

## Original Research Article

<https://doi.org/10.20546/ijcmas.2017.608.148>

## Formation of Electrically Conductive Bacterial Nanowires by *Desulfuromonas acetoxidans* in Microbial Fuel Cell Reactor

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### ABSTRACT

#### Keywords

Anaerobic sulfate  
respiration bacteria,  
*Desulfuromonas  
acetoxidans*,  
Conductive bacterial  
nanowires, Insoluble  
electron acceptor,  
Microbial fuel cell.

#### Article Info

##### Accepted:

17 June 2017

##### Available Online:

10 August 2017

In this investigation on Anaerobic Sulfate Respiration Bacteria (ASRB), it is reported that the obligate anaerobic microorganism, *Desulfuromonas acetoxidans* are capable of producing nano scale bacterial appendages for facilitating extracellular electron transfer. The nanowires were resistive and electrically conductive ( $1.88 \times 10^{-8} \Omega \cdot m$  and  $7.32 S \cdot m^{-1}$ ). They also permitted the ASRB to colonize the surface of the solid or insoluble electron acceptors, thereby making it possible for extracellular electron transfer to take place to the insoluble electrode in the MFC directly and without the need of mediators for electron shuttling. The maximum power density reached was  $7.9 Wm^{-3}$ , and nanowire production was stimulated whilst insoluble electron acceptors were present for cellular respiration to occur. The results suggest *D. acetoxidans* initiates the production of conductive nanowires in case of limited availability of a soluble electron acceptor ( $SO_4^{2-}$ ) for ASRB as an alternative means for facilitating electron transfer to the insoluble electron acceptors.

### Introduction

The microbial transformation of various organic and inorganic substances found to be present in the subsurface anoxic environment requires anaerobic micro-organisms to utilize inorganic ions as electron acceptors, such as  $Fe^{3+}$ ,  $SO_4^{2-}$ ,  $NO_3^-$ , and  $Mn^{4+}$  (Lovley, 1991; Ishii *et al.*, 2013). However, the dissimilatory reduction of iron and manganese oxides have received negligible attention in their capacity as solid electron acceptors in spite of their abundance in soils and sediments. Previously, electron shuttles or mediators (Lovely *et al.*, 1996) and *c*-type cytochrome in outer cellular membranes (Bond and Lovley, 2003) have been proposed as possible mechanisms for

electron transfer. Furthermore, EL-Naggar *et al.*, (2010), Reguera *et al.*, (2005) and Gorby *et al.*, (2006) among others suggest direct electron transfer to insoluble electron acceptors takes place by dissimilatory iron reducers, such as those from the *Shewanella* and *Geobacter* genera, by means of bacterial nanowires, and that there is a need for direct contact of cells with insoluble electron acceptors in order to reduce them. Investigating the need for bacterial cells to be in direct contact with insoluble electron acceptors could give insight into the mechanism by which extracellular electron transport to insoluble electron acceptors

occurs in nature. Similarly, nano scale bacterial appendages reduce iron during direct electron transfer to Fe (III) oxides or other solid electron receptors. The microbial fuel cell (MFC) device utilizes electrically catalytic microorganisms for generating electricity from organic and inorganic substances (Logan *et al.*, 2006). This makes it useful in evaluating extracellular electron transfer reactions that occur close to electrode surfaces, thereby acting as a naturally insoluble or solid-phase electron acceptor in the environment (Kiely *et al.*, 2011). Indirect electron transfer by mediators from cells to electrodes is one strategy formed for explaining extracellular electron transfer in MFC (Newman and Kolter, 2000). Mediators capable of functioning as electron shuttles include iron oxides, sulfate, phenazines and quinones. Another strategy is direct electron transfer from cell to electrode by means of the *c*-type cytochrome related with outer membranes (Chaudhuri and Lovley, 2003), and an alternative of interest to this study is direct electron transfer by means of filamentous structures such as bacterial nanowires (Gorby *et al.*, 2006). In the case of MFCs that are mediator-less, microorganisms enable direct electron transfer to the electrodes without the need for mediators, and the electrodes function as sole electron acceptors (Chaudhuri and Lovley, 2003; Gregory *et al.*, 2004).

Although many micro-organisms exist with the capability of electron donation to the MFC anode, and thereby of electricity production, only some iron reducing species have been discovered to produce nanowires as mediators during direct electron transfer to the electrode, such as *Geobacter sulfurreducens* and *Shewanella oneidensis* MR-I (Gorby *et al.*, 2006; El-Naggar *et al.*, 2010). ASRB (Anaerobic Sulfate Respiration Bacteria) present in highly reducing environments normally use soluble sulfate as a terminal

electron acceptor during the process of respiration to aid in their growth (Heidelberg *et al.*, 2004). It is by means of this process that ASRB contribute to the carbon and sulfur cycles considerably, as well as bioremediation of contaminated subsurface systems (Martins *et al.*, 2009). ASRB such as *Desulfobulbus propionicus* are capable of using other electron acceptors, such as Fe<sup>3+</sup> (Lovley *et al.*, 1993), NO<sup>3-</sup> (Marietou *et al.*, 2009), Mn<sup>4+</sup> (Myers and Nealson, 1988), and fumarate (Tomei *et al.*, 1995), i.e. other than sulfate. Notably, ASRB are also capable of being used as microbes for generating electricity in MFC (Cordas *et al.*, 2008; Zhao *et al.*, 2008). A number of strategies have been devised to facilitate electron transfer to date, from ASRB to solid electrodes in an MFC system. For instance, indirect electron transfer to electrodes has been demonstrated for ASRB by means of inorganic electrons as mediators functioning as shuttles to thereby generate electricity, such as sulfate or sulfide (Zhao *et al.*, 2008). In a mediatorless MFC, it has also been shown that when functioning as a microbial catalyst, ASRB facilitate electron transfer to the electrode by means of contact between the microbes and electrodes through a *c*-type cytochrome present in an outer cell membrane (Cordas *et al.*, 2008). However, the transfer of electrons by ASRB through microbial appendages directly has not been adequately addressed previously as it has for iron reducers (Reguera *et al.*, 2005; Gorby *et al.*, 2006; El-Naggar *et al.*, 2010).

