İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

CHARACTERIZATION OF MICROBIAL COMMUNITY OF CATHODE BIOFILM IN SINGLE-CHAMBER AIR-CATHODE MICROBIAL FUEL CELLS DECOLORIZING TEXTILE DYES

M.Sc. Thesis by
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Department: Advanced Technologies

Programme: Molecular Biology-Genetics and Biotechnology

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TEKSTİL BOYALARINI GIDEREN TEK ODALI HAVA- KATOT MİKROBİYAL YAKIT HÜCRELERİNDE KATOT MİKROBİYAL FİLM TOPLULUKLARIN BELİRLENMESİ

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FOREWORD

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ABBREVIATIONS

MFC : Microbial Fuel Cell
rRNA **:** Ribosomal Ribonucleic acid
ATP **:** Adenosine triphosphate
AQDS **:** anthraquinone-2,6-disulfonate
PEM **:** Proton Exchange Membrane
W **:** Watts
V **:** Volts
A **:** Amperes
E_{emf} : Electromotive force
OCP **:** Open Circuit Potential
COD **:** Chemical Oxygen Demand
DGGE **:** Denaturant Gradient Gel Electrophoresis
PTFE **:** Polytetrafluoroethylene
Pt **:** Platinium
DNA **:** Deoxyribonucleic acid
TAE **:** Tris-Acetate-EDTA
PCR **:** Polymerase Chain Reaction
dNTP **:** Deoxyribonucleotide triphosphate
NaOAc **:** Sodium Acetate
Tm **:** Melting Temperature
APS **:** Ammonium peroxosulphate
TEMED **:** Tetramethylethylenediamine
UPGMA **:** Unweighted Pair Group Method with Arithmetic Mean
BLAST **:** Basic Local Alignment Search Tool
RDP **:** Ribosomal Database Project
FISH **:** Fluorescence in situ hybridization
OTU **:** Operational taxonomic unit
SEM **:** Scanning electron microscopy
CSLM **:** Confocal scanning laser microscopy
B : Remazol Brillilant Blue BB Gran 133 (Dyestar GA10503)
S **:** Remazol Black RL (Dyestar C19143)
T **:** Remazol Turquoise Blue G 133 (Dyestar FA09807)
R **:** Reactive Red 195 (Setaş Kimya 5017)
Y **:** Reactive Yellow 145 (Setaş Kimya 5030)

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CHARACTERIZATION OF MICROBIAL COMMUNITY OF CATHODE BIOFILM IN SINGLE-CHAMBER AIR-CATHODE MICROBIAL FUEL CELLS DECOLORIZING TEXTILE DYES

SUMMARY

Microbial fuel cells represent a new approach for energy production with their capabilities of bioelectricity generation from organic matter using bacteria. Common dual-chamber designs comprise of anode and cathode departments separated by a proton exchange membrane. In recent studies, it was shown that absence of PEM improves voltage generation in MFC reactors by decreasing internal resistance of the system. Determination of the dynamics of microbial community in MFCs using various substrates can provide vast amount of information towards understanding the mechanism of better energy yield. There have been various studies about increasing the efficiency of MFC devices, yet, the microbiology of the electrode biofilms, both anode and cathode, remains to be better understood. Very few studies were performed on determination of the microbial community of cathode biofilm.

