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Rewiring the microbe-electrode interfaces with biologically reduced graphene oxide for improved bioelectrocatalysis

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ABSTRACT

The aim of this work was to study biologically reduced graphene oxide (RGO) for engineering the surface architecture of the bioelectrodes to improve the performance of Bioelectrochemical System (BES). *Gluconobacter roseus* mediates the reduction of graphene oxide (GO). The RGO modified bioelectrodes produced a current density of 1 mA/cm^2 and 0.69 mA/cm^2 with ethanol and glucose as substrates, respectively. The current density of RGO modified electrodes was nearly 10-times higher than the controls. This study, for the first time, reports a new strategy to improve the yield as well as efficiency of the BES by wrapping and wiring the electroactive microorganisms to the electrode surfaces using RGO. This innovative wrapping approach will decrease the loss of electrons in the microbe-electrolyte interfaces as well as increase the electron transfer rates at the microorganism-electrode interfaces.

1. Introduction

Bioelectrochemical systems (BES) are promising technologies for diverse applications with capabilities to operate in a broad range of environments including space and deep biospheres (Venkata Mohan et al., 2014). Apart from bioelectricity generation, BES finds applications in effluent treatment (Yeruva et al., 2015; Watson et al., 2015; Nancharaiah et al., 2016), desalination (Nikhil et al., 2016), metals removal and recovery (Nancharaiah et al., 2015) and production of value-added products (Roy et al., 2016). The use of the electroactive microorganisms or enzymes as electrocatalysts makes the bioelectrochemical processes economical and eco-friendly when compared with the chemical, electrochemical, and microbial processes. The use of electroactive microorganisms in BES helps in catalysing a broad range of substrates and accelerate the rates of electrocatalysis. BES suffers from low rates of bioelectrocatalysis when compared to other non-enzymatic electrocatalytic processes. Several approaches including the identification of new electroactive microorganism (Bhuvaneswari et al., 2013), new substrates (Thygesen et al., 2009; Selvaraj et al., 2016), surface display technology (Fishilevich et al., 2009), metabolic engineering approach (Gustavsson and Lee, 2016), improving the fuel cell configuration (Cui et al., 2016; An et al., 2016; Navanietha Krishnaraj et al., 2013; Navanietha Krishnaraj et al., 2015a), proton-exchange membrane (PEM) (Daries Bella et al., 2016) have been reported to improve the performance of BES. However, most of these strategies could not drastically increase the rates of bioelectrocatalysis.

Electron transfer resistance across electroactive microorganism-electrode interfaces is one of the major shortcomings impeding the rates of bioelectrocatalysis. Choice of an electrode material that aids the anchoring of bacteria, facilitating direct electron transfer, will greatly lessen the interfacial electron transfer resistance at the microorganism-electrode interfaces (Choi and Sang, 2016). Microbes have been shown to respire onto the electrodes with the aid of their inbuilt conductive membrane proteins such as cytochromes and conductive pili nanowires (Lovley, 2011). Harnessing the maximum number of electrons between the microbe and the electrodes has been a challenge. Conductive membrane proteins that are involved in receiving/transferring the electrons from/to electrodes are orientated on the surface of the microbial cells. A major fraction of the cell surface containing these proteins are exposed to the electrolyte, and are not attached to the electrode to mediate direct electron transfer. Technologies that could aid in trapping most of the electrons from the surface of entire microbial cells would significantly increase the yield of BES. Although electrode functionalizing strategies helped to increase the adherence of bacteria, increasing electron transfer rates, they did not attempt to trap the electrons produced by the microorganisms completely (Navanietha

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Krishnaraj et al., 2013).

Reports have been made on the use of materials with high conductivity and high specific surface area such as graphite, carbon foam, carbon felt, and carbon paper to increase the yield of electrocatalysis (Wei et al., 2011). These materials increase the yield and electron transfer rates, but loss of electrons on the microbe-electrolyte interface remains. In addition, certain carbon electrode materials fail to support the adherence of bacteria to its surface (Cornejo et al., 2015). Functionalizing the electrodes using biopolymers, nanomaterials, and conducting polymers have been reported to increase the biocompatibility of the electrodes thus improving the performance of BES (Karthikeyan et al., 2016). But but the biopolymer modified electrodes do not improve the electron transfer characteristics of the electrode. On the other hand, the use of nanomaterials and conducting polymers for modifying electrodes, pose toxicity to the microbial cells leading to a decrease in rates of microbial catalysis (Kang et al., 2008). These methods aim to improve the adhesion of microorganisms, or rates of electron transfer leading to improved electrocatalysis, but they fail to focus on trapping the maximum number of electrons that are produced by the microorganisms, leading to poor efficiency of the system.

