig 1

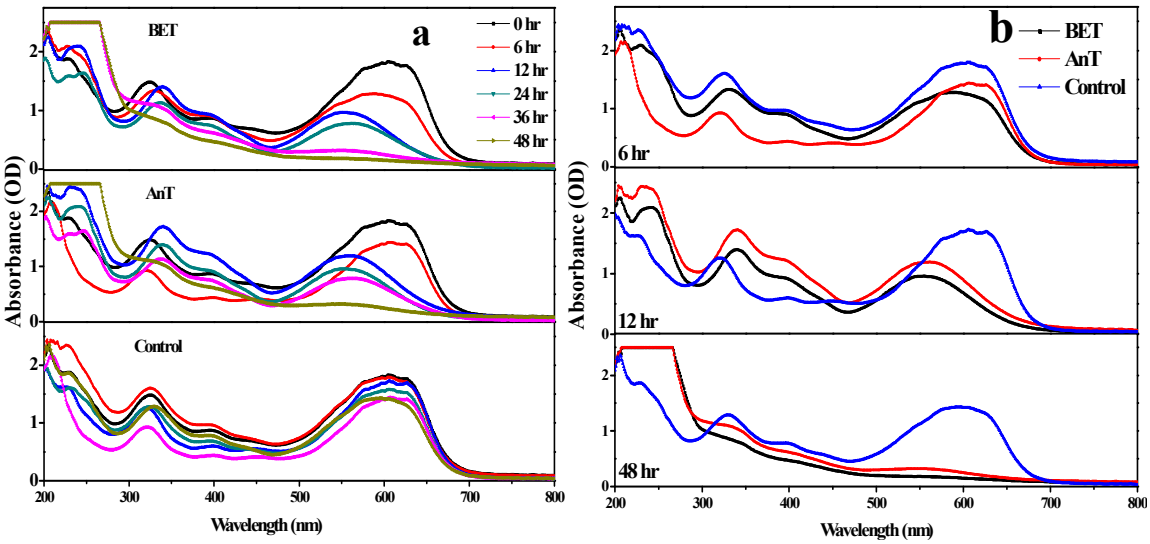

BET
a
0 hr
6 hr
12 hr
24 hr
36 hr
48 hr
AnT
Control
Absorbance (OD)
Wavelength (nm)
b
BET
AnT
Control
6 hr
12 hr
48 hr
Absorbance (OD)
Wavelength (nm)

Fig 2a

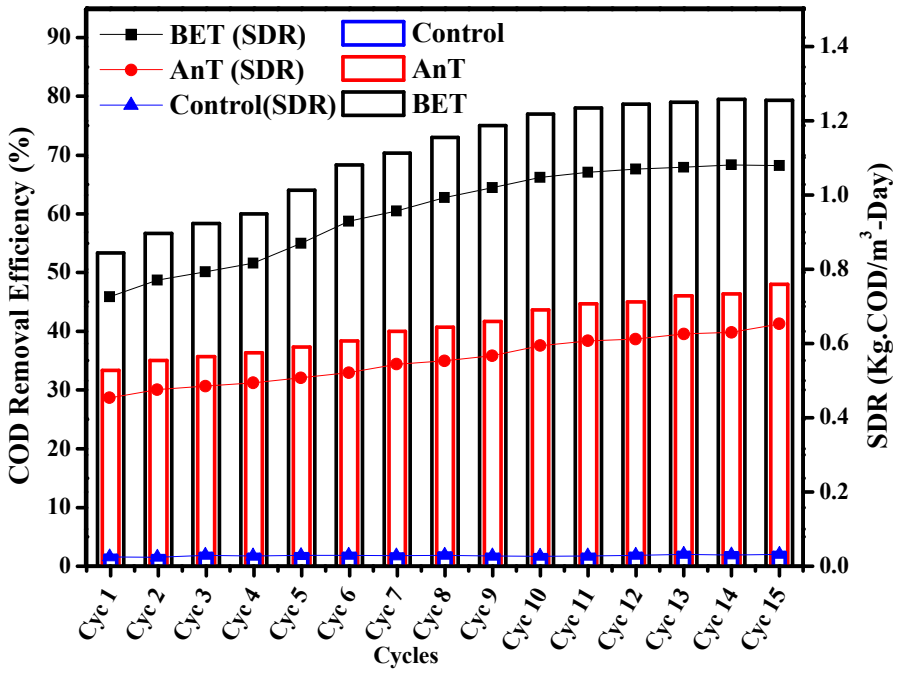

BET (SDR)
AnT (SDR)
Control(SDR)
Control
AnT
BET
COD Removal Efficiency (%)
SDR (Kg.COD/m3-Day)
Cycles
Cyc 1
Cyc 2
Cyc 3
Cyc 4
Cyc 5
Cyc 6
Cyc 7
Cyc 8
Cyc 9
Cyc 10
Cyc 11
Cyc 12
Cyc 13
Cyc 14
Cyc 15

