

Article

Evolving Microbial Communities in Cellulose-Fed Microbial Fuel Cell

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Abstract: The abundance of cellulosic wastes make them attractive source of energy for producing electricity in microbial fuel cells (MFCs). However, electricity production from cellulose requires obligate anaerobes that can degrade cellulose and transfer electrons to the electrode (exoelectrogens), and thus most previous MFC studies have been conducted using two-chamber systems to avoid oxygen contamination of the anode. Single-chamber, air-cathode MFCs typically produce higher power densities than aqueous catholyte MFCs and avoid energy input for the cathodic reaction. To better understand the bacterial communities that evolve in single-chamber air-cathode MFCs fed cellulose, we examined the changes in the bacterial consortium in an MFC fed cellulose over time. The most predominant bacteria shown to be capable electron generation was *Firmicutes*, with the fermenters decomposing cellulose *Bacteroidetes*. The main genera developed after extended operation of the cellulose-fed MFC were cellulolytic strains, fermenters and electrogens that included: *Parabacteroides*, *Proteiniphilum*, *Catonella* and *Clostridium*. These results demonstrate that different communities evolve in air-cathode MFCs fed cellulose than the previous two-chamber reactors.

Keywords: microbial fuel cell; cellulose; microbial community changes; air-cathode microbial fuel cell

1. Introduction

The microbial fuel cell (MFC) is a technology that converts carbon-rich waste into bioelectricity using the ability of certain microorganisms, called exoelectrogens, to transfer electrons outside the cell [1]. As there has been an increase in the global demand for renewable energy, exploiting biomass—which are biopolymers such as cellulose, chitin and starch—seems to be a promising part of the solution for the future. It may be possible for MFCs to compete with other methods of producing electricity from biomass, as it directly produces electricity rather than requiring multiple steps. Extensive investigations in the last years have shown that MFCs can be used for production of bioelectricity from different substrates, from simple sugars to complex wastes, including various types of wastewater [2,3]. Advances in new electrode materials and reactor configurations have increased power densities to as much as 1.5 kW/m³ [4]. As cellulose is the most abundant polysaccharide in nature, there have been several efforts undertaken to design efficient MFCs using cellulose as

a fuel [5–8]. The prerequisite for cellulose-fed MFCs is the appropriate choice of microorganisms able to decompose cellulose and produce a current. However, cellulose is a very difficult substrate for microorganisms to use in an MFC due to its water insolubility and complex spatial structure. Until now only one bacterial strain has been found to show both cellulolytic and electrogenic activity [7,9].

Generally, turning cellulose into electricity needs a syntrophic consortium where communication between species leads to metabolic cooperation enabling decomposition of a complex substrate, like cellulose, into volatile acids that can be further used by exoelectrogens for current production [10]. The type of substrate used in MFCs strongly affects bacterial consortium dynamics and influences the composition of a bacterial community and performance of the system [11,12]. One of the most important directions in development of MFC technologies, beside reactor design, is the investigation of improving current production by exoelectrogenic microorganisms. This is especially important for biomass-fed MFCs, where a consortium of bacteria are required to convert the biomass to chemical that can be used by the exoelectrogenic bacteria. These improvements may include efficient selection of strains from consortia, genetic engineering, or optimization of adapting conditions. For these reasons, the knowledge of bacterial communities that evolve in cellulose-fed MFCs may be crucial to enhancing the performance of MFCs fed with cellulosic substrates.

