An improvised microtiter dish biofilm assay for non-invasive biofilm detection on microbial fuel cell anodes and studying biofilm growth conditions

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Abstract

Microbial life is predominantly observed as biofilms, which are a sessile aggregation of microbial cells formed in response to stress conditions. The microtiter dish biofilm formation assay is one of the most important methods of studying biofilm formation. In this study, the assay has been improvised to allow easy detection of biofilm formation on different substrata. The method has then been used to study growth conditions that affect biofilm formation, viz., the effect of pH, temperature, shaking conditions, and the carbon source provided. Glass, cellulose acetate, and carbon cloth materials were used as substrata to study biofilm development under the above conditions. The method was then extended to determine biofilm formation on the anodes of a microbial fuel cell in order to study the effect of biofilm formation on power production. A high correlation was observed between biofilm formation and power density ($r = 0.951$). When the electrode containing a biofilm was replaced with another electrode without biofilm, the average power density dropped from 59.55 to 5.76 mW/m². This method offers an easy way to study the suitability of different materials to support biofilm formation. Growth conditions determining biofilm formation can be studied using this method. This method also offers a non-invasive way to determine biofilm formation on anodes of microbial fuel cells and preserves the anode for further studies.

Keywords Biofilm · Crystal violet · Microbial fuel cell · Anode · Power generation

Introduction

Most of the bacteria in nature occur as biofilms [1], which can be described as cellular aggregates embedded in a matrix of extracellular polymeric substances [2]. A biofilm confers several advantages for bacteria over planktonic mode of growth: the ability to tide over adverse environmental conditions of varying pH and low nutrients, resilience to withstand toxic substances in the environment, and a division of metabolic

labor that conserves energy, enabling it to thrive [3]. Biofilm formation occurs as a sequential process that constitutes aggregation of bacteria and their initial attachment onto a suitable substratum, formation of microcolonies, maturation of the biofilm, and finally dispersal [4]. Bacteria that are originally in a free-living state produce adhesins and extracellular matrix components that enable them to grow on a substratum and mature into a sessile aggregation of bacterial cells [5]. While biofilms are predominantly considered as agents of disease [6] and a nuisance in healthcare applications [7], they also help in beneficial tasks like transformation of environmental pollutants [8]. Biofilms aid biogeochemical cycles [9] and are crucial in wastewater treatment [10]. New avenues are being explored in the wake of environmental issues to provide a promising platform for wastewater treatment and energy generation. Microbial fuel cells (MFCs) are one such emerging technology that employ bacteria as catalysts to convert chemical energy present in biomass to electrical energy [11]. Bacteria housed in the anode chamber of MFCs oxidize organic/inorganic substrates provided as fuel and transfer the

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resulting electrons generated to the cathode, generating current [11]. Since microbes constitute the driving force behind operation of the MFC, the development of an electroactive biofilm on the anode surface is crucial. Electroactive biofilms developing in MFCs play a critical role not only in enhancing electron transfer to the anode and increasing the power output, but also in accelerating the oxidation of substrates [12]. It is thus crucial to monitor MFCs for the development of electroactive biofilms.

A deep understanding of biofilm formation and development is thus necessary not only to monitor and eliminate hazardous biofilms, but also to regulate and enhance beneficial electroactive biofilms in MFCs [13]. A common method to assess bacterial biofilm formation in a MFC involves disruption of the biofilm by vortexing and scraping, followed by determination of colony-forming units per milliliter [14]. This method has disadvantages of being laborious and time consuming. Moreover, it can be difficult to dissociate the bacterial clumps. When this method is applied, the anode needs to be sacrificed, which places limitations on the lifetime of a MFC [15]. A method that does not disrupt the anode is essential to study biofilm formation and to monitor its development.

Among the methods employed for studying biofilm development, the microtiter dish biofilm formation assay developed by Madilyn Fletcher [13] is an important method used to assess the development of bacterial biofilms [16]. It allows biofilm development on the walls of a microtiter plate and is used to study the growth of biofilms. The biofilm growing on the walls of the microtiter plate is stained with crystal violet and destained with acetic acid or ethanol to record absorbance at 550 nm. We have improvised this assay so as to enable simple and rapid detection of biofilms growing on the anode surface of a MFC in a non-invasive way so that the anode can be used in future experiments without compromising the functionality of biofilms. Using the improvised method, different substrata can be evaluated for their ability to support biofilm growth. Application of this method to study growth conditions affecting biofilm formation (pH, temperature, dynamic and static conditions, and the effect of carbon sources) has also been presented. Enabling easy, rapid, and non-invasive detection of biofilms on anode (acting as substratum for biofilm formation) by improvising the microtiter dish biofilm formation assay is the focus of this study.