The main goal of this study is to characterize bacterial species on the cathode biofilm of four identical single chamber air-cathode MFCs degrading five different reactive textile dyes using cultivation-independent methodology. Despite their identical inocula, electrode materials, architecture and operational conditions, maximum powers generated among the devices varied. Maximum circuit potentials of 0.47V , 0.49V, 0.59V and 0.45V were measured respectively among four identical fuel cells operating under same conditions. In all MFC reactors, microbial communities were highly dissimilar to starting inoculum. However, introduction of five textile dyes did not have similar effects on each cell. While in three of the cells similar profiles were obtained after introduction of second dye to the system, similarity values of microbial communities was fluctuating in the other cell. Changes in microbial communities also correlated with MFC potentials. Also different numbers of taxa was detected for each collected sample among the same MFC. Community members of the cathode biofilm were identified phylogenetically using 16S rRNA gene clone-library analyses which verified differences in communities. Clone library analysis showed the presence of diverse groups of Bacteria which belong to Alpha-, Beta-, Gamma-proteobacteria, Actinobacteria. *Aquamicrobium, Mesorhizobium, Ochrobactrum, Thauera, Paracoccus, Achromabacter, Chelatacoccus* and *Methanobacterium* affiliated phylotypes were detected as major phylotypes depending on clone frequencies. According to clone frequencies Rhizobiales division of Alphaproteobacteria emerged as the most abundant group as they occupied 87% of the analyzed clones while they were not detected in starting microbial consortia.

It can be postulated that cathode biofilm in single chamber MFCs are also active role players on setting MFC potential. Communities of this biofilm structure changes

temporally during system operation. Thus identification of microbial communities on cathode and their dynamics can provide useful insight towards more efficient performance in single chamber MFC reactors.

TEKSTİL BOYALARINI GIDEREN TEK ODALI HAVA- KATOT MİKROBİYAL YAKIT HÜCRELERİNDE KATOT MİKROBİYAL FİLM TOPLULUKLARIN BELİRLENMESİ

ÖZET

Mikrobiyal yakıt hücrelerinin (MYH) organik maddeyi bakteriler aracılığıyla yıkarak biyoelektrik üretimine olanak sağlamaları, enerji üretimine yeni bir bakış açısı getirmektedir.Tipik olarak tasarlanan iki odalı yakıt hücreleri anot, katot ve bu odaları ayıran proton değişim membranından oluşmaktadır. Yakın zamandaki çalışmalarda, proton değişim membranının tasarımdan kaldırılmasının sistemin direncinde düşüşü sağlayarak voltaj üretimine olumlu katkı sağladığı gösterilmiştir. Bu tip sistemlerde, farklı substratlar kullanan yakıt hücrelerde, mikrobiyal toplulukların dinamiklerinin belirlenmesi, daha yüksek verimde enerji üretilmesinde etken olan faktörlerin belirlenmesinde önemli bilgiler sağlayabilir. Bu bağlamda, MYH'lerin verimlerini arttırmaya yönelik birçok çalışmalar bulunsa da, katod ve anod elektrodlarında oluşan biyofilmlerin mikrobiyolojisinin daha iyi anlaşılması gerekmektedir. Özellikle katot biyofilmlerinin mikrobiyal topluluklarının belirlenmesi konusunda yapılan çalışmalar çok sınırlıdır.

Bu çalışmanın ana amacı, kültürden bağımsız yöntemler kullanarak, farklı Reactive tekstil boyalarını parçalayabilen hava-katot, tek odalı dört özdeş MYH'nin mikrobiyal topluluklarının belirlenmesidir. Aynı mikrobiyal toplulukla aşılanmalarına rağmen ve aynı tasarım ve elektrot malzemelerine rağmen, elde edilen maksimum güçler hücreler arası farklılık göstermiştir. Hücrelerdeki devre potansiyelleri sırasıyla 0.47V, 0.49V, 0.59V ve 0.45V olarak tamamen aynı koşullarda çalışmalarına rağmen değişkenlik göstermektedir. Bütün hücrelerde mikrobiyal toplulukların başlangıçtaki aşı topluluğundan farklılaştığı görülmüştür. Fakat beş farklı tekstil boyasının sisteme eklenmesi her hücrede aynı etkiyi göstermemiştir. Hücrelerin üçünde, ikinci boyanın sisteme eklenmesinden sonra mikrobiyal topluluklar arasındaki değişim düşük seviyededir fakat dördüncü hücrede topluluklar arası benzerlik değerleri dalgalanmaktadır. Mikrobiyal topluluklardaki değişim MYH devre potansiyellerindeki değişimle de örtüşmektedir. Aynı zamanda MYH'lerden alınan farklı örneklerde farklı sayıda taksa tespit edilmiştir. 16S Rrna genine dayalı klonlama yöntemleriyle katot biyofilmindeki mikrobiyal toplulukta bulunan türler bu farklılığı doğrulamaktadır. Oluşturulan klon kütüphanelerinin analizlerinde Alfa-, Beta- ve Gammaproteobakterilere ve Aktinobakterlere ait muhtelif bakteriyel gruplar tespit edilmiştir. Bu gruplar içerisinde *Aquamicrobium, Mesorhizobium, Ochrobactrum, Thauera, Paracoccus, Achromabacter, Chelatacoccus ve Methanobacterium* ilişkili türler klon frekanslarına gore ana türler olarak belirlenmiştir. Bunlara ek olarak, klon frekansları Alfaproteobakteriler içerisindeki Rhizobiales ailesine ait grubun katot biyofilmindeki mikrobiyal