Herein, the use of graphene for wrapping the microorganisms, which can aid in efficiently trapping the electrons from the entire surface of the microorganisms is reported. The extraordinary features of graphene, including high electrical conductivity and large surface area, make it a promising material for wiring the microorganisms to the electrode surface (Zhu et al., 2010). Chemical methods that employ reducing agents such as hydrazine (N_2H_4), dimethylhydrazine ($C_2H_8N_2$), and sodium borohydride ($NaBH_4$) may not yield biocompatible RGO that is desired for wrapping the microbial cells (Chua and Pumera, 2014; Barbolina et al., 2016; Qiu et al., 2017). Graphene produced by these chemical routes is also prone to lose its unique properties such as high conductivity that is crucial for electrochemical applications (Stankovich et al., 2006b; Stankovich et al., 2007). In addition, chemical reducing agents like hydrazine and hydrazine hydrate are toxic and explosive, and may pose several deleterious effects to the environment. On the other hand, reports are documented on the reduction of GO using microorganisms such as *Shewanella* sp. (Salas et al., 2010; Wang et al., 2011) and *E. coli* (Akhavan and Ghaderi, 2012; Gurunathan et al., 2013).

Biologically reduced RGO was shown to enhance the extracellular electron transfer in microbial fuel cells. Simultaneous reduction of GO and current generation in the anodic compartment of microbial fuel cell is also reported (Yuan et al., 2012). This study reports, for the first-time wiring the RGO wrapped microorganisms with electrodes, which reduces the loss of electrons in the microbe-electrolyte interfaces as well as increases the electron transfer rates at the microorganism-electrode interfaces, leading to improved electrocatalysis.

2. Materials and methods

2.1. Synthesis of graphene oxide

Graphite powder of size less than 20 μm, purchased from Sigma Aldrich, was used for the synthesis of GO. Graphene oxide was synthesized using the modified Hummers method (Hummers and Offeman, 1958). Briefly, 4 g of graphite and 2 g of sodium nitrate were added to a 250-mL flask. 100 mL of concentrated H_2SO_4 was slowly added. Stirring was continued for 30 min 0.6 g of $KMnO_4$ was added to the mixture with continuous stirring for another 30 min 14 g of $KMnO_4$ was added to the mixture. Stirring was continued for few hours till the temperature of the mixture comes down to room temperature. 180 mL of water was slowly dripped into the paste and the diluted suspension was once again stirred for another 15 min. Finally, 7 mL of H_2O_2 and 55 mL of water was added. The resulting bright yellow solution was filtered and the yellow brown filter cake was collected. The cake was washed repeatedly with 3% aqueous HCl. The synthesized GO was dried in a desiccator and is used for the synthesis of graphene.

Carbon felt with a geometrical area of 0.4 cm^2 was used for the experiment. The electrical connection was made with a brass rod. The fabricated carbon felt electrodes were cleaned, and were used as bare electrodes. Carbon felt electrode of same size was modified with RGO.

2.2. Reduction of GO

Reduction of GO was mediated by pure cultures of *G. roseus* (National Chemical Laboratory, NCIM #2524). The culture was inoculated into a growth medium containing tryptone (1 g), yeast extract (1 g), glucose (1 g), and $CaCO_3$ (1 g) in 100 mL of distilled water and incubated for 24 h at 37 °C. After 24 h, the broth containing the microbial cells was centrifuged, and the cells are washed with phosphate buffer (0.1 M, pH 7) to remove the debris and medium constituents. About 0.1g (wet weight) cells were dispersed in phosphate buffer containing sorbitol (5g/100 mL) as the electron donor (substrate) and 0.5g of GO as the electron acceptor. The conical flask was kept in the incubator at 37 °C for 24 h for the reduction of GO. The Scanning Electron Microscopy (SEM) images of the bacteria wrapped with RGO during the reduction process were observed. *G. roseus* reduced graphene oxide was collected and purified by washing repeatedly with deionized water to remove the cell debris. The purified samples were collected and washed in the following sequence: 18 MΩ-cm water, 80% ethanol, 18 MΩ-cm water, 1 N HCl, and 18 MΩ-cm water (Millipore) (Salas et al., 2010).