Materials and methods

Microtiter dish biofilm assay Domestic wastewater (5 mL) collected from a sewage treatment plant in Prasanthi Nilayam township was inoculated in 95mL nutrient medium containing (per liter) NH_4Cl , 0.31 g; $\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}$, 5.38 g; Na_2HPO_4 , 8.66 g; KCl , 0.13 g (pH 7) [17]; and incubated at

37 °C. Biofilm growth was estimated as per the standard microtiter dish biofilm assay [18]. Subsequently, this procedure was modified to allow biofilm estimation on various substrata (Fig. 1). Three different materials, viz., carbon cloth, cellulose acetate, and glass (0.25 cm² each), were introduced in the culture to serve as substrata for biofilm growth. These materials were selected based on their differing hydrophobicity: carbon cloth, a commonly used electrode material in MFCs, is hydrophobic, cellulose acetate used in the study was weakly hydrophilic, while glass used was moderately hydrophilic. These materials were placed in separate flasks of the microbial culture immediately after inoculation. Biofilm growth on each of these materials was determined at 6, 12, 18, and 24 h after inoculation as follows. The materials introduced in the microbial culture for biofilm attachment were rinsed with distilled water and directly immersed in 300 µL of 0.1% crystal violet for 30 min. The additional step of growing biofilms in 96 well plates and washing them was eliminated to aid biofilm detection on the substrata and to save time. After staining with crystal violet, the substrata were thoroughly washed with distilled water and destained with 200 µL of 30% acetic acid. A total of 100 µL of the destaining solution was transferred to a new microtiter dish plate and absorbance was measured at 550 nm. The assay performed with carbon cloth, cellulose acetate and glass immersed in sterile medium served as the control.

Effect of glucose and acetate 0.25% glucose and acetate were added to the growth medium in separate flasks and the medium was inoculated as described above. Carbon cloth, glass, and cellulose acetate (0.25 cm² each) were introduced in the microbial cultures containing glucose and acetate. Microtiter dish biofilm assay was done to determine biofilm growth on all the three materials introduced in the cultures containing glucose and acetate. The results were compared against the standard protocol of the microtiter dish biofilm assay done without addition of extraneous materials to the microbial culture.

Dynamic and static conditions Carbon cloth, cellulose acetate, and glass (0.25 cm² each) were introduced in separate flasks in the microbial culture. The culture flasks containing all the three materials were subjected to dynamic shaking and static condition at 37 °C. Shaking conditions (112×g) were maintained in an incubator shaker (Orbitek). Biofilm growth on the three materials was analyzed in dynamic and static conditions on each of the three materials.

Effect of pH on biofilm growth Carbon cloth, cellulose acetate, and glass (0.25 cm² each) were introduced in the microbial culture at pH 6, 7, 8, 9, and 10. Biofilm growth on all the materials at each of these pH values was estimated by the assay as mentioned above.

Fig. 1 Schematic diagram of the microtiter dish biofilm formation assay and the modifications to the traditional procedure

Effect of temperature on biofilm growth The microbial culture was maintained at 25 °C, 30 °C, 35 °C, and 40 °C. Biofilm growth at all the temperatures was assessed by crystal violet assay on glass, cellulose acetate, and carbon cloth.

Microbial fuel cell A five-chambered microbial fuel cell with a central cathode (500 mL) and four anodes (250 mL each) was constructed using carbon cloth electrodes (0.5 cm² anode in all chambers; 12 cm² cathode). Cation exchange membrane (Membranes International) was used as the separator. Microbial culture grown from inoculating domestic wastewater in nutrient medium [17] was used as the anolyte in all anode chambers while aerated tap water was used as the catholyte. Power density was calculated for all the four chambers using resistors ranging from 300 to 15 Ω. The improvised crystal violet assay was done for each of the anodes at 6, 12, 18, and 24 h as described above. The anode in one of the chambers containing the biofilm was then replaced with a fresh carbon cloth electrode of the same size and power generated was calculated.

