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RESEARCH ARTICLE

Role of Pseudomonas aeruginosa biofilm formation in mediator less Microbial fuel cell

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Abstract

The research includes Gram-positive bacterium with rod-shaped cells was isolated from the anodic compartment of a microbial fuel cells (MFCs). The isolate was identified to be one of the Bacillus species dominating the anodic microbial community of the MFC, During the closed circuit experiments, biofilm formation from anode blocks indicated that the two species investigated the viability was higher adjacent to the electrode. In culture experiment with Pseudomonas spp. and Bacillus produced more current compared to the pure cultures, Pseudomonas spp. and Bacillus spp. separately generated 2.5 and 0.2 mA respectively while together the highest current generated was 4.6 mA. And levelled off among 5-7 after which it began to decrease. The biofilm height of Pseudomonas spp. was light pellicle on medium surface and on tube wall and that for Bacillus spp. was light pellicle on tube walls and at medium surface. Both developed smaller micro-colonies over the top of the biofilm layer. Biofilm form in Gram negative are flatter and more uniform than the Gram positive. We suggest that the most active part of the biofilm, playing a major role in the extracellular electron transfer (EET) process, the electrode. The decreasing viability away from the anode can rather be attributed to limitations for the electron transfer towards the electrode than substrate limitation. The overall concept that the Gram-positive Bacilli use electron shuttles produced by other bacteria is intriguing. The interaction of Bacillus spp. with Pseudomonas spp., more specifically with secondary metabolites produced by Pseudomonas spp., and the role of other factors such as quorum-sensing molecules and/or other mediators should not be underestimated and need to be focused on in further studies.

INTRODUCTION

Microbial fuel cells (MFCs) use bacteria as catalysts to oxidase organic and inorganic matter and generate electrical current. The most widespread proposed use of MFCs, and now the broader term Bio-electrochemical Systems (BESs) (Allen and Bennetto, 1993; Lovley, 2006), is for electricity generation during wastewater treatment (Aelterman et al., 2006; Bond and Lovley, 2003). Irrespective of the goal, the cornerstone of BESs is the capacity of microorganisms to perform or participate in extracellular electron transfer (EET).In this process, microorganisms effectively pump electrons outside the cell, using direct or indirect mechanisms, towards the electron acceptor, i.e. the anode, which is insoluble and exterior to the cell. They also provide us with a platform to perform more fundamental research such as that presented in this paper. Direct EET occurs via electron flow through outer membrane proteins (Hernandez and Newman, 2001) or potentially through electrically conductive bacterial appendages such as nanowires (Gorby, et al., 2006; Reguera et al., 2005) that make physical contact with the anode or other bacteria in the vicinity. Indirect EET involves exogenous (e.g. humics) (Rabaey et al., 2005a) or endogenous (e.g. phenazines) (Perneel et al., 2007; Fernandez and Pizarro, 1997) soluble molecules (called mediators or redox shuttles) that act to shuttle electrons through the extracellular aqueous matrix from the cells to

the anode (Hernandez et al., 2004). Although there is some evidence that increased current production in Gram-positive bacteria in an MFC is achieved through redox shuttles (Rabaey and Verstraete, 2005; Pham et al., 2006) other information pertaining to their role in EET is limited (Hernandez et al., 2004; Pham et al., 2006; Kim et al., 1999). Generally, Gram-positive bacteria on their own make limited current in comparison to the Gram-negative (Marshall and May, 2009).

Biofilm is an extracellular polymeric substance (EPS) encased, surface adhering microbial community. Conventional theory categorizes biofilm structure around three basic stages of development, initial attachment, maturation and detachment (Tapia et al., 2009). The EPS physically immobilize the bacteria while at the same time provide them opportunity for cell to cell contact and communication. Moreover, electron transfer is constrained by the distance over which electrons need to travel to the electron acceptor and therefore, having a greater understanding of biofilm structure and development in BESs may provide us with more of an insight this area.