This study assesses the utility of nanoscale bacterial appendages produced by ASRB as filaments for facilitating the transfer of electrons to insoluble electron acceptors directly. It hypothesized that the bacterium *D. acetoxidans*, selected as representative of ASRB based on it having a well-characterized genome and its prevalence in diverse anoxic environments (Devereux and Mundfrom, 1994), produces nanowires only when there

are insoluble electron acceptors present to facilitate extracellular electron transfer, as opposed to  $\text{SO}_4^{2-}$  as the soluble electron acceptor. Furthermore, distinguishing characteristics of the nanowires are identified with respect to their morphological features and functions by using an MFC inoculated with *D. acetoxidans* as the chief ASRB.

## Materials and Methods

### Microorganism, media and cultivation

The ASRB *D. acetoxidans* was obtained from a Germanic collection of micro-organisms in Braunschweig, Germany. The cells underwent preharvestation within a growth medium in strict anaerobic conditions at 37°C. This medium contained (per liter): 0.5 g of  $\text{K}_2\text{HPO}_4$ , 1.0 g of  $\text{NH}_4\text{Cl}$ , 1.0 g of  $\text{Na}_2\text{SO}_4$ , 0.1 g of  $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ , 2.0 g of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2.0 g of sodium lactate, 1.0 g of yeast extract, 1.0 mg of resazurin, 0.5 g of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.1 g of sodium thioglycolate, 0.1 g of ascorbic acid, and 1 mL of a trace element solution. Prior to sterilization at 121°C by autoclaving for 15 minutes, the pH 7 of the medium was adjusted with 0.1 M NaOH. The cells were harvested by centrifugation for 20 min at 10,000g and 4°C, then washed twice in a buffer solution of 50 mM of phosphate at pH 7, and seeded in the anodic MFC chamber.

A mixed culture of ASRB was then isolated from an anaerobic sewage digestion of sludge, which was collected from a treatment plant of domestic wastewater by subculturing the bacteria in a selective medium of ASRB. The medium was composed of 2 g/L of sodium lactate, 0.3 g/L of sodium citrate, 0.1 g/L of yeast extract, 4.5 g/L of  $\text{Na}_2\text{SO}_4$ , 0.06 g/L of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 1.0 g/L of  $\text{NH}_4\text{Cl}$ , 0.5 g/L of  $\text{KH}_2\text{PO}_4$ , 2.0 g/L of  $\text{MgSO}_4 \cdot 4\text{H}_2\text{O}$ , 0.5 g/L of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.3 g/L of disodium ethylene diamine tetra acetate, and 0.2 g/L of  $\text{K}_2\text{CrO}_4$ . The mixed culture of ASBR was retained for a week under strict anaerobic conditions

before being subcultured. The microbial inocula were washed with the phosphate buffer solution (50 mM at pH7), and a mixed ASRB culture was seeded in the MFC anodic chamber.

### Bacterial nanowire formation

The *D. acetoxidans* developed anaerobically in the growth medium at 37°C. The cells were given 50 mM of sulfate as soluble and 50 mM of Fe (III) oxide ( $\alpha\text{Fe}_2\text{O}_3$ ) as insoluble electron acceptors. The cells were grown in the growth medium without an electron acceptor as a control experiment.

### MFC set up and operation

Experiments were conducted with the electrodes as solid electron acceptors in a dual-chamber MFC, similar to the arrangement of Alshehri *et al.*, (2016) with some modifications. A Nafion 117 proton exchange membrane (PEM; Dupont, USA) separated the two chambers. The working volume was 100 mL, and the total volume was 200 mL. The cathode chamber was then filled with a catholyte solution involving 30 mM Tris buffer solution (pH 7) and continuously purged using water-saturated air.

The pure culture of *D. acetoxidans* was seeded in the anode chamber, and a mixed ASRB culture was added in a separate experiment. Electron donor was given through a supply of organic substrate as fuel, and with the exception of the electrode, no electron acceptor or electron-shuttling mediator was used. The anode chamber medium was purged with a  $\text{N}_2$  and  $\text{CO}_2$  mixture in a ratio of 9:3 (v/v) in order to maintain the anaerobic conditions. Graphite felt with a surface area of 30  $\text{cm}^2$  was used as the cathodic and anodic electrodes (GF series, Electrosynthesis, USA). The electrodes were then connected to a platinum wire with a 150  $\Omega$  external resistance.

## Monitoring and calculation

When the MFC reached a steady state, automatic measurements of the voltage were taken using a digital multimeter (Sanwa CD800a, Japan) connected to a personal computer and Picolog software (Pico Technology Limited) at one second intervals. The corresponding current was based on equation  $I=E/R_{ext}$ , where:  $I$  is current (mA),  $E$  is voltage (mV) and  $R_{ext}$  is external resistance. The power ( $P$ ) was obtained by  $P=IE$ . The current density and the power density have been normalized based on the projected surface area of the anode via equations  $I_{An}=I/A_{An}$ , where  $I_{An}$  is current density and  $A_{An}$  is the surface area of anode,  $P_{An}=E^2/A_{An}R_{ext}$ , where  $P_{An}$  is power density. The polarization curve was obtained at different external resistance (50 - 1000 $\Omega$ ). Internal resistance was derived from the polarization curve as the slope. Coulombic efficiency ( $CE$ ) was derived from the equations  $C_p=It$ ,  $C_{max}=FfS_{COD}V_{An}$ , and  $CE=C_p/C_{max}$ , where  $C_p$  is the coulombs of energy produced,  $t$  is the time of stable voltage output,  $C_{max}$  is the theoretical maximum coulombs,  $F$  is Faraday's constant (96.485 C/mol of electrons),  $f$  is a factor of 1mol electrons/8g COD,  $S_{COD}$  is substrate concentration g COD/L, and  $V_{An}$  is a net volume of anolyte (mL). All the experiments were replicated twice at 37 °C under the same conditions.

## Phylogenetic analyses

Purity of the culture was done by DNA extraction and PCR amplification of the 16S rRNA gene with the aid of a forward primer (5'- ACC GTT AGA TGG CTC TAC TTG GGC AGA TTC GCT -3') and a reverse primer (5'-TGC CGC TGA AGC AGG TTC ACC TCC TAC GGC A-3'), and subsequent sequencing of the purified PCR products. Phylogenetic analysis was then done using BLAST (NCBI) and the standard DNA

sequencing program, and PCR amplification was done similarly for the mixed culture of ASRB using the forward and reverse primers as listed in table 1. The neighbor-joining method (Daly *et al.*, 2000) was then used during the phylogenetic analysis based on six ASRB predominant subgroups.