Fig 2b

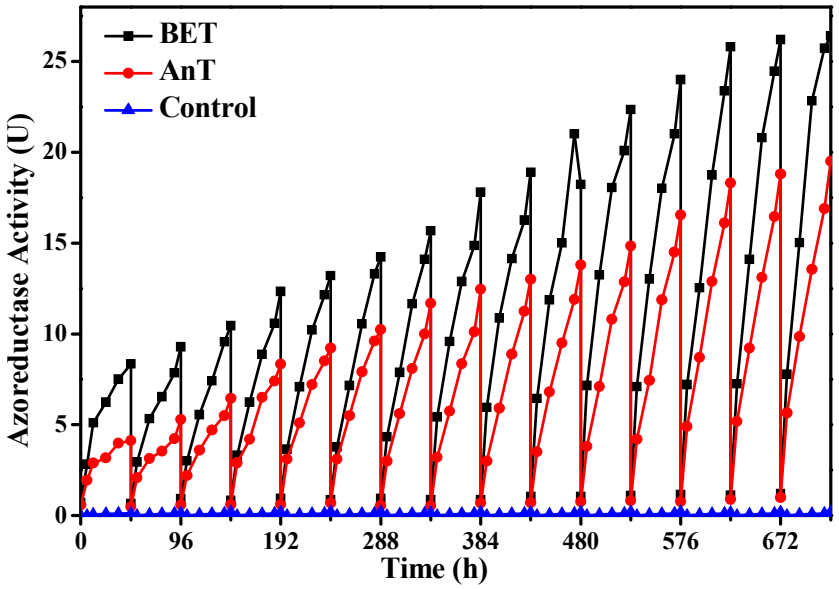
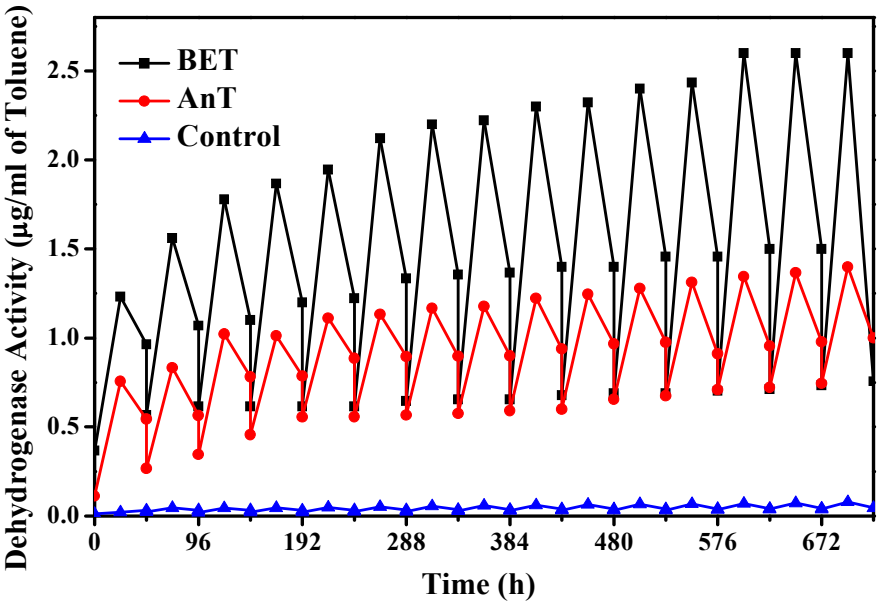

BET
AnT
Control
Azoreductase Activity (U)
Time (h)
0
5
10
15
20
25
0
96
192
288
384
480
576
672

Fig 3a


BET
AnT
Control
Dehydrogenase Activity (µg/ml of Toluene)
Time (h)
0.0
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1.0
1.5
2.0
2.5
0
96
192
288
384
480
576
672