This study aimed (i) to investigate the viability, structure and current production of Gram-positive and -negative pure culture and co-cultures biofilms when growing on a closed circuit (current flowing) and open circuit (soluble electron acceptor provided) anode (ii) to investigate whether bacteria in co-culture generate different levels of current than pure cultures and (iii) to investigate biofilm structure and development between pure and cocultures on the anode. To approach these goals two types of well-known bacteria (Pseudomonas and Bacillus) were used.

2. Materials and methods

2.1 Sources of fresh water

Freshwater sediment was collected from Shishin Sewage Treatment Station located at Tikrit city, Iraq. The bed of a burn before it drained into reservoir. Samples were collected in a 10 L bucket and transferred to a 20 L carboy for transporting back to the laboratory where they were stored at 4°C. Health and safety regulations were observed throughout.

2.5 Pure cultures and media

Pure cultures of Pseudomonas spp. and Bacillus spp. were used, these cultures were all grown in a Luria-Bertani medium containing 10.0 g/L NaCl, 10.0 g/L tryptone, 10.0 g/L yeast extract [36], 20 mM of sodium acetate. The catholyte 0.5% NaCl. Cultures were pre-grown to mid exponential phase (determined by OD 600 nm measurement). For the experiments bacterial inoculate were standardized for cell numbers by estimating the inoculum density. The inoculum for Pseudomonas spp. and Bacillus spp. was estimated from total cell counts and added to the medium to achieve a final inoculum density of 1.29 x 107 cells ml-1.

2.2 MFC Reactor operation

MFC reactors consist of two chambers (anode & cathode) joined with salt bridge, Graphite electrodes are most commonly used for MFC (Allen and Bennetto, 1993; Reguera et al., 2005; Pham et al., 2006). Although there are other materials used for the electrode to allow better conductivity, the most important idea is based on the size of these electrodes: the greater the surface area of the anode and cathode in any situation, the higher the power density (Hernandez and Newman, 2001; Hernandez 2004; Kim 1999), there will also be more room for the microorganisms or mediators to transfer electrons via direct or indirect physical contact.

Anodes and cathodes of the reactors were flushed prior to the experiment in order to create aerobic conditions. Then the anode was filled with filtered autoclaved waste water with the addition of glucose 0.5% and sodium acetate 3mM as a substrate with final COD 555 mg/L. aerobic autoclaved media(wastewater), with soluble electron acceptor for the closed circuit experiments, while the cathode was filled with (5% NaCl). The anodes were then inoculated with the pure cultures of (Bacillus spp, Pseudomonas spp., mixed culture of Bacillus & Psudomonas spp. and blank as control negative) anodes and cathodes are c connected to ovameter. Continuous experiments were run for seven days with blocks taken for current measure at (0, 4, 8 12, 24, 72 and 144) hours under aerobic conditions.

2.3 Anode sampling

At the end of each experiment whole anodes of MFC reactor were removed and the biofilm was removed from the anode by cotton swab in a sterile plastic tube containing 30 ml of 0.85 % NaCl solution. Diluted cell suspensions (10-1 to 10-6) were inoculated onto luria-bertani agar and incubated aerobically at 37°C for 2 days transferred to 50 ml sterile. The anode biofilm was sloughed off from the anode by suspending the anode in physiological saline for 20 minutes and the biomass suspension was stored at -20°C and used for further microbial analysis.

2.4 Biofilm formation

To test biofilm production overnight cultures were used to inoculate liquid MSgg medium (100 mmol l^1 MOPS pH 7.0, 0.5% glycerol, 0.5% glutamate, 5 mm potassium phosphate pH 7.0, 50 µg ml^{-1} tryptophan, 50 µg ml^{-1}

phenylalanine, 2 mmol l^{-1} $MgCl_2$, 0.7 mmol l^{-1} $CaCl_2$, 50 µmol l^{-1} $FeCl_3$, 50 µmol l^{-1} $MnCl_2$, 2 µmol l^{-1} thiamine, 1 µmol l^{-1} $ZnCl_2$) (Branda et al., 2001), and cells grown at 37 °C in static conditions for up to 48 h. Cells forming a solid layer at the liquid–air interface were considered as biofilm producers. Pseudomonas spp., the putative strain known to be capable of producing pyocyanin at elevated levels and bio-surfactants, can serve as soluble electron shuttles for the Gram-positive Bacillus spp. to use or that they at least invoke extracellular electron transfer by Bacillus spp., and generated currents up to 4.6 mA.