2.3. Characterization of RGO

The synthesized GO and RGO were characterized by UV spectroscopy, Fourier Transform Infrared (FT-IR) spectroscopy, Laser Raman spectroscopy, and X-ray powder diffraction. FT-IR spectra of the GO and RGO were recorded in a spectral range of 400–4000 cm^{-1} using FT-IR Spectrometer (Bruker Optik GmbH, Germany, TENSOR 27). The samples for FT-IR were prepared by grinding the dry powdered sample with KBr. The powdered samples were smeared on a clean SiO_2/Si substrate that was used for the Raman measurement. X-ray powder diffraction spectra of both the samples were recorded to observe the diffraction features (Bruker). The synthesized GO and RGO were characterized by Transmission electron microscopy (FEI, Tecnai 20 G2). The samples for TEM characterization were prepared by placing the aqueous suspension (~0.02 mg/mL) of GO or RGO samples on the Cu grids and allowed to dry. About 0.5 mg RGO (in 1 mL of phosphate buffer (0.1 M, pH 7)) was drop casted on the carbon felt electrode. Adhering bacteria onto the RGO electrode had the tendency to wrap the RGO on its surface. This leads to natural wiring of the microbial conductive proteins on the surface to the electrodes. The bare and modified electrodes were analysed using SEM.

2.4. Formation of biofilm

The biofilm of a mixed culture containing *Acetobacter aceti* and *G. roseus* was formed on both bare and modified electrodes in phosphate buffer solution, containing glucose (0.2 g/30 mL of buffer) and the mixed cultures were incubated under anaerobic conditions. Biofilm was allowed to form until the electrode reached a stable negative potential. The electrodes with biofilms were used for studying the efficacy of the functionalized electrodes on microbial electrocatalysis of ethanol. Similarly, natural biofilm formed from a food waste sample was formed on bare and modified electrodes with the food waste sample. The electrodes with natural biofilm were also used for analysis of microbial electrocatalysis of glucose.

2.5. Electrochemical studies

The bare and modified electrodes containing the biofilms were

gently rinsed with phosphate buffer solution. Cyclic voltammograms were recorded in the potential range −1 to +1 V at 5 mV/s. Normal calomel electrode (NCE) and Pt foil were used as the reference electrode and the counter electrode, respectively for electrochemical studies. Phosphate buffer (0.1 M, pH 7) was used as the electrolyte. Anaerobic conditions were maintained by purging nitrogen during the electrochemical investigations. The bioelectrocatalysis of ethanol using the biofilm of *A. aceti* and *G. roseus* was evaluated with additions of 25 mM ethanol. Bioelectrocatalytic activity of the natural biofilm was analysed with additions of 25 mM glucose. (Navanietha Krishnaraj et al., 2015b) Further, long-term Chronoamperometry studies were performed for both bare and modified electrodes with the biofilms. Ethanol and glucose were used as the substrates for the amperometry studies for acetic acid bacteria biofilm (*A. aceti* and *G. roseus*) and the food waste based natural biofilm, respectively. Electrochemical Impedance Spectroscopy (EIS) experiments were also carried out in the BES with five different electrolytes. EIS was performed in the three-electrode mode in the frequency range of 20 Hz to 10 mHz with an AC amplitude of 10 mV.

2.6. Scanning electron microscopy

The biofilms formed on bare carbon felt and RGO modified carbon felt electrodes were characterized using SEM. A small piece of the bare and modified carbon felt electrodes with biofilm were dried in a desiccator under aseptic conditions and gold sputtered for analysing the effect of RGO wrapping on biofilm formation.

3. Results and discussion

3.1. Reduction of graphene oxide

The change in colour from brownish yellow to black was observed on reduction of GO, and it is used as a yardstick to validate the reduction of graphene oxide. This color change indicates the restoration of electronic conjugation. The reduction of GO causes a decrease in polar functionality on the surface of the sheets which in turn increases the hydrophobicity of the material leading to the formation of black coloured precipitate from brown coloured solution (Stankovich et al., 2006a). GO has a maximum absorption peak at about 231 nm which is due to the π-π^* transition of aromatic C–C bonds. Another peak at 301 nm is observed which is due to n-π^* transition. *G. roseus* RGO had the maximum absorption peak at 280 nm. *G. roseus* mediated GO reduction is evident from the red shift of maximum absorbance peak from 231 nm to 280 nm. The shift suggests that the electronic conjugation within RGO sheets is restored after the reduction of GO (Li et al., 2008).