Fig 3b

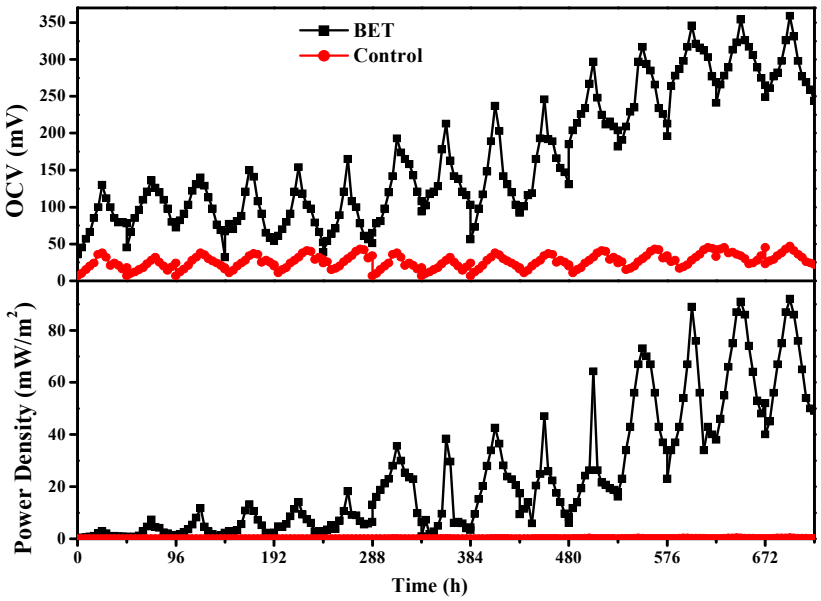

Fig 4a

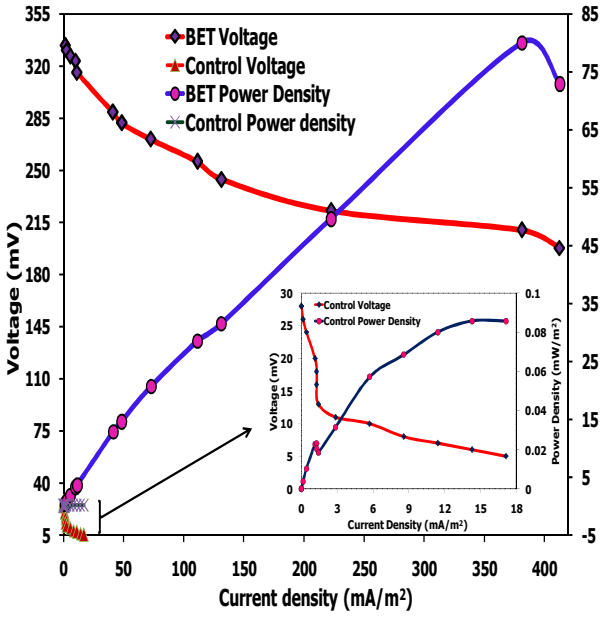

Fig 4b

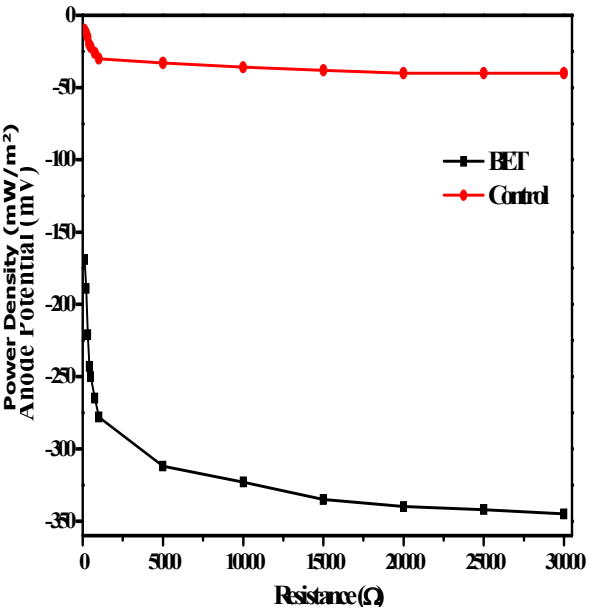

Fig 4c

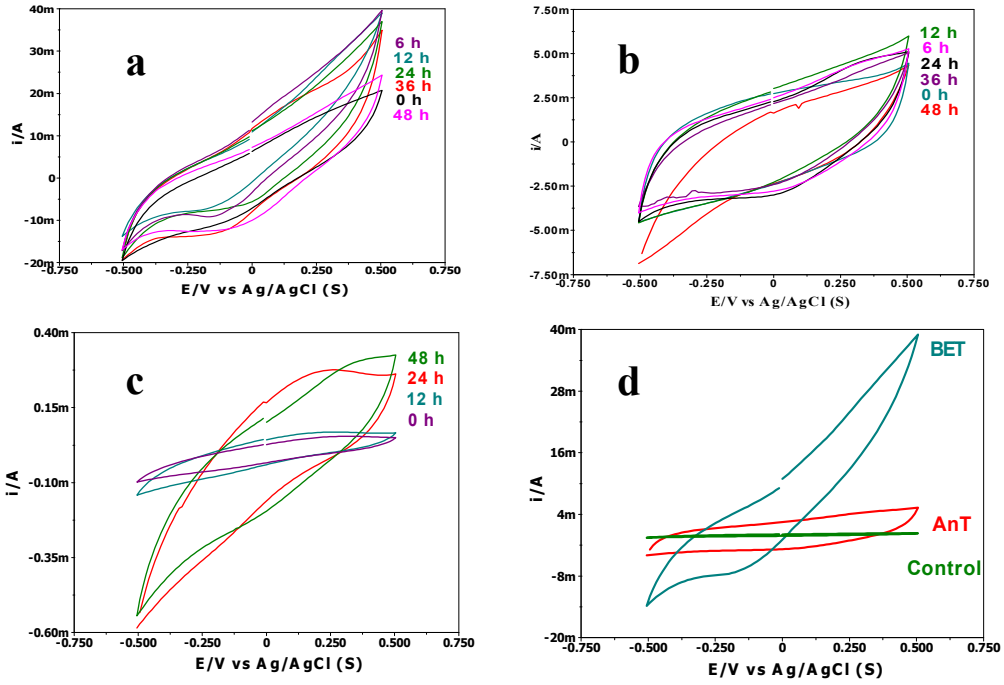
a
6 h
12 h
24 h
36 h
0 h
48 h
i/A
E/V vs Ag/AgCl (S)
b
12 h
6 h
24 h
36 h
0 h
48 h