Results and discussion

Microtiter dish biofilm assay Biofilm growth was recorded in terms of absorbance at 550 nm from the microtiter dish biofilm formation assay. All the three materials, viz., carbon cloth, glass, and cellulose acetate supported biofilm growth (Fig. 2). Compared to the standard protocol of biofilm estimation by crystal violet assay where biofilms are grown in microtiter plates, enhanced biofilm formation was observed with the use

of extraneous materials as substrata. Biofilm formation was significantly higher in carbon cloth as compared to glass and cellulose acetate materials, which may be due to the hydrophobicity of carbon cloth. Initiation of bacterial cell attachment to a substratum depends largely on the nature of the substratum. Hydrophobic interactions play a key role in the attachment of bacterial cells onto a substratum and hence the hydrophobicity of the material used as substratum for biofilm growth determines the extent of biofilm development [19]. In a study on biofilm formation on marine cellulose acetate debris, it was found that increasing hydrophobicity of the cellulose acetate contributed to a greater biofilm formation [20]. Properties of bacterial cells like surface charge and appendages also

Fig. 2 Biofilm growth recorded at 550 nm on glass, cellulose acetate, and carbon cloth introduced in microbial culture

Fig. 3 Growth conditions affecting biofilm formation: **a** Effect of glucose (0.25%) and acetate (0.25%). **b** Effect of shaking conditions. **c** Effect of pH. **d** Effect of temperature

determine the strength of adhesion [21]. In addition to the nature of the substratum, bacterial cells and the composition of the extracellular polymeric substance also play key roles in determining the extent of biofilm formation [22].

The microtiter dish biofilm assay is a simple, reliable, and high-throughput method for the study of biofilms [23]. Crystal violet stains living biomass as well as the extracellular matrix

of the biofilm [24]. Washing, drying, and staining the 96 well microtiter plate before and after biofilm formation in the standard microtiter dish biofilm assay vary from lab to lab, and differences in these procedures tend to make comparison of results difficult. In the improvised method presented in this study, we have eliminated the additional step of growing biofilms in 96 well microtiter plates, thus quantifying the biofilm growth on the substratum directly and making the assay less cumbersome. Another major advantage of this procedure is that different materials could be tested for their susceptibility for biofilm growth. Accordingly, materials having suitable properties to enhance or minimize biofilm growth could thus be selected for the desired application. For example, materials exhibiting a low propensity to aid biofilm growth can be used in surgical equipment to minimize risk of infections, while those used in wastewater treatment systems can be carefully engineered to enhance biofilm growth. Clinical culture techniques employed in hospitals cannot easily detect biofilm growth [25]. Long-term use of implants and intravascular devices is prone to biofilm growth that is difficult to detect, which provides a serious impediment in healthcare [26]. This method can be used to distinguish between different kinds of bacteria that produce strong, moderate, or weak biofilms [27]. Multiple bacterial strains that exhibit

Fig. 4 Power density curves recorded for the four anode chambers in the multi chambered microbial fuel cell

Table 1 Biofilm growth on the anodes of the microbial fuel cell (recorded in terms of absorbance at 550 nm) and the corresponding power densities

Time (hours)	Anode 1		Anode 2		Anode 3		Anode 4	
	A ₅₅₀	Power density (mW/m ²)	A ₅₅₀	Power density (mW/m ²)	A ₅₅₀	Power density (mW/m ²)	A ₅₅₀	Power density (mW/m ²)
6	1.003	24.25	1.045	28.25	0.970	25.48	1.084	32.54
12	1.214	36.54	1.157	38.15	1.198	37.25	1.164	41.51
18	1.654	47.54	1.865	49.54	1.784	46.55	1.712	50.85
24	1.987	60.21	2.054	64.55	2.000	62.74	2.012	67.98

differences in biofilm formation can be analyzed using this method [28]. Growth conditions that affect biofilm formation, like presence of nutrients, osmolarity, temperature, and pH, may also be monitored using this method.

Effect of glucose and acetate Biofilm growth was enhanced in the presence of glucose and acetate. Compared to acetate, the presence of glucose gave a better biofilm growth (Fig. 3a). The extent of biofilm growth was maximum on carbon cloth among the three substrata used. Availability of nutrients is a major factor in biofilm formation. It has been observed previously that high nutrient loads enhance the ability of *Pseudomonas aeruginosa* to produce biofilms [25]. Increasing glucose concentrations correlated with increase in *P. aeruginosa* [29] and mixed-culture biofilms [30]. Stepanovic et al. [31] have shown that the presence of glucose significantly enhances the adherence and attachment of various strains of *Staphylococcus aureus* cells to form biofilms. They have also stated that biofilm formation enhanced by glucose was independent of the concentration of glucose used. Enterococci were able to improve their biofilm formation rate

upon addition of glucose [32]. In another study conducted to assess biofilm formation on glass and teflon surfaces, it was found that biofilm formation was strongly enhanced by introducing low concentrations of acetate [33]. Conversely, there are also reports of high glucose levels disrupting biofilm formation by promoting cell detachment [34]. Culture conditions have a great influence on the ability of microbes to form biofilms. Carefully controlled culture conditions with adequate nutrients can be used to monitor biofilm growth.