The FTIR spectrum clearly show removal of oxygen-containing groups of GO in *G. roseus* RGO. The FTIR spectrum of GO show several peaks in the range of 900–1500 cm^{-1}, which may be attributed the presence of functional groups like hydroxyl and epoxy groups. A dominant broad peak at 3384 cm^{-1} is due to the vibration and deformation peaks of hydroxyl groups. A peak at 1390 cm^{-1} also arises from the hydroxyl group. The peaks at 1038 cm^{-1} and 1119 cm^{-1} are due to stretching of the C–O bond. The other peak at 1649 cm^{-1} is due to the C=O bond. These peaks decreased significantly after *G. roseus* mediated reduction.

Raman spectroscopy is used as a tool to assess the reduction process of GO. Using Laser Raman Spectroscopy, the vibrational, rotational modes (for liquids) and other characteristics of GO and RGO can be analysed. Graphitic materials frequently display two distinct bands, known as the D band and the G band. The D band is associated with disorder in the graphene structure, whereas the G band is a first order band resulting from in-plane vibrations of sp2 bonded carbon atoms. Change in the relative intensities of G and D bands in the Raman spectra of GO after reduction reflects changes in the electronic conjugation state, and the intensity ratio (I_D/I_G) can provide an estimate of defect density (Childres et al., 2013). From the Raman spectrum of GO and RGO, the I_D/I_G ratio of GO and RGO were found to be 1.12 and 0.87 respectively, implying the occurrence of a reduction reaction of the GO. The XRD pattern of RGO shows a major peak at a 2θ value of 23–24 degrees with interplanar spacing of 3.7 Å which is attributed to RGO, as reported by Park et al. (2011). The TEM image clearly shows the slightly folded sheet of RGO after purification. The use of *G. roseus* for the reduction of silver nitrate has been reported in the literature (Navanietha Krishnaraj and Berchmans, 2013).

3.2. Electrochemical studies

The *G. roseus* RGO was used for wiring the microorganisms with the carbon felt electrode and the efficacy of the wrapping and wiring strategy to enhance microbial electrocatalysis was investigated. The RGO functionalized electrode was assessed with two different biofilms. The biofilm formed with a coculture of *A. aceti* and *G. roseus* was used for electrocatalysis of ethanol, and biofilm formed from food waste-based microorganisms (called natural biofilm) were used for the electrocatalysis of glucose. The carbon felt electrode unmodified with RGO was used as a control. The cyclic voltammograms of bare and modified electrodes with biofilms of coculture containing *A. aceti* and *G. roseus* displayed redox peaks due to the presence of electroactive moieties in biofilms (Fig. 1a and b). The cyclic voltammogram of the acetic acid bacteria biofilm displayed a well-defined broad peak at a potential of 0.09 V. In the case of *A. aceti* and *G. roseus* biofilm formed on the modified electrode, a clear peak at 0.643 V was observed. Additionally, a small peak could be seen at 0.09 V. The redox peaks in electrodes with RGO wrapped biofilm indicates that the electroactivity of the biofilm is not affected by the RGO. It also confirms the biocompatibility of RGO to the biofilm. The redox peaks in the voltammograms at 0.09 V correlates with the membrane bound pyrroloquinoline quinone (PQQ) containing enzymes that are involved in oxidation of a wide range of substrates. The alcohol dehydrogenase (ADH) *A. aceti* and *G. roseus* contains quinoheme protein-cytochrome *c* complex that mediates the oxidation of electron and transfer to ubiquinone embedded in the membrane phospholipids. The quinoheme protein-cytochrome C complex that is bound to the periplasmic side of the cytoplasmic membrane was shown to mediate direct electron transfer from the microorganism to the electrode at the electrode-electrolyte interface. Presence of electroactive functional groups such as quinones and flavins are also reported in the literature (Karthikeyan et al., 2009).