Previous studies on cellulose-fed MFCs have usually been conducted with two-chamber systems, where the power densities produced were generally low, and dissolved oxygen was completely excluded from the anolyte (Table S1, [13–22]). Rezaei et al. [9] used a bacterial consortium from paper recycling wastewater in a two-chamber MFC with carbon electrodes and ferricyanide catholyte. The maximum power density obtained in a cotton linter cellulose-fed MFC was 18 mW/m². Cellulose from cotton linters was used as a substrate in another two-chamber MFC study with carbon paper electrodes, which was inoculated with sludge from a wastewater treatment plant [17]. The maximum power density was 12 mW/m² when dissolved oxygen was used as the final electron acceptor. Unfortunately, there was no investigation into the bacterial consortia that evolved in these two studies. Rismani-Yazdi et al. [15] used a bacteria consortium from cow rumen in a two-chamber MFC with graphite electrodes and ferricyanide catholyte and obtained 55 mW/m² when the cellulose from cotton linters was a substrate. An analysis of the bacterial communities after operation in a MFC system revealed the community was dominated by *Firmicutes* and *Betaproteobacteria*. However, there was no information about the original inoculum, so changes in consortium dynamics were not shown. Ishii et al. [18] used an Avicel cellulose as a substrate in a two-chamber MFC inoculated with rice paddy field soil from which 10 mW/m² power was obtained. They found a different bacterial consortium after operation, as *Rhizobiales*, *Clostridiales* and *Chloroflexi* were predominant. In another two-chamber MFC fed wheat straw hydrolysate, *Bacteroidetes* and *Alpha-*, *Beta-* and *Deltaproteobacteria* were found to be dominant in the community [16]. Thus, there were no common bacterial communities identified with these complex substrates.

Single-chamber air-cathode MFCs can produce higher power densities than two-chamber MFCs with dissolved oxygen, due to the higher internal resistance of the two-chamber reactors. However, oxygen diffusion through the air cathode can adversely affect power production by anaerobic microorganisms, and the infusion of oxygen can alter bacterial communities compared to completely anaerobic systems. For example, only 2–10 mW/m² was obtained for an air-cathode single-chamber MFC fed with corn stover, inoculated with domestic wastewater [19]. The power production was increased to 475 mW/m² when a more readily biodegradable substrate (diluted hydrolysate of corn stover) was used as the substrate as oxygen could be more effectively scavenged using this substrate [20]. Unfortunately, no bacterial identification was made in those studies. Many other reports using air-cathode MFCs have used soluble and readily biodegradable fermentation end products, not particulate substrates, and the resulting consortia is almost always found to be dominated by *Deltaproteobacteria*. For example, Kiely et al. [23] reported that a microbial community developed in air-cathode MFCs fed with succinic acid was dominated by *Geobacter sulfurreducens*, while *Geobacter* sp. were also reported to dominate consortia where acetate was used as a substrate [24,25]. *Geobacter* sp.

was also found to be the most abundant when MFCs were fed more complex substrates, such as potato wastewater [26].

Although a number of papers available in the literature regard the bacterial composition of the consortia operating in MFCs, there are only few reports on direct biocurrent production from cellulose, and still little is known about the community evolving in MFCs with air cathodes. Thus, the identification of cellulose-degrading and electrogenic microorganisms that develop as a result of MFC operation on cellulose substrate is lacking. The aim of this work was to investigate the bacteria genera in a consortium before and after operation in an air-cathode, cellulose-fed MFC, which brings new data about the consortium evolving in the presence of cellulosic substrate. The influence of a cellulose substrate on changes in the bacterial community was examined after stable power was generated in this system. The results of the investigations allow for a better understanding of the communities that develop in MFCs during bioelectricity production from complex substrates like cellulose.

2. Materials and Methods

2.1. MFC Construction and Operation

Single-chamber MFCs were constructed in duplicate as previously described by Logan et al. [27], based on a design used by many laboratories around the world [28]. The cube-shaped MFC was made from Plexiglas by drilling a cylindrical chamber (4 cm long by 3 cm in diameter, 28 mL in volume). The anode was carbon fiber brush (2 cm long, 2.5 cm diameter) placed in the center of the chamber. The air cathode (7 cm² area) was carbon cloth with four polytetrafluoroethylene (PTFE) diffusion layers, a Pt catalyst and the Nafion binder, as previously described [29]. The consortium of anaerobic bacteria was isolated from cow manure collected from a farm near Warsaw, Poland. The cow manure was filtered and suspended in 50 mM phosphate buffer-saline (PBS) solution at a 25% concentration. Water insoluble cellulose fibers (Sigma C6288) from cotton linters, were used as the substrate (1%). The scheme of possible use of cotton linters and their residues after isolation of cellulose fibers is presented in Figure S1 [30].