## Scanning electron microscopy

The surfaces of the graphite felt electrodes (both bare and treated) and the biofilm formation present on these surfaces during runs of the MFC were examined with scanning electron microscope (SEM, JSM-5410LV, JEOL Ltd., Japan). Distilled water was used to rinse the electrodes, fixed firstly with 2% of glutaraldehyde for 2 hours at 4°C. It was then rinsed 3 times using 0.05 M of sodium cacodylate buffer (pH 7.2) for 10 minutes at 4°C, and fixed further with 1% of osmium tetroxide in 0.05 M sodium cacodylate buffer for 2 hours at 4°C. Mill-Q water was then used to rinse the fixed electrode twice at room temperature before dehydrating with increased ethanol concentrations (30%, 50%, 70%, 80%, 90%, and 100%) for 10 minutes each at room temperature. After gently washing the electrode with 100% hexamethyldisilazane for 15 minutes at room temperature and making it air-dried overnight, the electrode was then coated before the SEM analysis with platinum.

## Atomic force microscopy (AFM)

After centrifugation of the microbial samples collected from MFC for 5 minutes at 3000 g and removal of the supernatant, distilled water was used to wash the pellet, and an aliquot sample (20 $\mu$ L) was applied onto a highly oriented pyrolytic graphite (HOPG) plate as a substrate (10 $\times$ 10 $\times$ 1.5 mm, grade ZYA, Ted Pella, USA). This sample was then air-dried and washed with distilled water 3 times. The

HOPG plate loaded with the cells was subjected to examination using Atomic Force Microscope (AFM, XE-100 series, Park System Scanning Probe Microscope, Park System, Santa Clara, USA) in either the contact or non-contact modes. In the case of the contact mode (conducting probe AFM), a nominal spring constant of 0.2 N/m was applied to a conductive cantilever (CDT-CONTR 3M-T, Park System) with a platinum-coated probe (Park system). The current response profile was obtained by applying a +5.0V bias voltage, and the current responses to sweep bias voltages in the range -1 V to +1 V were measured to form an *I-V* (current-voltage) curve. The formula  $p = R \times A / L$  was used to calculate the nanowire resistivity where  $p$  = resistivity ( $\Omega\text{m}$ ),  $R$  = resistance ( $\Omega$ ),  $L$  = length of the nanowires, and  $A$  = cross-sectional area of the nanowires ( $\text{m}^2$  was calculated using AFM height measurement), and the formula  $\sigma = 1/p$  was used to calculate the conductivity was calculated as the inverse of resistivity, where  $\sigma$  = electrical conductivity ( $\text{S}\cdot\text{m}^{-1}$ ) and  $p$  = resistivity ( $\Omega\text{ m}$ ).

### **Analytical techniques**

A Petroff-Hauser counting chamber was used to determine the cell density, and quantification of the cellular proteins was done as previously described (Chaudhuri and Lovley, 2003). The Bradford method was used with bovine serum albumin as standard to extract and measure the protein attached to the electrode (Quick Start Bradford Protein Assay, Bio-Rad, USA).

Determination of the substrate concentration was made by using visible spectroscope, then ion chromatographe (Cordas *et al.*, 2008). The sulfide concentration was determined using a silver/sulfide ion selective electrode. Quantification of the  $\text{Fe}^{2+}$  was done by a ferrozine method (lovley and Phillips, 1986).

Purity testing of the cell cultures was done through PCR amplification of the 16S rRNA gene.

## **Results and Discussion**

### **Bacterial nanowire formation**

Examination of the bacterial nanowire formation was done by growing a pure culture of *D. acetoxidans* under strict anaerobic conditions in the presence or absence of electron acceptors. Sulfate or Fe (III) oxide was utilized by the *D. acetoxidans* for the growth as the sole electron acceptor. This led to an increase in its biomass, whereas almost no growth was evident in the absence of an inorganic electron acceptor (Fig. 1). Dissimilatory reduction occurred of sulfate to sulfide, and of Fe (III) oxide to  $\text{Fe}^{2+}$  (Fig. 2), and the production of nanowires during this period was confirmed by AFM. In the presence of Fe (III) oxide, nanowire formation around the cell was induced markedly in numbers, as the Fe (III) oxide served as an electron (Fig. 3A). This finding confirms earlier demonstrations of bacterial nanowire production by iron reducing *G. sulfurreducens* and *S. oneidensis* when they were grown on poorly crystalline Fe (III) oxides (Reguera *et al.*, 2005; Groby *et al.*, 2006). With *D. acetoxidans*, only a single filament was produced when grown on the soluble electron acceptor ( $\text{SO}_4^{2-}$ ).

This filament appeared as a polar flagellum for cell motility (Fig. 3B), and the detection of a single filament was in the absence of an inorganic electron acceptor (Fig. 3C). Microbial nanowire formation in numerous quantities can therefore be stimulated (as it clear from these results) for allowing contact with insoluble electron acceptors, as for metal oxides present in surface systems, and for transferring electrons for dissimilatory reduction directly.

## MFC performance

For examining the performance of MFC, *D. acetoxidans* was seeded in its anode chamber to function as a microbial catalyst. There was no provision of any inorganic electron acceptors or electron-shuttling mediators with the exception of the graphite felt as a solid electrode. When the circuit was connected (Fig. 4), a current was produced, and the maximum power density was  $7.9 \text{ W/m}^3$ . The consumption of organic fuel as substrate was due to cell growth and production of electric current. This was indicated by a gradual increase in the cell's biomass in the anode chamber over time (Fig. 5). Dense biofilms were formed continuously by the cells on the anode surface, and at the end of the MFC operation, the biomass which reached  $0.08 \text{ mg-protein/cm}^2$ -anode surface was found to be higher than previously reported observations (Bond and Lovley, 2003; Chaudhuri and Lovley, 2003). The electron transfer rate calculated based on the bacterial populations attached to the anode surface was  $2.7 \text{ } \mu\text{mol-electrons/mg-protein/min}$ , and the value of the coulombic efficiency (CE) was found to be 93% on substrate oxidation. The results show *D. acetoxidans* to be capable of directly transferring electrons to the solid electrode as the electron acceptors. Measurements of the electrical conductivity were taken to further confirm the result.