The cyclic voltammograms of the biofilms in both bare and RGO electrodes showed enhanced current response on addition of 25 mM of ethanol. In the unmodified electrode, the current increased from 0.590 mA to 0.668 mA on addition of 25 mM of ethanol. On further addition of 25 mM of ethanol the current increased to 0.833 mA. In the case of the electrode with RGO wrapped microorganisms, the peak at 0.643 V displayed an increase in current from 0.538 mA to 1.564 mA, which on the second addition rises to 1.657 mA. The rise in current could also be observed in the peak at 0.09 V on addition of ethanol as substrate, increasing from 0.229 mA to 0.386 mA; and to 0.615 mA with the second addition of 25 mM of ethanol. Investigations with the biofilm formed from food wastes showed that the *G. roseus* RGO modified electrode significantly increased the electrocatalysis of glucose (Fig. 1c and d).

The bioelectrocatalytic investigations with unmodified electrode showed that the current density increased by 0.1165 ± 0.068 mA/cm^2 with addition of 20 mM of ethanol. In the case of RGO modified electrodes, the current density increased by 1.01 ± 0.02 mA/cm^2 with addition of 20 mM of ethanol.

The unmodified electrode with natural biofilm displayed a broad peak at a potential of 0.268 V. In the unmodified electrode, the current increased from 3.12 mA to 3.21 mA on addition of 25 mM of glucose at 0.268 V. On further addition of 25 mM of glucose, the current increased to 3.3 mA. In the modified electrode, the current increased significantly from 0.412 mA to 0.450 mA, which on the second addition rose to

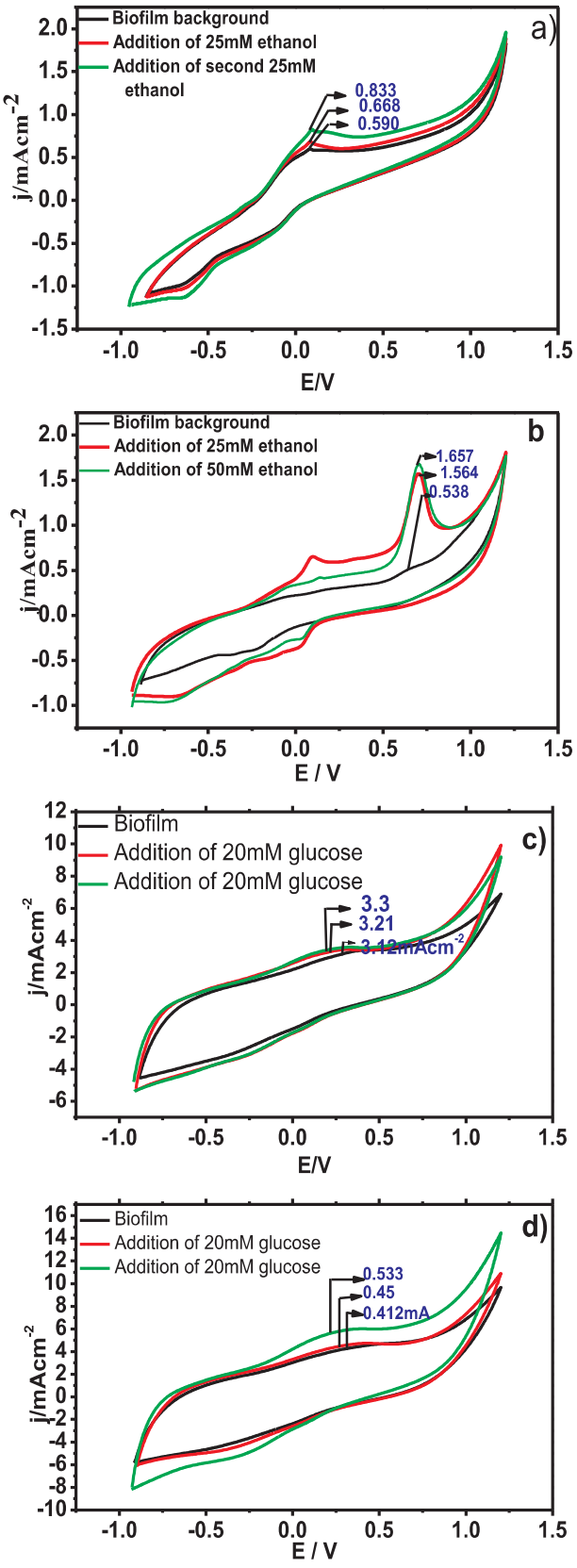

Fig. 1. Cyclic voltammograms showing the bioelectrocatalysis of ethanol on (a) bare carbon felt electrode, (b) *G. roseus* RGO electrode; and showing the bioelectrocatalysis of glucose on (c) bare carbon felt electrode (d) *G. roseus* RGO electrode.