i/A
E/V vs Ag/AgCl (S)
c
48 h
24 h
12 h
0 h
i/A
E/V vs Ag/AgCl (S)
d
BET
AnT
Control
i/A
E/V vs Ag/AgCl (S)

Fig 5a

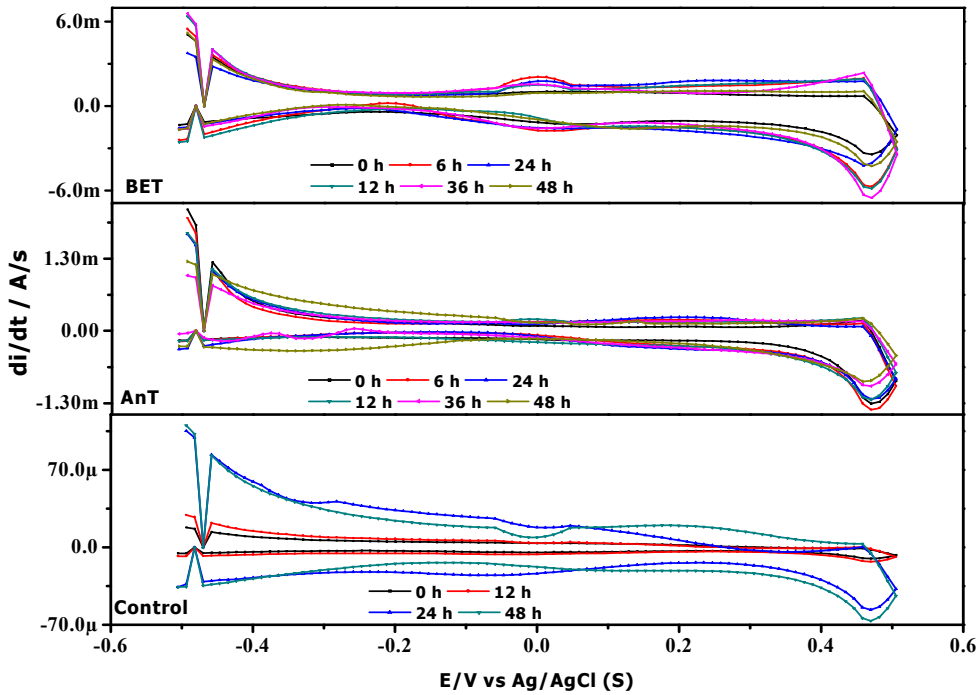
BET
0 h 6 h 24 h
12 h 36 h 48 h
AnT
0 h 6 h 24 h
12 h 36 h 48 h
Control
0 h 12 h
24 h 48 h
di/dt / A/s
E/V vs Ag/AgCl (S)