Current and power generation in the MFC were determined by measuring the voltage (U) every 20 min with across fixed external resistance (1000 Ω , unless noted otherwise) with a self-made Arduino-based automated measuring system connected to a computer. Current (I) was calculated from Ohm's law ($I = U/R$) and power (P) was calculated as $P = IU$. Current density and power density were normalized to the projected surface area of the cathode (7 cm²). In order to obtain the polarization curves using the single cycle method the external resistance was changed over a range 10²–10⁴ Ω , with 20 min per resistor.

2.2. DNA Extraction, Polymerase Chain Reaction (PCR) and Illumina Sequencing

Cow manure samples (fresh inoculum and 30 days after stable power production in MFCs) were frozen after collection and stored at -80 °C. For DNA extractions, the samples were thawed on ice and vortexed independently. DNA extraction, PCR and Illumina sequencing were made according to the protocol described by Liang et al. [31].

2.3. Determination of Volatile Acids Production

Samples were quantitatively analyzed for acetic acid content using static headspace gas chromatography. Analyses were performed on Agilent 7697A Headspace Sampler coupled to gas chromatograph Agilent 7890A with a flame-ionization detector and a split/splitless injector. Prior to analysis, 5 mL of sample was transferred into a 20 mL headspace vial. Headspace sampler conditions were as follows: oven temperature 50 °C, oven equilibration time 20 min, loop temperature 65 °C, temperature of the transfer line 70 °C, time of pressurization 0.2 min, time of loop fill 0.2 min, time of loop equilibration 0.05 min, injection time 0.7 min. gas chromatography (GC) conditions were as

follows: injector—splitless (250 °C), detector: FID (250 °C), oven: initial temperature program was 60 °C. This temperature was held for 6 min and then increased 10 °C min⁻¹ to 230 °C with a final isothermal period of 5 min; flow rate of carrier gas (helium) through the column was 1.3 mL min⁻¹. Separation was performed on capillary column Stabilwax-DA (Restek, Bellefonte, PA, USA) with modified polyethylene glycol phase (30 m × 0.25 mm I.D. × 0.25 μm film thickness). Acetic acid was quantified with reference to a standard calibration curve. The same procedure was used to determine valeric, isovaleric, butyric and isobutyric acids. All analyses were performed in triplicate. The obtained data were analyzed using ChemStation software (Agilent Technologies, Santa Clara, CA, USA).

3. Results

3.1. Performance of MFCs Fed with Cellulose and Production of Volatile Acids

After inoculation of the MFC, the voltage fluctuated between 0 and 50 mV for the first 12 days of operation (Figure 1a). After this, the voltage began to stabilize and slowly increase to reach a peak of 175 mV in the first fed-batch cycle. Solution replacement generated repeatable cycles that lasted approximately 6 days. These observations are in accordance with the previous reports that cellulose hydrolysis is the slowest step in degradation process [21]. The measurements of volatile acids revealed a high concentration of acetic acid (Figure 1b). Acetate was the dominant intermediate and it reached a maximum of 220 mg/L after 7 days, and then decreased after 15 days of MFC operation. The concentration of other acids (valeric, isovaleric, butyric and isobutyric acids) was remarkably lower, and did not exceed 5 mg/L over the whole period of study (data not shown). In previous investigations acetate was also found to be the main intermediate produced during cellulose degradation, and its concentration was observed to decrease with electricity production [16,21].