3. Results and discussion

A Gram-positive bacterium with rod-shaped cells was isolated from the anodic compartment of an MFC. The isolate was identified to be one of the Bacillus species dominating the anodic microbial community of the MFC, furthermore, applying the same procedure, also Pseudomonas sp. strains as Gram-negative were found. Using three cultures (two were pure and the third was mix, closed circuit (in the presence of anode to cathode current) and batch experiments were run for seven days each in an MFC as shown in (Figure. 1)

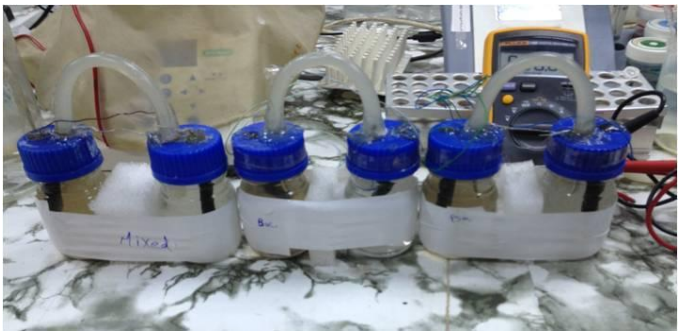
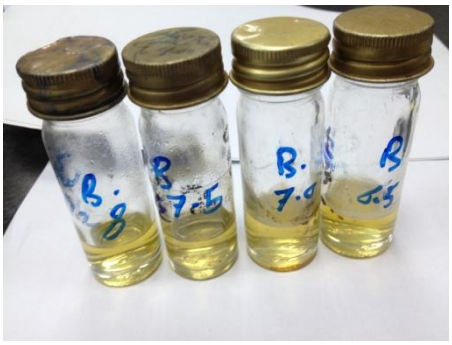


Figure (1): two-chambers of microbial fuel cell example of microbial fuel cell, cells producing electricity and biofilm formation

During the closed circuit experiments, biofilm formation from anode blocks (Fig. 2) indicated that the two species investigated the viability was higher adjacent to the electrode.



A

B

Figure (2): Biofilm formation by species isolated from dominant MFC bacteria on A. Luria- Bertani medium, B. MSSgg minimal medium

This study designed depending on microbial interaction, and aspects such as power efficiency was considered. However, overall, mechanistic principles that are true for this system can be also applicable in larger systems. Beside one study (Park et al., 2001), no electrochemically active Gram-positives have been described thus far. It is a query how these bacteria can interact with electrodes while they have a rigid cell wall with peptidoglycan layers. In a recent study (Milliken and May, 2007), Gram positives that were not electrochemically active were triggered to produce current by the addition of humic acids or their analogs. However, there is little known about the true mechanism through which Gram-positive bacteria demonstrate electrochemical activity. According to that, from the

results depicted in Table. 1, it can be seen that Bacillus spp. was not or barely electrochemically active. Pseudomonas spp., the putative strain known to be capable of producing pyocianine at elevated levels and bio-surfactants, can serve as soluble electron shuttles for the Gram-positive Bacillus spp. to use or that they at least invoke extracellular electron transfer by Bacillus spp., and generated currents up to 4.6 mA (Table 1).
Co-culture experiment with Pseudomonas spp. and Bacillus produced more current compared to the pure cultures (Table 1). For example, Pseudomonas spp. and Bacillus spp. separately generated 2.5 and 0.4 mA respectively while together the highest current generated was 4.6 mA. And levelled off among 5-7 after which it began to decrease.