Fig 5b

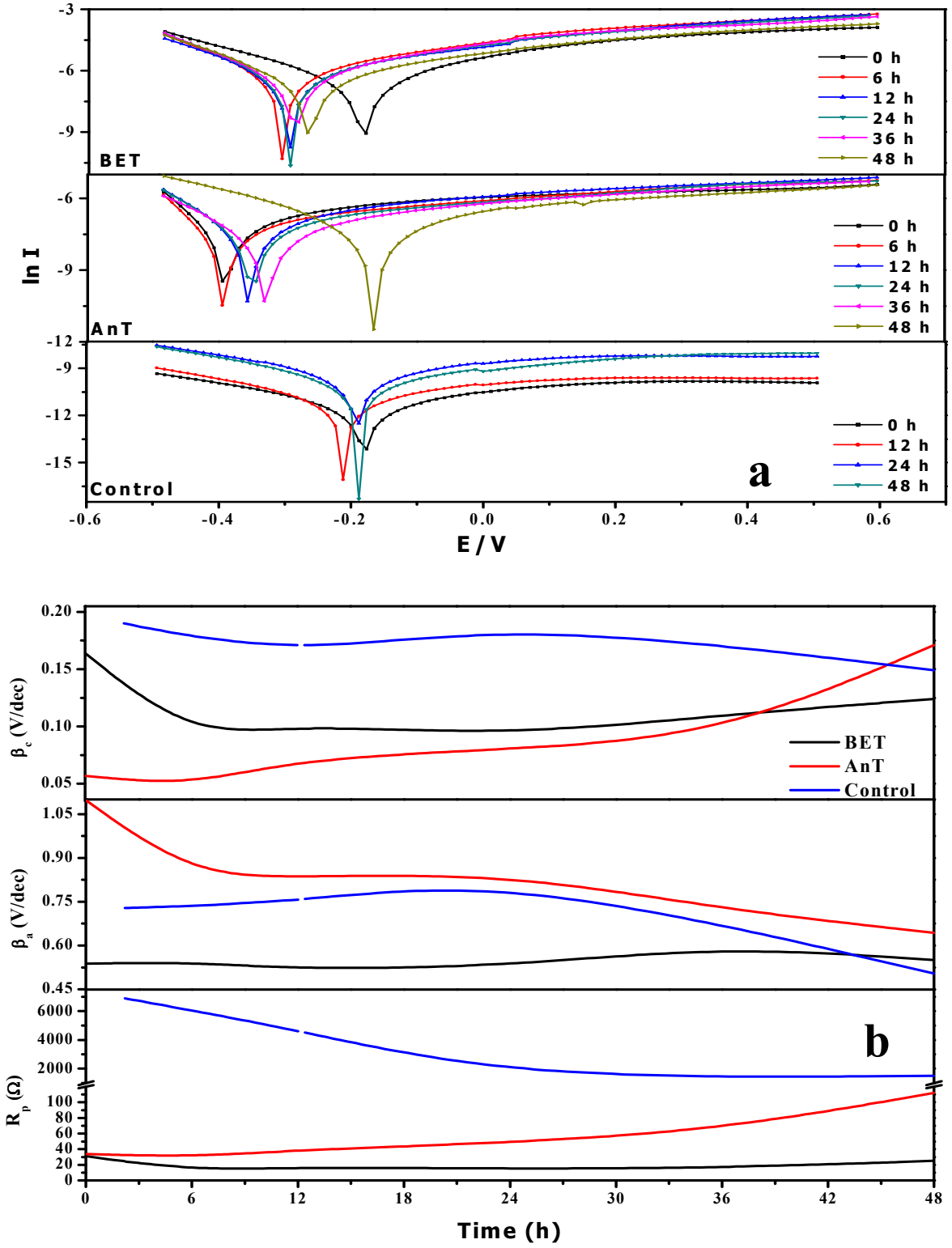
ln I
E / V
BET
AnT
Control
0 h
6 h
12 h
24 h
36 h
48 h
a
β_c (V/dec)
β_a (V/dec)
R_p (Ω)
BET
AnT
Control
b
Time (h)

Fig 6