topluluğun %87'sini oluşturduğu tespit edilmiştir. Bu grup başlangıç aşı topluluğunda bulunmamıştır.

Sonuçlar tek oldalı MYH'lerde katot biyofilminin de devre potansiyelinin belirlenmesinde aktif rol oynayabileceğini göstermektedir. MYH'nin çalıştırılması süresince, biyofilmdeki mikrobiyal topluluk zamana bağlı olarak değişim göstermiştir. Bu sebeple katottaki mikrobiyal topluluğun ve dinamiklerinin belirlenmesi daha verimli performansların elde edilmesine dair önemli bilgiler verebilir.

1. INTRODUCTION

Most important element to sustain our lives is energy. Either being metabolically or in larger scale without energy life would not exist. In this context, need for the energy sources are inevitable. Rapid increase in world population also points out that there is a necessity for the discovery of alternative energy sources. Fossil fuels, that are currently used as primary energy source are limited with their reserves and the they are also the most important reason for the global warming climate changes and environmental pollution. Therefore, there is a significant need for finding sustainable and novel energy sources.

Microbial fuel cell is a potential means of providing the opportunity of producing clean and sustainable energy from biodegradable compounds, and in the meantime the possibility of bioremediating biological waste. MFC has many advantages when compared to other technologies which uses organic compounds as a main source of energy. These advantages are high conversion efficiency, ability to work at low temperatures, elimination of aeration process and potential for widespread application areas (Liu et al., 2004).

1.1 Aim of the study

The aim of this study was to investigate bacterial communities of mediator-less microbial fuel cells responsible of decolorization of five different reactive textile dyes.

Cathode microbial communities have been discussed to have an effect on MFC performance on recent studies, but very few studies has correlated cathode biofilm with energy generation efficiency.

Thus, four identical MFCs' cathode biofilms were monitored to track changes in bacterial communities using molecular biology methods while five different reactive textile dyes together with acetate were utilized as potential substrates in the reactors.

Microbial community profiles of the samples from cathode biofilm were compared and abundant strains was investigated using 16S rRNA based phylogenetic techniques.

2. MICROBIAL FUEL CELLS

2.1 History

Microbial fuel cells (MFC) comprise a novel approach towards generation of electricity using bacteria. Potter demonstrated for the first example of current generation from bacteria (Potter, 1911), and early 1990s was when the interest and work towards the area intensified. Allen and Bennetto were the first researchers to define microbial fuel cells as devices that produces electricity from carbohydrates. (Allen and Bennetto, 1993) However, in this study, for electricity production use of mediators were necessary to transport electrons from bacteria to fuel cell electrodes. Turning point in MFC technology was the discovery of mediatorless fuel cells which revolutionized this technology farther beyond. (Kim et al., 1999)