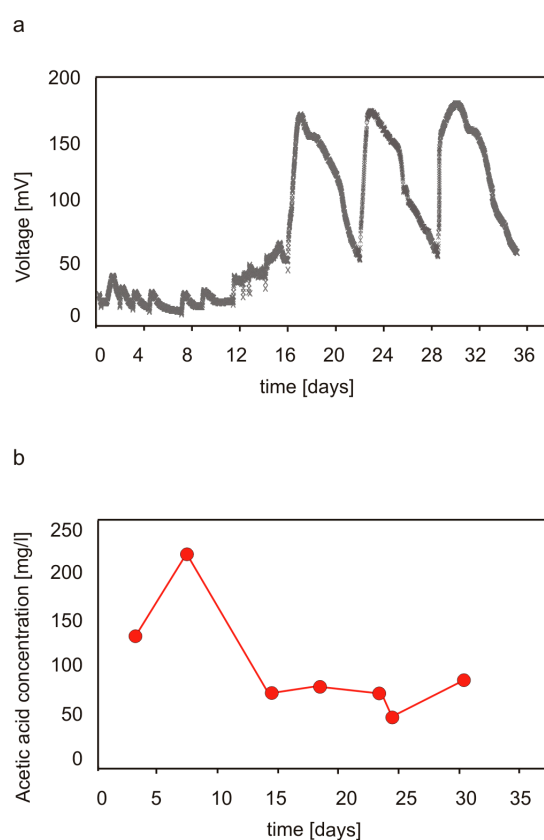


Figure 1. Voltage output (a) and acetate concentrations (b) over time in a cellulose-fed microbial fuel cell (MFC).

Power density was measured after three repeatable voltage cycles (1 month of MFCs operation). Polarization data revealed the maximum current produced in the reactors was 331 mA/m^2 ($R = 100 \Omega$) and the maximum power production was 44 mW/m^2 ($R = 1000 \Omega$), (Figure 2). During the next 30 days of observation, the power density averaged $36 \pm 10 \text{ mW/m}^2$. This power density was low relative to that typically produced with acetate, but more similar to previous results when using insoluble cellulose as a substrate [5].

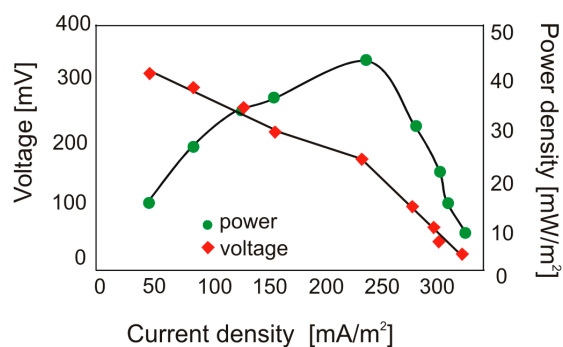


Figure 2. Polarization and power density curves of cellulose-fed MFCs.

3.2. Microbial Community Analysis

The microbial communities were examined on the anode of the MFC, and compared to the inoculum. The main phylum that was enriched on the anode after 30 days of stable operation in MFC was *Bacteroidetes*, which increased from 0.8% in the inoculum to 34% in the MFC (Figure 3). *Bacteroidetes* have previously been reported to dominate the anode consortium of a two-chamber MFC fed wheat straw hydrolysate, and were also found in two-chamber acetate-fed MFCs [16,32] as well as in a single-chamber MFC [33]. The most abundant phylum was *Firmicutes*, with 32% in the inoculum and 50% after 30 days. This observation is in accordance with a previous study where *Firmicutes* were found a dominating anodic communities of a cellulose-fed MFC [15]. The relative abundance in two phyla: *Proteobacteria* and *Actinobacteria* remained at comparable levels in the inoculum and operation of the MFC. The presence of these two phyla was also observed in a two-chamber MFC fed cow manure [34].

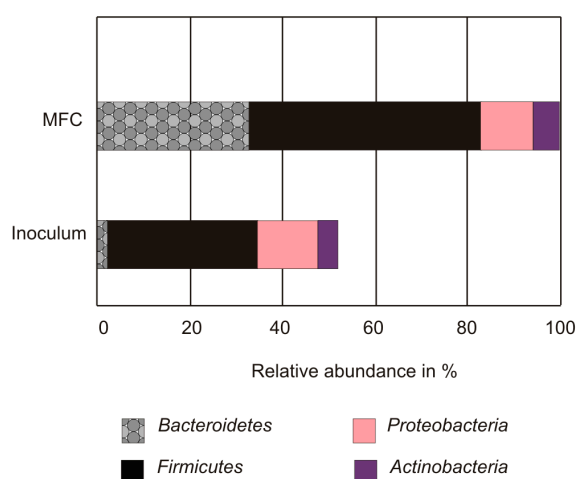


Figure 3. The relative abundance of different bacteria (at the phyla level) present in the inoculum before and after operation of the MFC.