Dynamic and static conditions Biofilm growth was greater in shaking conditions rather than static conditions. The results are depicted in Fig. 3b. According to Moreira et al. [25], one of the decisive factors influencing biofilm formation is shear force in dynamic conditions. Shaking conditions have a strong influence on the oxygen transfer rates to cells [35]. Effective transfer of oxygen and nutrients to cells due to shaking may be promoting biofilm growth. Studies on *Candida albicans* biofilms have shown that cells incubated under shaking conditions formed large amounts of exopolymeric matrix that led to formation of stable biofilms [36]. When the same cells were incubated under static conditions, production of exopolymeric substances was reduced.

Effect of pH on biofilm growth An increase in pH from 6 to 8 suited biofilm growth. Best growth of biofilms for all the materials was observed at pH 8 (Fig. 3c). Biofilm growth did occur at pH 9, but not as much. A further increase in pH to 10 disrupted biofilm growth. In a study conducted on biofilms [27], it was found that biofilms are stable structurally in alkaline conditions and that disruption occurred in acidic conditions. Villaverde et al. [27] reported that at pH 8.2, biofilms exhibited maximum nitrification ability.

Effect of temperature Microbes exhibit a temperature-dependent capacity for biofilm formation. Biofilm growth was best observed at 30 °C. Increase in temperature to 35 °C and 40 °C lead to decrease in biofilm formation (Fig. 3d). It has been observed previously that expression of the promoter *agfD* occurs effectively at 30 °C, which is responsible for formation of fimbriae, cellulose, and extrapolymeric material contributing to biofilm formation [37]. Temperatures above

Fig. 5 Effect of biofilm on power generation: replacement of anode containing biofilm with a fresh anode without biofilm lead to a decrease power density. When the original anode was reintroduced, power density was restored

35 °C lead to faster depletion of nutrients and changes in pH, leading to reduced biofilm formation [37].

Microbial fuel cell As conditions across the anode chambers were similar, all the anode chambers produced power densities (normalized with respect to anode) that were comparable (Fig. 4). Table 1 gives the maximum power density produced in all anode chambers along with the corresponding biofilm growth. Power density for all anodes peaked at 70 Ω . Biofilm growth on each of the anodes of the microbial fuel cell was also comparable. A strong correlation was observed between biofilm formation and power density ($r = 0.951$). Replacement of biofilm containing electrode with fresh electrode led to a sharp decrease in power density (Fig. 5). Biofilm-coated anodes are acclimatized to MFC conditions and give a higher power density compared to freshly introduced electrodes without biofilm growth. The average power density reduced from 59.55 to 5.76 mW/m² upon replacement of the biofilm anode with a fresh carbon cloth anode without biofilm. Upon reintroduction of the original carbon cloth electrode containing the biofilm, power density was restored to its original value.

According to Kumar et al. [38], the first step towards electricity generation in microbial fuel cells is acclimatization of exoelectrogens to working conditions and formation of an anodic biofilm on the surface of the anode. Biofilms use extracellular electron transfer (EET) to communicate with their external environment [39]. In direct electron transfer (DET), microbes develop conductive pili called nanowires or employ outer-membrane cytochromes (OMCs) and other related proteins to transfer electrons to the anode [40]. It has been noted that formation of pili is essential for electroactive biofilm formation [4]. In mediated electron transfer (MET), redox molecules are used as shuttles by microbes to transfer electrons to anode [40]. The redox molecules are either produced by the microbes, or are externally added compounds like methylene blue [41]. Irrespective of the mechanism, however, formation of an electroactive biofilm is essential for efficient operation of MFCs. Thick exoelectrogenic biofilms growing on the surface of anode in MFCs generate higher power densities as compared to MFCs whose anodes do not contain exoelectrogenic biofilms [42]. Microbes that are incompetent at biofilm formation in anodes are unable to generate power densities that are significant [4]. Bacteria that form substantial biofilms on the anode generate higher power densities compared to bacteria that form thin biofilms on the anode [43, 4]. An enriched consortium of exoelectrogenic bacteria that formed biofilms produced 22% more power than a pure culture of *Geobacter sulfurreducens* that was limited in its ability to form a biofilm [44].

The performance of the anodic biofilm in microbial fuel cells is a critical factor driving its operation. This method can be employed in microbial fuel cells to assess biofilm growth on the anode in a non-invasive way. Since the biofilms are not disrupted or killed after evaluation, the anode can be

preserved and used in future experiments like imaging without affecting the lifetime of the MFC. Different anode materials can also be evaluated using this method to determine the extent of biofilm formation. This assay is an easy to perform, high-throughput, time-saving, and low-cost method that can be used under a wide range of growth conditions.

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Compliance with ethical standards

Conflict of interest All the authors declare that they have no conflict of interest.

Research involving human participants and/or animals This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent N/A

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