2.2 General Principles of MFC Technology

Microorganisms oxidize organic energy source and as a result electrons are released through their respiratory chains which helps production of energy for the microorganism in the form of ATP. Terminal electron acceptor accepts the electrons for the coupling reduction reaction. Different molecules like oxygen, nitrate or sulfate can act as terminal acceptors that can easily diffuse through membranes and their products can diffuse out of the cell. Recently some bacteria are identified to transfer electrons out of the cell. Those bacteria are called “exoelectrogens”, and they can be utilized in some reactors for generation of electricity. These microorganisms can harvest part of their microbial energy for generation of electricity. In this case iron oxide and some other metal oxides act as terminal electron acceptors during current generation. The process of electron generation is named as “electrogenesis” and the reactor in which exoelectrogens perform this process is called a “microbial fuel cell”. (Logan, 2008)

Basic MFC reactor comprise of cathode, anode, ion or proton exchange membrane and an electrical circuit as shown in Figure 2.1. Oxygen must be kept away from microorganisms to prevent the inhibition of electricity production. To allow this separation usually a membrane is used to separate the reactor into two chambers. While the anode chamber houses the microorganisms to grow, cathode chamber provides the oxygen for the reaction (Figure 2.1). Two electrodes on both chambers are connected by a resistance or some electrical device that consumes power. During complete MFC reaction, protons that are produced in anode chamber diffuses through membrane to the cathode chamber where they react with oxygen and reduced to water by the electrons extracted from the substrate. (Logan, 2008)

In MFC movement of electrons towards anode occurs by various ways. Among these are electron mediators or shuttles, direct transfer of elecrons to electrode via the membrane, and through the bacterial nanowires. (Rabaey et al., 2004, Bond et al., 2003, Regiera et al., 2005)

Some chemical mediators like neutral red or anthraquinone-2,6-disulfonate (AQDS), are also necessary for the microorganisms that are not capable of transferring their electrons directly to the electrode. (Logan, 2008)

After electrons reach anode, they flow through a wire to the cathode. As a result, power is generated from potential differences between the electodes (in Volts) and flow of electrones (in Amperes). During this stage, protons move to cathode chamber through the ion exchange membrane.

In cathode chamber, reduction reaction of the electron acceptor takes place.

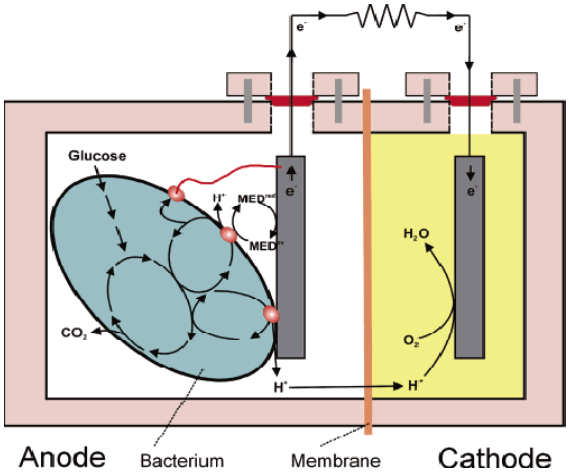


Figure 2.1: General components of MFC operation. (Logan et al.,2006)

2.3 MFC Materials

MFCs are composed of three main components; anode, cathode and ion exchange membrane. Anode and cathode must be present in all types of MFCs, while the membrane can be omitted. In fact, their high costs and resistance to transfer make them less preferable today. Advancements in of anode material search helped development of very efficient anode designs such as the graphite fiber brush. (Logan, 2008) In cathode however, need for an efficient catalyst to catalyze the redox reaction causes increases in fuel cell construction costs. (Logan, 2008)

2.3.1 Anode materials

Anode materials should have various properties for high efficiency. Anode material needs to be highly conductive, non-corrosive, possess high specific surface area, high porosity, should be non-fouling and inexpensive. However, the most important property is conductiveness of the material. Carbon-based papers and clothes are commonly used materials for anode. (Logan, 2008)

2.3.2 Cathode materials

Selection of the best cathode material is the hardest challenge foroptimization of MFC performance. Cathode department must provide the best conditions for electrons, protons and oxygen react to form water and complete the circuit. In order to maintain this, cathode should be conductive, must contain a catalyst, and it should interact both with liquid environment and air. Keeping these drawbacks aside, similar materials such as carbon paper, cloth or graphite can be used in the presence of a suitable catalyst.