At the genus level, in the *Bacteroidetes* phylum, the most abundant genera were *Parabacteroides* (relative abundance of 39%) and *Proteiniphilum* (33%) (Figure 4a). Amongha *Firmicutes*, the most

abundant genera were *Clostridium* and *Catonella* (both with a relative abundance above 20%), (Figure 4b). *Clostridium* genus was previously found to be dominant in the community of a single-chamber MFC fed with lignocellulosic substrate [35], a single-chamber MFC fed with sucrose [36], and two-chamber MFC fed with acetate [37]. The highest relative abundance of genera in the bacteria consortium evolved after working in the MFC system was observed for *Parabacteroides* (13%), *Proteiniphilum* (11%), *Catonella* (11%) and *Clostridium* (10%) (Figure 5). The genera *Parabacteroides*, *Proteiniphilu*, and *Catonella* were present in the fresh inoculum in low amounts or below detection limits (Figures 4 and 5). In case of *Clostridium*, its relative abundance in consortium after 30 days in the MFC was sustained at the level of the original inoculum (10%). The genus *Comamonas* decreased in relative abundance from 10% in inoculum to 2% after operation of the MFC system.

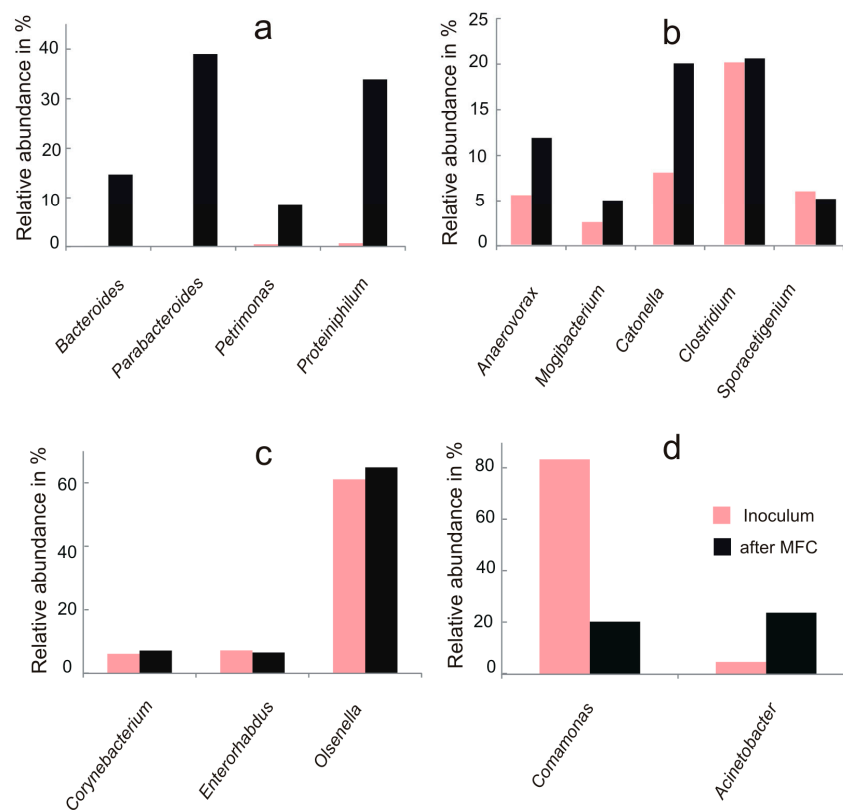


Figure 4. The relative abundance of bacteria genera in different phyla: (a)—*Bacteroidetes*; (b)—*Firmicutes*; (c)—*Actinobacteria*; (d)—*Proteobacteria*.

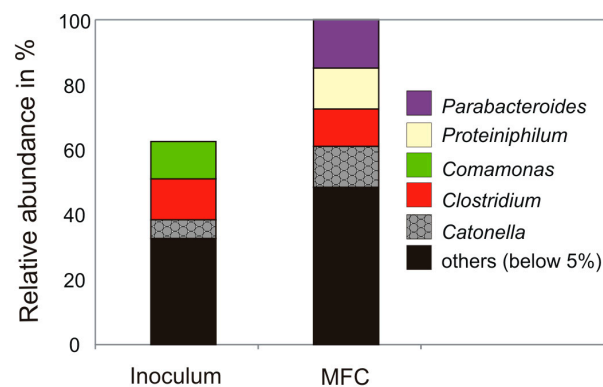


Figure 5. Relative abundance of dominating bacteria genera in fresh inoculum and after work in MFC system.