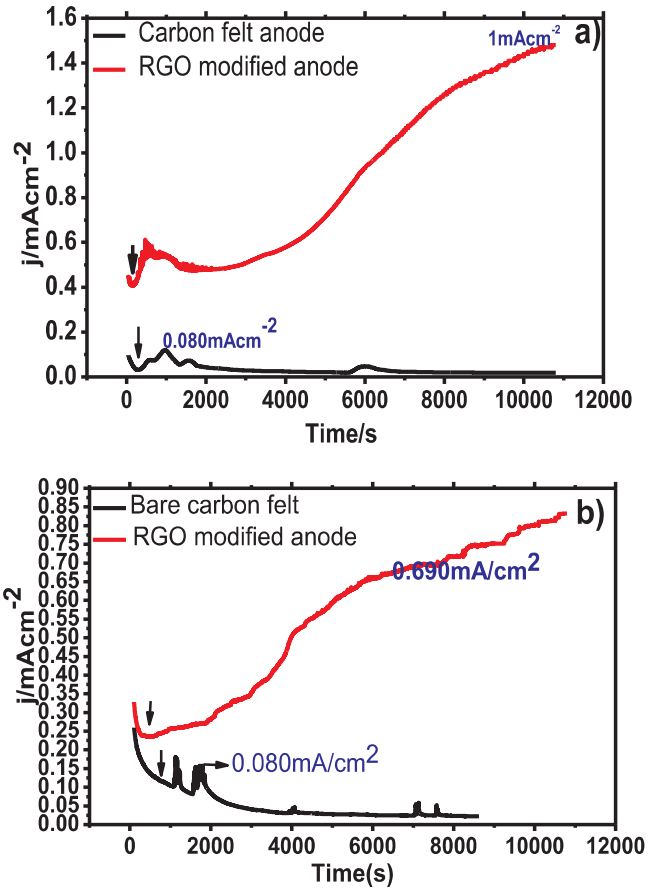

Fig. 2. Long term chronoamperometry studies (a) bioelectrocatalysis of ethanol using *A. aceti* and *G. roseus* (b) bioelectrocatalysis of glucose using natural biofilm from food waste.

0.533 mA at 0.308 V. From the cyclic voltammetry studies with the two different biofilms it is evident that wrapping the microorganisms with RGO, and wiring them onto electrodes aid bioelectrocatalysis, leading to development of improved bioelectrodes and BES for practical applications. Overall, in the bioelectrocatalysis of glucose using natural biofilm, the current density increased by 0.105 + 0.221 mA/cm^2 and 0.0605 ± 0.031 mA/cm^2 respectively.

The long term chronoamperometry was performed for bare and RGO electrodes with two different biofilms to find the maximum sustainable current produced. The half cell studies show that the RGO electrode better electrocatalytic properties when compared to the bare carbon felt electrode. In the case of studies with biofilm of acetic acid bacteria (*A. aceti* and *G. roseus*), the maximum current density of 0.080 mA cm^{-2} was produced with an applied potential of 0.6 V (vs NCE) by the unmodified electrode (Fig. 2a). Modifying the electrode with RGO significantly enhanced the power output to 1 mA cm^{-2} with an applied potential of 0.6 V (vs. NCE). Applying a lower potential of 0.2 V resulted in a current density of 0.007 mA cm^{-2} and 0.121 mA cm^{-2} with bare and RGO modified electrodes respectively. The studies with food waste based natural biofilm on unmodified electrode produced a maximum current density of 0.080 mA cm^{-2}. RGO electrodes with natural biofilms produced a maximum current density of 0.69 mA cm^{-2} (Fig. 2b). The power output was increased by nearly 8.6 times in this case. The salient features of RGO such as high conductivity and high specific surface area along with biocompatibility offered by the microbial synthesis procedure helped in enhancing the power output significantly.