2.4 MFC Design

As it was mentioned in the previous sections, there is a variety of options available to be used as anode and cathode materials for MFCs. However, the main issue is the selection of the materials and construction of the best design for optimum performance. Laboratory- scale studies suggested wide range of design oppurtunities depending on the aim of the study. If the main goal is to study the suitability of a certain microorganism or different substrate for power generation, two-chamber

systems are preferred.(Logan, 2008) In this type of systems internal resistance is so high that power outputs remain at lower values. Yet, the main target in most of the recent studies is to increase the power outputs, to affiliate industrial use of these systems, and thus, some chemicals such as ferricyanide and permanganate are used on cathodes. (Logan, 2008)

2.4.1 Air-cathodes MFCs

Air-cathode MFCs are designed in a way that cathode must be exposed to both air, and the culture medium containing bacteria and/or bacterial biofilms A few examples of the air-cathode MFC designs were shown below.

2.4.1.1 Cube reactors

Prospect of MFC technology points out designs with air-cathodes, which eliminates the cost for the aeration. In 1989, first application of air-cathode was mentioned. (Sell et al., 1989) Air-cathode MFCs have great importance to study aspects for power generation. Most widely used design was developed in Penn State University which is a single-chamber, air-cathode cube reactor, and it has been cited in numerous publications. (Liu and Logan, 2004) It is made up of Acrylic or Lexan and a small chamber is drilled through to place cathode and anode at opposite ends. Size can be scaled up and down but kept at minimum for lab-scale research. In order to empty and fill the reactor there are two openings on MFC which are closed with stoppers during operation of the reactor. Most common materials used for cathode and anode is carbon cloths, but in cathode platiniumum catalysts are used and Nafion is heat pressed as proton exchange membrane (Figure 2.2). Wires are used to maintain electron transfer from anode to cathode and screw bars hold the reactor together. Finally external resistor completes the design.

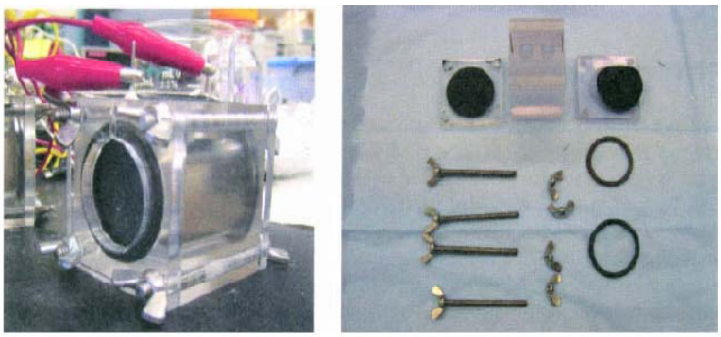


Figure 2.2: Unassembled and assembled MFC design of Liu and Logan (2004)

Performance tests of the design with glucose as substrate demonstrated power output 494±21mW/m^2 without a proton exchange membrane (PEM) and 262±10mW/m^2 with PEM. (Liu and Logan, 2004). Domestic wastewater generated 146±8mW/m^2 without PEM in comparison to 28±3mW/m^2 With PEM (Liu and Logan, 2004). Negative effect of a membrane on power generation was clearly shown. Analysis with other substrates such as acetate and butyrate reached 506mW/m^2 and 305mW/m^2 respectively. (Liu et al,. 2005)