Table (1): Comparison of current generation and biofilm in pure and co-cultures

Bacterial strain	Current generation (mA)	Morphological characteristics of biofilm
Bacillus spp	0.4	light pellicle on tube walls and at medium surface
Pseudomonas spp	2.5	light pellicle on medium surface and on tube wall
Pseudomonas+ Bacillus (co-culture)	4.6	pleated pellicle on the walls and on medium surface, non-fragmented
Blank	0.1 - 0.2	No biofilm

During the pure culture of a closed circuit experiments the heights of the pure cultures biofilms were less than that of the co-cultures experiments (Table 1). For example, the biofilm height of Pseudomonas spp. was light pellicle on medium surface and on tube wall and that for Bacillus spp. was light pellicle on tube walls and at medium surface. Both developed smaller micro-colonies over the top of the biofilm layer. Biofilm form in P. aeroginosa are flatter and more uniform than the Bacillus (Table 1). During these pure culture experiments species Bacillus spp. delivered low current throughout while the P. aeroginosa produced a much higher current as shown in Table 1.

In Bacillus spp. form biofilm slower than co-culture; Pseudomonas and Bacillus spp., with pleated pellicle on the walls and on medium surface, non-fragmented respect to that in Bacillus alone, Moreover, the biofilms became less dense developing while prolonged biofilm development revealed less coverage of the electrode giving way to the formation of channels and loss of biofilm mass, similar to that observed in the Pseudomonas or in co-culture (Pseudomonas & Bacillus) biofilm (Figure 4).

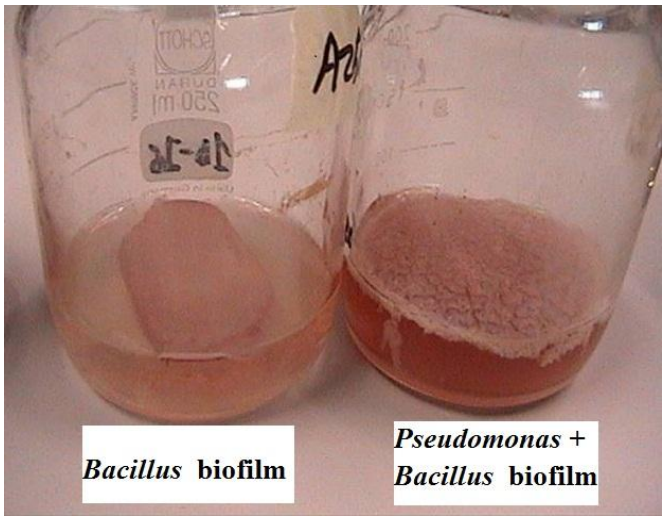


Figure (4): Biofilm formation by pure culture Bacillus and co-culture (Bacillus and Pseudomonas)

With slight variations between biofilm cultures (Figure 4). This may suggest that the most active part of the biofilm, playing a major role in the extracellular electron transfer (EET) process, the electrode. The decreasing viability away

from the anode can rather be attributed to limitations for the electron transfer towards the electrode than substrate limitation. At the current densities observed, it appears unlikely that proton accumulation limited the biofilm performance, as observed previously (Han- Shin, and Park, 2013). During these experiments the G- biofilms remained viable while the thinner G+ biofilms rapidly lost viability as seen in figure 1.

In conclusion the overall concept that the Gram-positive Bacilli use electron shuttles produced by other bacteria is intriguing. The interaction of this species with Pseudomonas spp., more specifically with secondary metabolites produced by Pseudomonas spp., is proposed as the most reasonable explanation for their presence and dominance in the microbial community of the MFCs. Moreover, this bacterial interaction could be a base to develop approaches to improve the anodic electron transfer in a MFC. Nevertheless, the role of other factors such as quorum-sensing molecules and/or other mediators should not be underestimated and need to be focused on in further studies

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