TEM analysis clearly showed that the RGO gets wrapped around the surface of the microorganisms leading to enhanced power output,

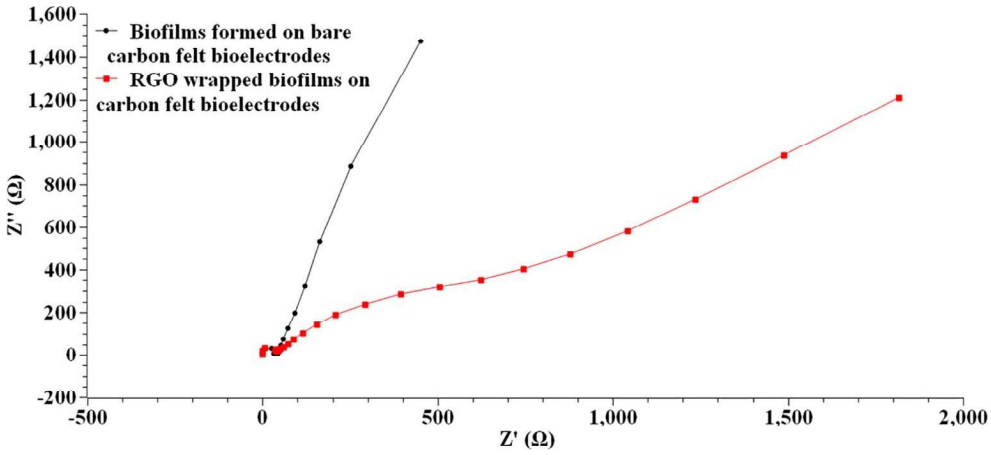

Fig. 3. Nyquist plot of the biofilm formed on the (a) bare carbon felt electrode and (b) RGO modified carbon felt electrode.

arising from the known high electrical conductivity and low impedance RGO.

3.3. Electrochemical impedance analysis

Electrochemical Impedance Spectra (EIS) of the biofilms formed on the bare and the RGO electrode provide insights on the effect of RGO functionalization on solution resistance and polarisation resistance. Although microorganisms have a few membrane bound conductive proteins that are involved in electron transfer, most of their components are non-conductive, leading to increased resistance. Formation of several layers of biofilm and the polymer matrices also contribute towards increased resistance across the electrode-electrolyte interface leading to a decrease in microbial electrocatalysis. Fig. 3 shows Nyquist plots of the bare and the RGO electrode with biofilm. The sum of solution resistance (Rs) and polarisation resistance (Rp) could be obtained from the low frequency region in the real axis of the Nyquist plot. The inversely proportional relationship between Rp and exchange current density provides valuable information on the biofilm formation on the electrode at the electrode-electrolyte interface as well as the electron transfer rates (Manohar et al., 2008). In the case of the biofilms formed onto the carbon felt anode, Rp value was found to be 8000Ω. However, it significantly decreased to 2000Ω on using this wrapping strategy. The decreases in Rp in RGO electrodes clearly depicts the high conductivity of RGO and decreased electron transfer resistance at electrode-electrolyte interface.

3.4. SEM analysis

The biocompatibility is evident from electron microscopic observations of the electrodes with biofilms. SEM images of RGO functionalized electrodes, bare and RGO electrodes with biofilms show that the density of biofilms was very high in the modified electrodes when compared to the unmodified felt electrode. The biological route for RGO synthesis contributes to the biocompatibility of *G. roseus* RGO and the modified electrode. Unlike the chemically reduced GO which renders toxicity to microorganisms, this biologically synthesized reduced GO aids and supports the growth of microorganisms.

4. Conclusion

The study reported the microbial reduction of graphene oxide and characterized the reduced graphene oxide using UV spectroscopy, FTIR spectroscopy, Laser Raman Spectroscopy, XRD, and TEM. In addition, a simple strategy for wiring microorganisms to electrodes is developed by tailoring the surface architecture of electrodes. The biocompatibility of functionalized electrodes and good electron transfer characteristics of the RGO promote trapping of the electrons from the surface of the microorganism by a wrapping mechanism. The electroanalytical investigations showed that the functionalized electrodes significantly enhanced the rates of microbial electrocatalysis by overcoming the bottlenecks in microbial electron transfer reactions at electrode-electrolyte interfaces.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2018.02.001.

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