2.4.1.2 Dual-chamber air-cathode MFC

Two chamber systems are used to investigate effects of different membranes on MFC performance (Figure 2.3). Dual-chamber helps to try different membrane types without bonding them to cathode. For this purpose different type of cation, anion and ultrafiltration membranes were tested for production of highest power densities. (Kim et al., 2007)

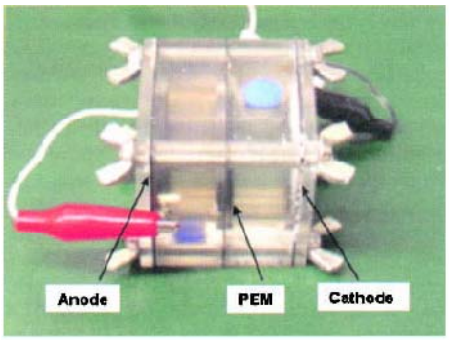


Figure 2.3: Two-chamber air cathode MFC

2.4.2 Single Chamber MFC

In this MFC design, cathode tube concentric with eight graphite anode was used in acrylic material. It was used to study simultaneous wastewater treatment and electricity generation. The reactor produced 26mw/m^2 while it removed 80% of the COD. (Liu et al., 2004)

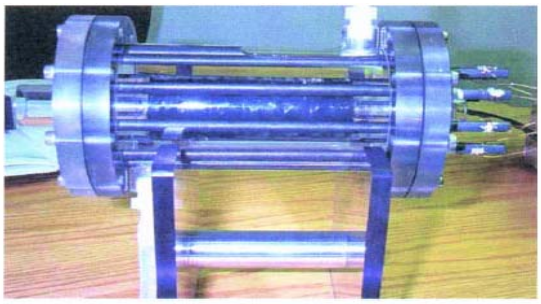


Figure 2.4: Single Chamber MFC reactor

2.4.3 Flat Plate MFC

Flat plate MFCs were designed to maximize power production (Figure 2.6). When two electrodes are put closer to each other, it is known that internal resistance of the system decreases. Keeping this in mind, flat MFCs minimizes the distance between anode and cathode for increased electricity generation. (Logan, 2008)

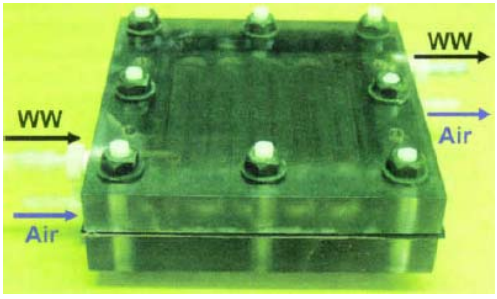


Figure 2.5: Flat-plate MFC designed by Min and Logan (2004)

2.5 Measurement of MFC Performance

Performance in MFC reactors are measured in terms of voltage, current, power and other parameters.

2.5.1 Voltage and Current

The most conventional parameter for electrical devices is the potential difference or voltage. It can be measured easily using voltmeters. Voltage depends on the resistance or load on the circuit, and the flow rate of electrons, current. Ohm's law defines this relation (V=I.R), and it can be used to calculate the current.

Generally, an average MFC reactor can reach voltage values 0.3V-0.7V . Current can also be measured using ampermeters. However, in lab scale, the value of current is very low, so it is calculated using Ohm's law.

Voltage concept in MFCs is harder to understand than any other fuel cells. It takes significant amount of time for stable generation of voltage because there is certain period needed for bacteria to form biofilms on the electrode surface or synthesize necessary structures for electron transfer. (Logan, 2008)

Maximum voltage that can be generated using MFC is limited by thermodynamic relationship of electron acceptors (oxidizers) and donors (substrates).