## Morphological and electrical conductivity of the nanowires

The morphological features of the nanowires and their conductivity were analysed by AFM using a conducting probe on cells collected from the MFC anode and applied to the HOPG surface. Thin bacterial nanowires were observed in the cell topography, which were of nano scale thickness like wires (Fig. 6). Using non-contact mode AFM for additional characterization, the length, width and height of the nanowires were measured and found to

be an average of  $3 \text{ } \mu\text{m}$ ,  $7\text{-}13 \text{ nm}$ , and  $5\text{-}8 \text{ nm}$ . These nanowire dimensions were similar to those produced by *S. oneidensis* ( $>10 \text{ } \mu\text{m}$  length,  $5\text{-}15 \text{ nm}$  width, and  $\sim 9 \text{ nm}$  height) (Gorby *et al.*, 2006), and *G. sulfurreducens* ( $\sim 20 \text{ } \mu\text{m}$  length, and  $3\text{-}5 \text{ nm}$  width) (Reguera *et al.*, 2005).

Measurement of the current around the cells and the nanowires, and the current profile across the nanowires including the HOPG background showed evidence of a current response from the nanowires (Fig. 7). The *I-V* curve indicated electrical conductivity of the nanowires from the MFC (Fig. 8), and the response of the electrical current to the applied voltage was supposed to be linear within the range of swept voltage. Measurement of the bias voltage of conductivity on the HOPG as a background current ( $\sim 9 \text{ pA}$ ) showed it was negligible for analyzing conductivity. The linear *I-V* curve pattern was consistent with findings from earlier experiments on nanowires, such as on *G. sulfurreducens* and *S. oneidensis* (Reguera *et al.*, 2005; El-Naggar *et al.*, 2010). The response of the electric current along the nanowires was almost constant. This is similar to the study on *S. oneidensis* (El-Naggar *et al.*, 2010) in which there was almost uniform electrical conductivity along the nanowires, non-linear electric current in response to applied voltage, and small fluctuations in conductance in the *I-V* curve.

The fluctuations in conductance in the case of *S. oneidensis* might be explained by the lack of adequate contact of the nanowire with the conductive tip, as these can become separated when in the low force range by a few angstroms. For the nanowires produced by *D. acetoxidans*, the resistivity and conductivity were  $1.88 \times 10^{-8} \text{ } \Omega\text{m}$  and  $7.32 \text{ S}\cdot\text{m}^{-1}$ , respectively. The obtained data was comparable to resistivity of nanowires produced by *S. oneidensis* ( $1 \times 10^{-8} \text{ } \Omega\text{m}$ ) (El-Naggar *et al.*, 2010) m and conductivity of

nanowires produced by *G. sulfurreducens* (~ 5 S·m<sup>-1</sup>) (Malvankar *et al.*, 2011). The nanowires produced by *D. acetoxidans* are thus electrically conductive and capable of facilitating electron transfer to the solid electrode directly without the need for electron shuttling mediators.

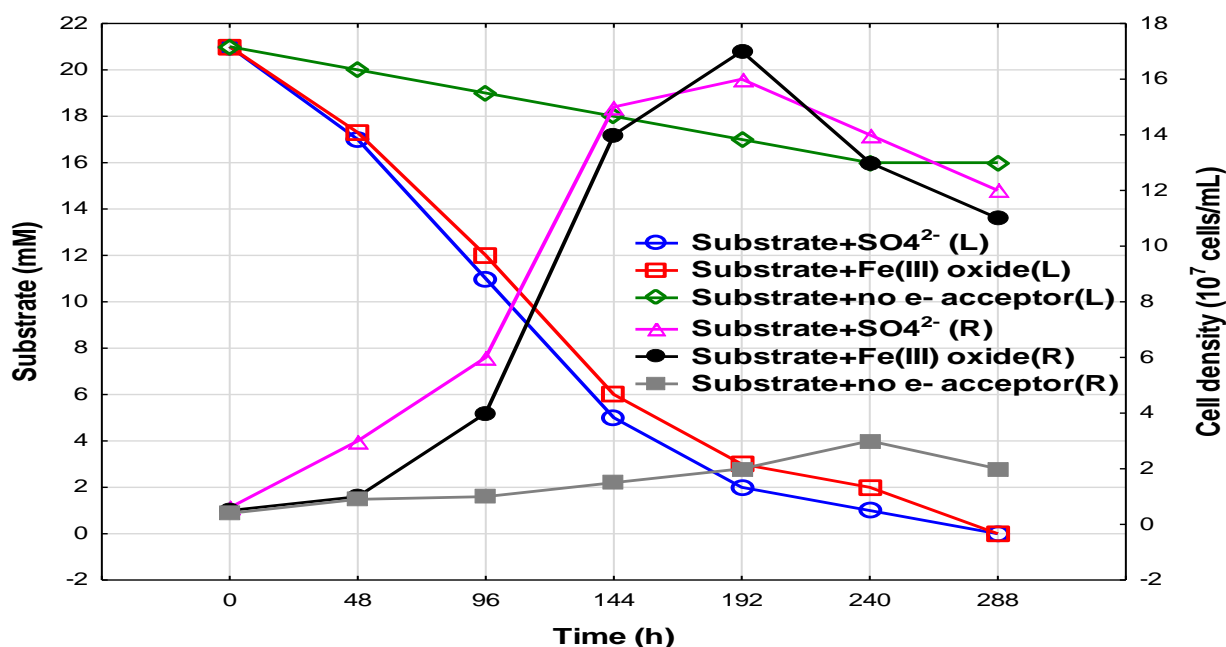
### Electrode characterization

Observation of the formation of biofilm on the MFC anode surface was made by SEM (Fig. 8A) with rod-shaped cells measuring 1.6-4.0 μm in length and 0.4-0.6 μm in width. Nanowires were produced by the bacteria that colonized the anode surface for connecting to other cells and for attaching to the surface of the solid electrode. Electron transfer among the cells may have been facilitated in part by these nanowires. Phylogenetic identification by PCR amplification was performed for confirmation of the purity of anode biofilm culture. For 16S rRNA gene fragments from the anode biofilm, the PCR amplification showed a prominent band (Fig. 9) with a