Although a high abundance of fermenters producing acetate were found here, no *Deltaproteobacteria* were identified. This observation is different from earlier works, as representatives of this class, especially *Geobacter* sp., were found to be dominant in acetate-fed MFCs [38] and in MFCs fed complex wastewaters [26]. Acetate produced in the MFC was likely utilized by Clostridia, which can produce current in MFCs. For example, *C. klyuveri* and *C. untunense* were previously reported to produce current using acetate or butyrate [39–41]. Acetate oxidation could be also performed by *Clostridium* sp. in a syntrophic association with fermenters [42].

4. Discussion

The current and power generation obtained from the cellulose-fed MFCs here (331 mA/m² and 44 mW/m²) were low compared to tests using acetate as a substrate, but comparable to several previous tests where water insoluble cellulose was also used. For example, Ahmad et al. [5] reported maximum current densities of 124–359 mA/m² using commercially available insoluble cellulose (Sigmacell and MN301), with the current depending on the specific MFC configuration and inoculum source. A maximum power density of 59 mW/m² was obtained in two-chamber cellulose-fed MFCs with ferricyanide in the catholyte and inoculated with a coculture of *C. cellulolyticum* and *G. sulfurreducens* [13]. The maximum power density in two-chamber MFCs fed with cellulose and inoculated with Rumen bacteria consortium was 55 mW/m², also using ferricyanide in the catholyte as an electron acceptor [15]. Power densities with ferricyanide catholytes are expected to be higher than those using air cathodes, due to the more favorable reduction reaction with ferricyanide than oxygen.

The microbial communities found in this investigation are consistent with some other studies where *Geobacter* sp. have not been predominant [18,23]. Lack of *Deltaproteobacteria* despite acetate production may be caused by simultaneous fermentation and electrogenesis running in the reactors. *Deltaproteobacteria* were usually identified in cellulose-fed MFCs when hydrolysis was kept separately from electrogenesis [16]. When these two processes ran in the same reactor simultaneously, the communities were dominated by *Clostridium* sp. and no representatives of *Deltaproteobacteria* were found [15,18,19].

Here, the relative abundance of *Firmicutes* and *Bacteroidetes* phyla significantly increased after operation of the MFC system compared to controls, suggesting members of these phyla played an important role in cellulose degradation and current generation. The representatives of *Bacteroidetes* have already been reported to be abundant in anodic communities as fermenters [36]. Among *Bacteroidetes* the most abundant genera were *Parabacteroides* and *Proteiniphilum*, which are known as degrading polysaccharides and xylans to acetate and succinate [35,43]. Members of *Bacteroidetes* have also been shown to be enriched in enzymes targeting carbohydrates what allows to utilize cellulose, xylans, pectin and galaxans [44].

The production of electric current in MFCs has been associated with the abundance of *Firmicutes* in MFCs fed with cellulose [15], wastewater [45,46], acetate [47], glucose [48], or activated sludge [49]. It has been shown that *Firmicutes* play a crucial role in current production with acetate as electron donor as they can transfer electrons to the electrode [15]. Members of this phylum have been previously been demonstrated to be able to transfer electrons to solid electron acceptors e.g., iron and manganese, as a part of their respiratory metabolism [50,51]. However, as reported in these studies, the abundant genera is varied. In our study, the most abundant genera among the *Firmicutes* were *Clostridium* and *Catonella*. Representatives of *Catonella* are known from their fermentative activity with acetic acid as the main product [52], whereas *Clostridium* genus members have both fermentative and electrogenic activity and have been shown to degrade unusually wide range of substrates including cellulose [53]. *Clostridium* were also reported to ferment glycerol, xylose or pentosans into acetate [54–56]. The strain *Clostridium butyricum* has been shown to ferment glucose to acetate, butyrate, CO₂ and H₂, but also lactate, formate and ethanol [57,58]. Simultaneously, *C. butyricum* was found to have membrane-bound cytochromes for direct electron transfer. Park et al. [59] isolated *C. butyricum* from a wastewater-fed MFC proved its exoelectrogenic activity. Another representative

of Clostridia, a fermentative bacterium *C. beijerinckii* isolated from sediment water, was also found to be capable of reducing Fe (III) [60]. The abundance of Clostridia here in the inoculum and after operation was sustained on the level 10%, but this genus was not the most abundant in the anodic microbial population. However, it has been noted that ability of microorganisms to produce high power densities does not necessarily correlate with their high abundance in the anodic biofilm [61], as abundances can be affected by biomass yields and other factors [62,63].