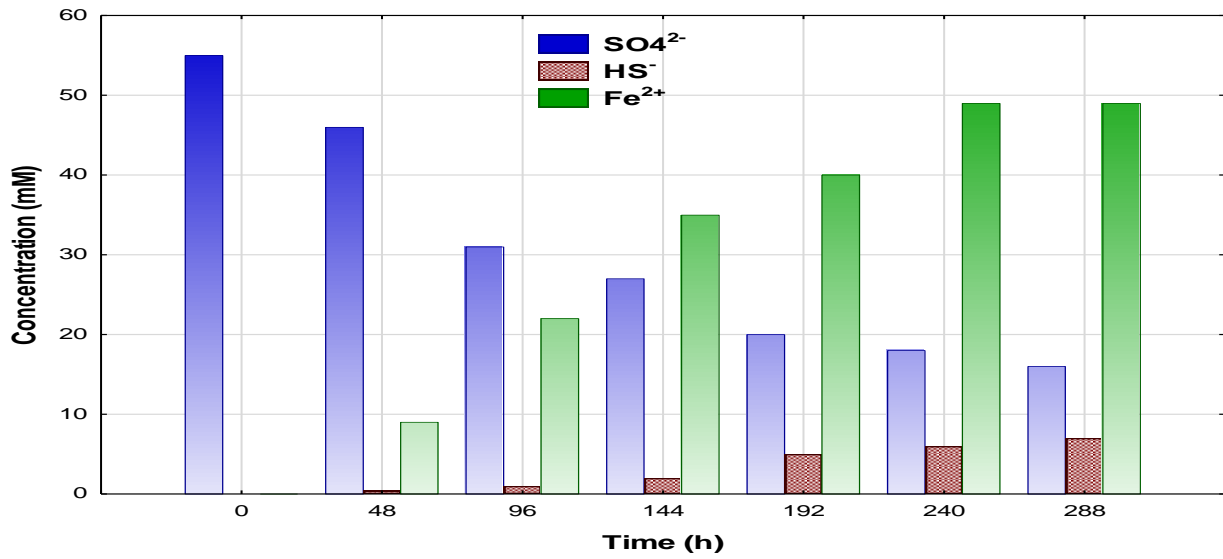
sequence identical to that of *D. acetoxidans* (GI: 444138542), thus confirming that the bacterial strain used was a pure culture, and that the current production was catalyzed by a single strain in the case of *D. acetoxidans*. Examination of the microbial nanowire formation when using a mixed ASRB culture as a microbial catalyst under the same conditions in the MFC as for *D. acetoxidans* revealed the maximum power density to be 7.0 W/m<sup>3</sup>, and a CE value of 92% on substrate oxidation, which was lower than when obtaining a pure *D. acetoxidans* culture.

At the end of the MFC operation, the density of the attached biomass was 0.086 mg-protein/cm<sup>2</sup>-anode surface, and the electron transfer rate of 2.2 μmol-electrons/mg-protein/min was lower than for *D. acetoxidans*. This was taken to indicate that electron recovery from organic fuel and electron transfer to the electrode are more effective for a single strain of *D. acetoxidans* to a slight degree relative to mixed ASRB culture.

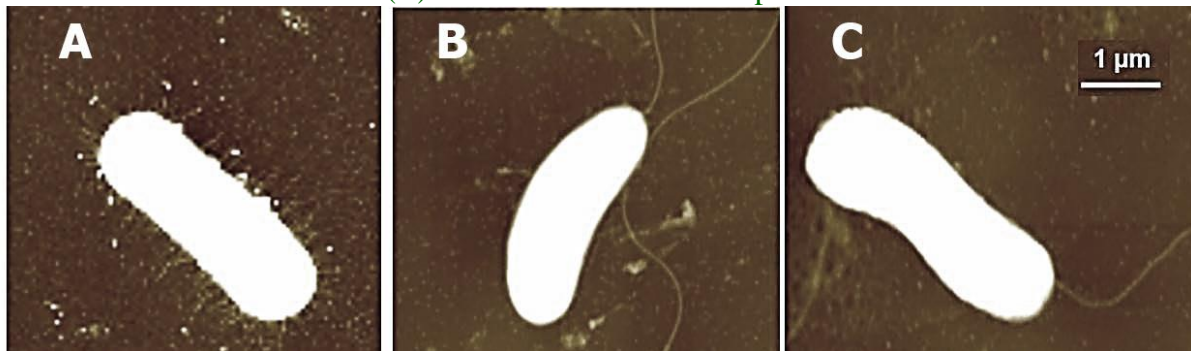
**Fig.1** Substrate consumption and cell growth during the growth of *D. acetoxidans*



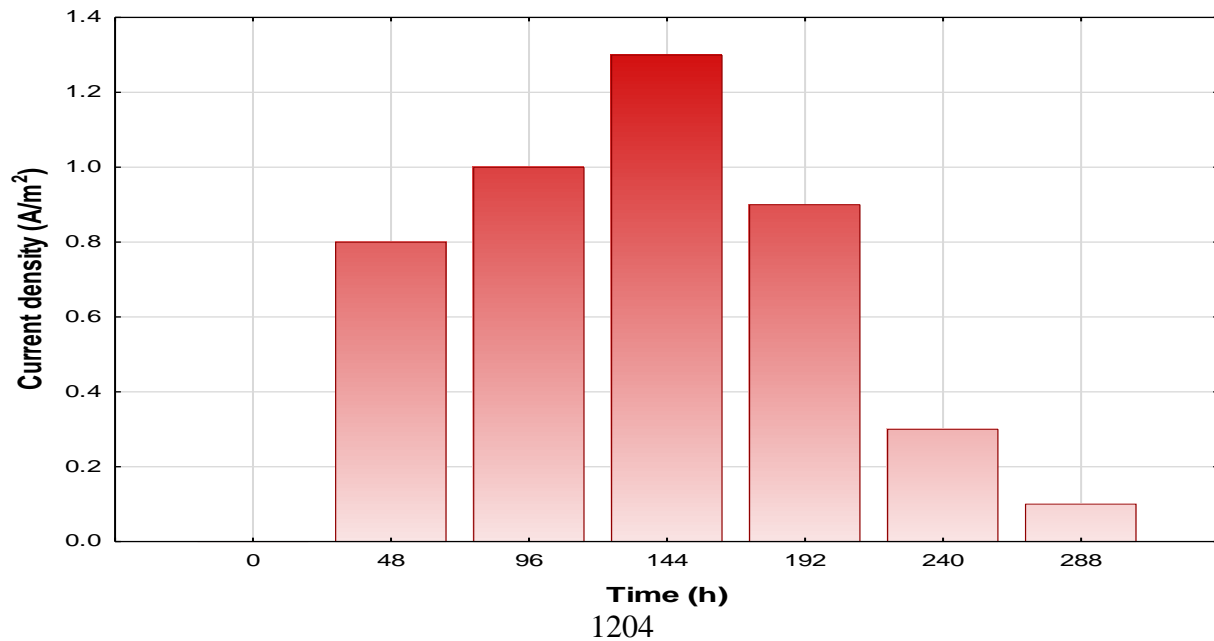
**Fig.2** Reduction of  $\text{SO}_4^{2-}$  and generation of  $\text{HS}^-$  and  $\text{Fe}^{2+}$  during the growth of *D. acetoxidans*



**Fig.3** AFM images (non-contact mode) of nanowires produced by *D. acetoxidans* (A) on Fe (III) oxide as an electron acceptor, (B) with  $\text{SO}_4^{2-}$  as an electron acceptor, and (C) Without an electron acceptor

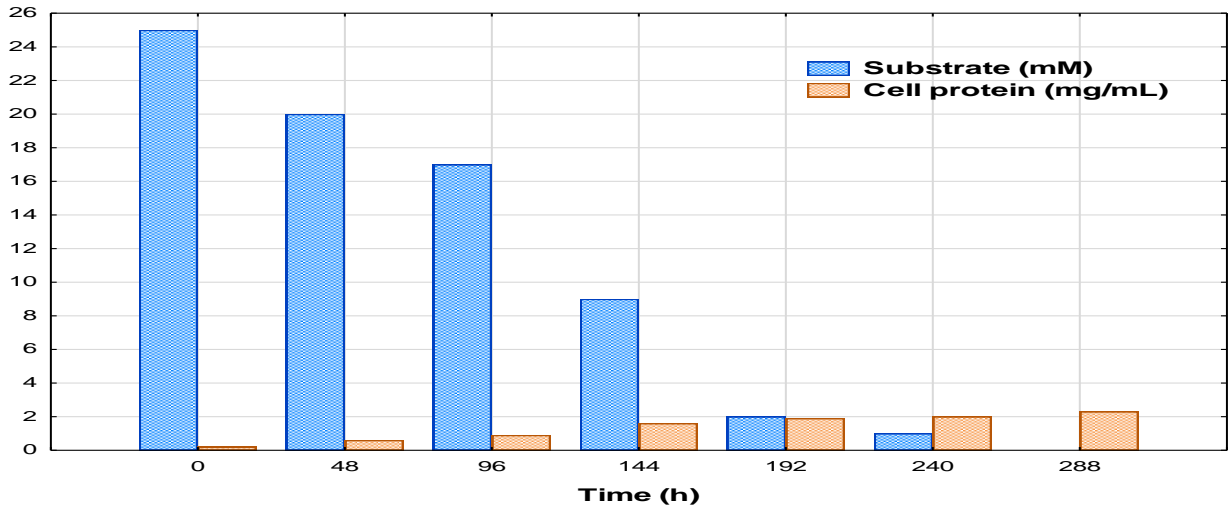


**Fig.4** Production of a current density by *D. acetoxidans* in the MFC

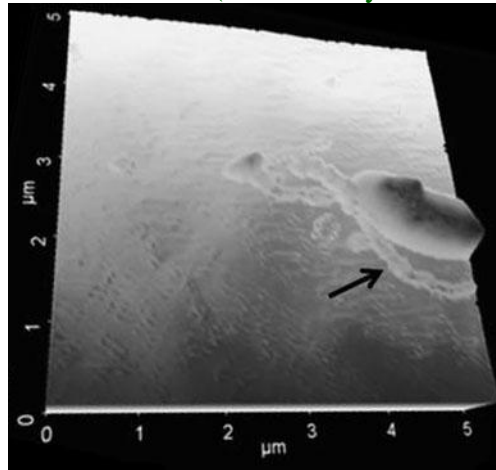




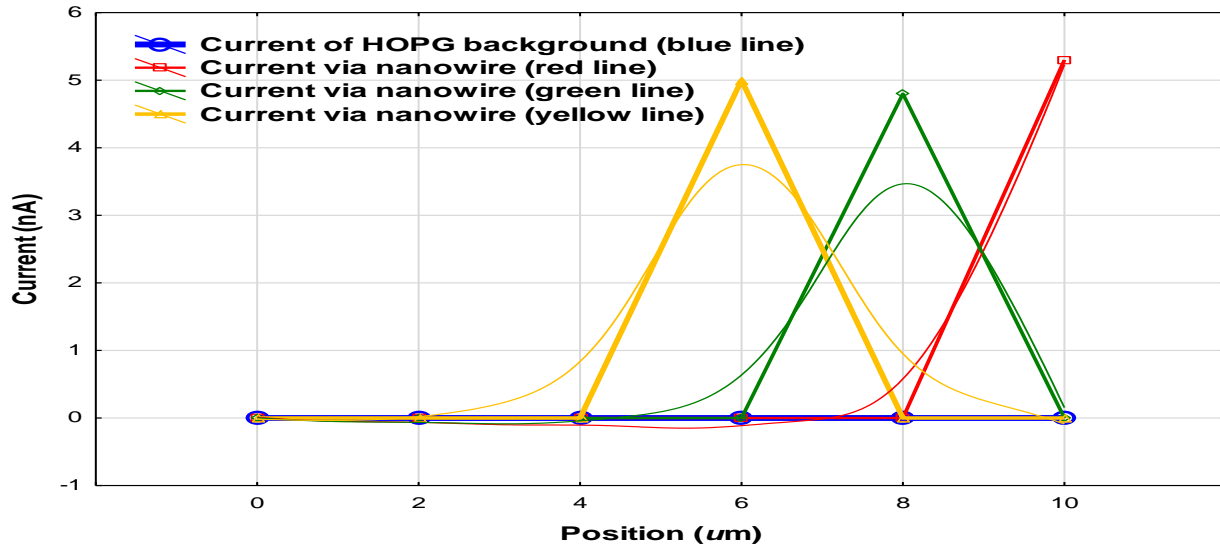
**Fig.5** Behavior patterns pertaining to the substrate and cell protein in the anode chamber



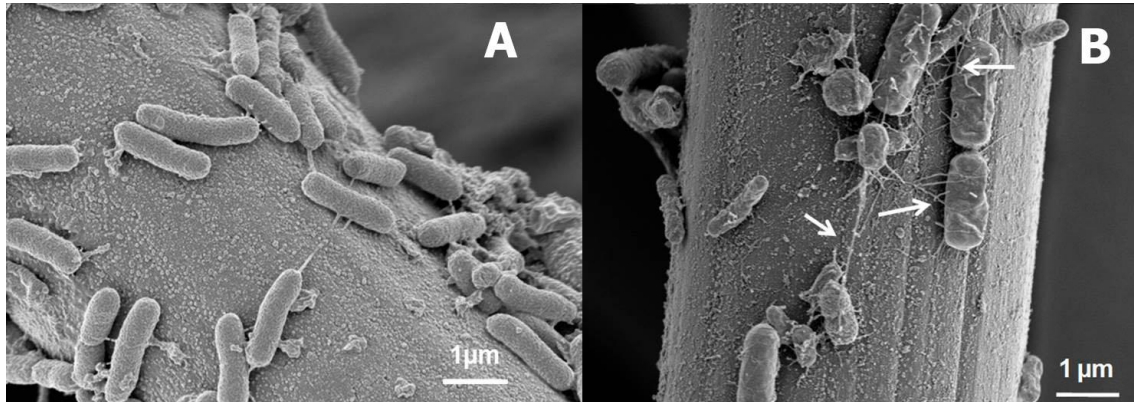
**Fig.6** Conducting probe AFM result shows, 3-dimensional topography of Bacterial nanowire (indicated by black arrow)