Not all genera present in the inoculum with previously identified exoelectrogenic activity were enriched in the investigated MFCs. *Comamonas*, a genus for which exoelectrogenic strains have been recently reported [64], decreased in relative abundance during MFC operation (Figure 4d). This observed decrease suggests that not all potentially electrogenic genera can adapt to cellulose-fed MFC conditions. The most abundant genera that developed in the course of MFC operation (*Parabacteroides*, *Proteiniphilum*, *Catonella*) were fermenters having the ability to degrade polysaccharides, which suggests that degradation and fermentation of cellulose were the main processes occurring in the cellulose-fed MFCs that led to the relative abundances of different bacteria. While fermentative genera contribute little to power production, their main function was presumably the conversion of cellulose into acetate that was then oxidized by the exoelectrogens, likely various Clostridia strains [65].

Cellulose-fed MFCs are currently in an initial phase of development. The investigated reactor volumes are below 1 L for current tests with cellulosic substrates, and the obtained power densities, when insoluble cellulose is used as a substrate usually do not exceed 200 mW/m² (Table S1). As the major limitation in converting cellulose to electricity is decomposition of this polysaccharide, attention needs to be focused on optimizing cellulose degradation and fermentation to the products that could be oxidized by exoelectrogens [66]. This will be the first step towards improving power generation, which will make it possible to scale-up cellulose-fed MFCs also by refining reactor materials and design [67,68]. Extremely important issue is the optimization of conditions for cellulolytic and electrogenic microorganisms. This was proven by Cheng et al., who managed to obtain power production ca. 1 W/m² from cellulose, when the bacterial consortium from domestic wastewater was properly acclimated and used in a microbial electrolysis cell [22]. Further identification of particular strains responsible for current production in the MFCs is also needed and would help to optimize growth conditions for exoelectrogens. A better understanding of microbial community dynamics operating under cellulose-fed MFCs conditions may translate to more efficient power generation in MFCs. That in turn might contribute to decrease in cellulose waste production, reduce amount of cellulose in landfills, and provide an ecofriendly alternative to electricity generation using fossil fuels.

5. Conclusions

During air-cathode MFC operation on cellulose, the bacterial consortium evolved towards the fermenters decomposing cellulose—*Bacteroidetes* and exoelectrogenic *Firmicutes*. The main genera developed were *Parabacteroides*, *Proteiniphilum*, *Catonella* and *Clostridium* with no *Deltaproteobacteria* though acetic acid was produced as main intermediate. The results suggest *Deltaproteobacteria* may not develop in reactors where cellulose fermentation and electrogenesis run simultaneously.

Further investigations should clarify whether low power production in cellulose-fed MFCs is related to slow cellulose degradation efficiency by the fermentative genera, low concentrations of acetate needed for current generation, or rather by low exoelectrogenic activity of Clostridia rather than *Geobacter*.

Supplementary Materials: The following are available online at www.mdpi.com/1996-1073/11/1/124/s1. Table S1: Review of cellulose-fed MFCs.

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Author Contributions: Renata Toczyłowska-Mamińska designed experiments, made the majority of the measurements with MFCs, analyzed the data and wrote the paper. Monika Kloch made measurements of work parameters of MFCs. Karolina Szymona and Patryk Król helped in developing the voltage automated measuring system. Karol Gliniewicz made PCR and Illumina sequencing. Katarzyna Pielech-Przybylska determined volatile fatty acids. Bruce Logan provided MFC reactors, consulted the results of experiments and helped to improve the paper.

Conflicts of Interest: The authors declare no conflicts of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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