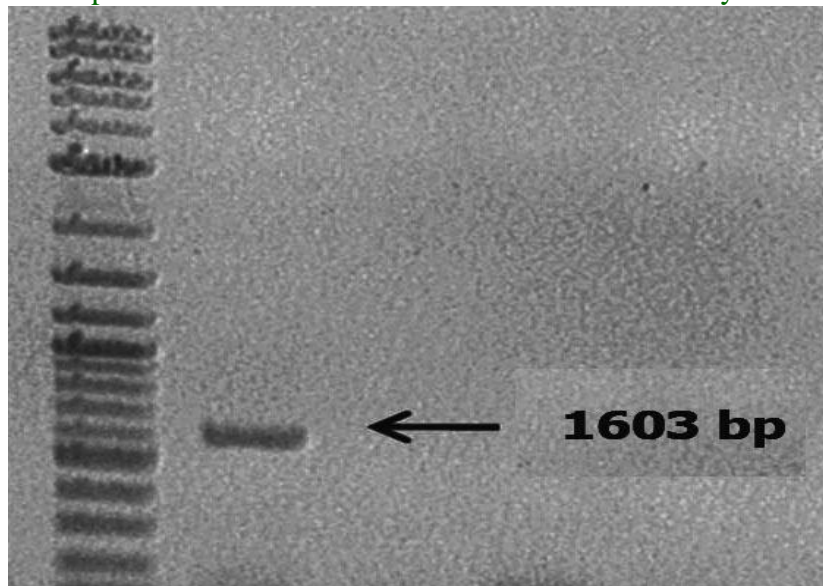
**Fig.7** Conducting probe AFM results, as current profile following the Yellow, green, red, and blue lines



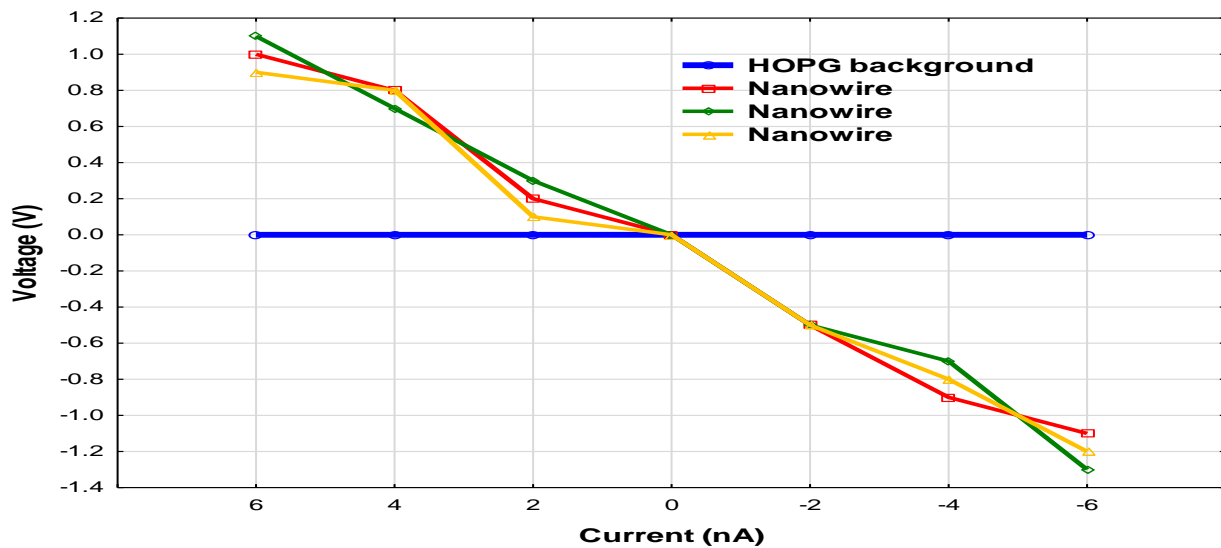
**Fig.8** (A) SEM images of the biofilm and nanowires of *D. acetoxidans*, (B) The biofilm and Nanowires web (indicated by arrows) formed by the mixed culture of ASRB



**Fig.9** PCR amplification of 16S rRNA from a biofilm formed by *D. acetoxidans*



**Fig.10** Current responses of nanowires compared to those of a background (Indicated by the yellow, green, red, and blue)



**Table.1** The 16s rRNA-targeted PCR primer sequences specific to Six predominant subgroups of ASRB

Primer sequences	Sequences 5'–3'	Specificity	Annealing temperature (°C)	Expected size of product (bp)
ADM250	ACT TAG GCG ATA ACG GCT C	Group 1	58	876
AOM872	CCC ATA GCA ACA GCT AGC AC			
AEB901	GTA CGC AAC GAT TCA CTG TCC TG	Group 2	66	1820
AFB1507	GGA GTA CGT TAG GTG CCC TGG ATA			
AAM369	GAT CTA ATG CCG GTC AG GAA	Group 3	64	940
AGM2206	CTC ATT AAG ATG AGT CTG TCA ATT			
ASA117	AGC CTG CCT AAT TCA GAT CTG G	Group 4	60	1738
ASD873	CGC GTT GCG GAG TCG CTG CCC T			
ASC1115	GAT CTG CCA CAC TTG AAC TGA CA	Group 5	65	940
ASC1865	CCG GCA GTA TCT TTA GAG TCC			
ADV889	GTG GCC GCG TCC CAT TAG C	Group 6	61	583
AFV808	GTC CTA CAC CTA GGA TCC ATC			

The latter formed dense biofilms tangled with many nanowire webs on the anode surface during the MFC operation (Fig. 8B). PCR amplification was performed for conducting phylogenetic analysis of the biofilm based on 6 predominant ASRB subgroups (Table 1). The products contained a prominent band, which indicated a matching of the primer sequences with the ones that were specific for the 6<sup>th</sup> group. Identification was made on this basis that the bacteria were a species of the genus *Desulfovibrio*, which include of *D. acetoxidans*.

*D. acetoxidans*, taken to be representative of sulfate reducers in subsurface environments, was shown in this study to be capable of electrically conductive nanowire production in soluble electron acceptor (SO<sub>4</sub><sup>2-</sup>) limiting conditions of only insoluble electron acceptor (Fe(III) oxide or solid electrode) availability. Previous demonstrations have been made using the iron reducing bacteria *G. sulfurreducens* and *S. oneidensis* as being capable of producing electricity when having an electrode as a sole electron acceptor in an MFC without mediation (Bond and Lovley,

2003; Gorby *et al.*, 2006). It was proposed that these bacteria are capable of transferring electrons to electrodes by interacting with the electrode surface directly through the bacterial nanowires (Gorby *et al.*, 2006; El-Naggar *et al.*, 2010), and the role of the nanowires has been demonstrated in biofilm formation, and electron transfer route in Fe (III) oxide reduction (Reguera *et al.*, 2005; Gorby *et al.*, 2006; Leang *et al.*, 2010).

This study also showed *D. acetoxidans* to be capable of producing microbial nanowires for facilitating direct dissimilatory extracellular transfer of electrons to the solid electrode. This was supported by current produced when catalyzed in the MFC system by the transfer of electrons from the cells to the electrode. The growth of *D. acetoxidans* cells on the insoluble electron acceptor (Fe (III) oxide) produced many bacterial nanowires thinly around the cells. When the *D. acetoxidans* cells were grown either on a soluble electron acceptor ( $\text{SO}_4^{2-}$ ) or without electron acceptors, a single thin filament was observed which appeared to be the bacterial nanoscale flagella. The results are consistent with those obtained by Childers *et al.*, (2002) for *D. acetoxidans* grown on  $\text{SO}_4^{2-}$  which produced a single nano sized flagellum, and the results suggest nanowire formation by *D. acetoxidans* occurs when there are limited soluble electron acceptors ( $\text{SO}_4^{2-}$ ).

As an alternative method of extracellular transfer of electrons, the nanowires function as electron mediators by means of direct contact with insoluble Fe (III) oxide. The iron reducers' *G. sulfurreducens* and *S. oneidensis* however, have been demonstrated directly transferring electrons to poor crystalline Fe (III) oxides via bacterial nanowires (Reguera *et al.*, 2005; Gorby *et al.*, 2006). Furthermore, when cultivated under electron acceptor-limiting conditions, nanowire production capability has also been reported for *S.*

*oneidensis*, *Cyanobacterium synechocystis*, and *Pelotomaculum thermopropionicum* (Gorby *et al.*, 2006). Measurement of conductivity while in AFM contact mode shows that *D. acetoxidans* produce nanowires that are effective as electrical conductors. These nanowires exhibit *I-V* curves showing them to be more conductive than the single filaments produced when growth of *D. acetoxidans* cells occur either with or without a soluble electron acceptor ( $\text{SO}_4^{2-}$ ) (Fig. 10). Further examination of the nanowire capability of facilitating extracellular electron transfer in a mediator-less MFC was made by using a solid electrode as the insoluble electron acceptor. The electrons were transferred by the *D. acetoxidans* directly to the solid electrode without mediating electron shuttling.

*D. acetoxidans* produced a higher maximum power, in comparison to the results for power in other studies for *S. oneidensis*  $0.25 \text{ W/m}^3$  and *G. sulfurreducens*  $0.35 \text{ W/m}^3$  (Bond and Lovley, 2003; Lanthier *et al.*, 2008). The electron transfer in the case of *D. acetoxidans* is therefore more effective relative to what has been recorded for nanowires of the other two iron reducers *S. oneidensis* and *G. sulfurreducens*, and this rate was also higher than when 2.2 of flat gold and 2.4  $\mu\text{mol-electrons/mg-protein/min}$  carbon cloth were used as an electrode by Richter *et al.*, (2008).

The electrical conductance between the biofilm and electrode seemed almost as high when a pure culture of *D. acetoxidans* was used in the MFC relative to the use of a mixed ASRB culture. This is confirmed further when it was noted that the electron transfer rate to the electrode was not much different for a pure culture of *D. acetoxidans* and a mixed ASRB culture. The latter belongs to the *Desulfovibrio* genus which includes *D. acetoxidans*, and those are present predominantly in nature, were isolated from

the anaerobic digestion of the sludge obtained from domestic wastewater.

Biofilms were formed as a cell network when the ASRB were clumped together, and this network was reinforced by the nanowires which stimulated electron transfer through cell-to-cell interactions. The production of current was enhanced by means of electrically conductive bacterial nanowires produced by *D. acetoxidans*, which developed different morphological features according to the different growth conditions. The presence of extracellular electron acceptors was found to be a particularly important growth facilitating factor, and the nanowires produced by the *D. acetoxidans* coming into contact with tiny Fe (III) oxide particulates were themselves tiny in size and numerous in quantity. When the large surface of a graphite electrode was used by these cells as the sole electron acceptor, the filaments produced had longer and thicker dimensions, which provided greater contact with the solid electrode. When growth occurred with soluble electron acceptors or without electron acceptors, only a single filament was produced by the *D. acetoxidans* similar to the flagellum in most bacteria, as used for motility.

This study concludes that ASRB includes *D. acetoxidans* (an ASRB found predominantly in soil and sediments) generate nano scale bacterial appendages that are electrically conductive. Bacterial nanowires facilitate electron transfer to the extracellular electron acceptors, such as insoluble materials. The nanowires allow for the bacteria to extend their capability of transferring electrons. This contributes to the microbial impact on the speciation greatly, as well as the cycling of inorganic nutrients present in anaerobic subsurface systems. The findings of this study could prove useful in exploiting ways of stimulating the transfer of electrons to an electrode directly and without the need for

mediating electrons, which can potentially enable them to catalyze the production of current effectively in MFC.

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**How to cite this article:**

Alshehri, A.N.Z. 2017. Formation of Electrically Conductive Bacterial Nanowires by *Desulfuromonas Acetoxidans* in Microbial Fuel Cell Reactor. *Int.J.Curr.Microbiol.App.Sci.* 6(8): 1197-1211. doi: <https://doi.org/10.20546/ijcmas.2017.608.148>