Total circuit potential of the reactor is calculated by the equation: $E_{emf} = E^0 - [(RT/nF)\ln(\Pi)]$

E_{emf} , the maximum electromotive force can be calculated with an equation above where E^0 is the standard cell electromotive force, R = 8.3 1447 J/mol-K the gas constant, *T* the absolute temperature (K), *n* the number of electrons transferred, and *F* = 96.485 C/mol is Faraday's constant. The reaction quotient is the ratio of the activities of the products divided by the reactants raised to their respective stoichiometric coefficients.

Total potantial can be calculated in any fuel cell by the difference of cathode and anode potentials.

Although different substrates are used as electron donors, open circuit potential (OCP) of tthe system is usually measured around -0.3V. For example, when acetate is used ***2*** HCO_3^- + 9 H+ + 8e- + CH,COO- + 4 H,O reduction reaction occurs. E^0 value for acetate is 0.187V. If anode potential is calculated using maximum electromotive force formula and acetate concentration is 1g/L (16.9mM), the equation results in -0.3V. This value is also very close to measured OCP values when acetate is utilized. (Logan, 2008) This shows that cells also help to achieve maximum potential values by changing their environment accordingly.

However, at cathode department, maximum potentials are not easy to reach. When oxygen is used E^0'cat is calculated as 0.805V under conditions suitable for MFC operation. So in air-cathode system when 1g/L acetate is used as a substrate maximum voltage should be 1.105V from the difference of anode and cathode potentials. However, this theory practically is not observed in reactors. In air-cathode designs OCP of the cathode is measured around 0.4V, and even with Platinium catalyst it is measured around 0.25V during operation. The reason for this failure in generating high potentials is caused from insuccessful reduction of oxygen to water. Sometimes instead of water production, hydrogen peroxide can be produced when only two electrons are transferred from oxygen molecules. E^0 for hydrogen peroxide production can be calculated as 0.695V but under operating conditions in which solubility of oxygen inside the fuel cell is considered 0.328V is calculated. This

value is closer to measured OCP value of 0.425V in a membrane-less MFC. Production of hydrogen peroxide instead of water may have some advantages and disadvantages but its long term effect during operation is yet to be explored. (Logan, 2008)

2.5.2 Power

Power is another parameter to evaluate MFC performance. It is a function of voltage and current and can be calculated as P=VI. Power itself is not correct indicator of the performance and it is normalized to the anode or cathode surface are or the reactor volume. Since the growth of microorganisms on the electrode surface is necessary for power generation, amount of surface available has significant effect on it. On the other hand in some cases power can be normalized to the cathode area or membrane surface area as well. (Logan, 2008)

2.5.3 Other parameters

In most cases evaluation of MFC performance using voltage and power output data does not provide reliable comparison of different designs. Therefore in many research, power output values are expressed in mA/m^2 or mW/m^2 of electrode surface. Even this type of evaluation, which are common for chemical fuel cells, are not enough since in MFC certain amount of space is filled up by bacteria as well. Studies point out that there is no agreement on how to express MFC performance. So far different combinations of reactor volume, exchange membrane, anode area or cathode area are used. Some researchers demonstrate reactor performance in terms of reactor volume which could be a possible alternative for future studies. (Rabaey and Verstraete, 2005)

Lastly, coulombic efficiency and energetic efficiency are parameters for MFC performance. Coulombic efficiency measures the ratio of electrons transferred to anode to the electrons available in the biodegradable substrate. Energetic efficiency is similar but this time energy of the electrons that are transferred to anode is important. Thus it also includes voltage and current. (Rabaey and Verstraete, 2005)

2.6 Substrates used in MFCs

Until today, studies have shown variety of organic substrates that are suitable for utilization in MFCs.

Researchers so far utilized carbohydrates, volatile fatty acids, alcohols, amino acids, proteins and even inorganic compounds in microbial fuel cells as energy source. (Cheng et al., 2007, Clauwaert et al., 2008c, He et al., 2005, Heilmann and Logan, 2006, Ishii et al., 2008, Liu et al., 2005b, Logan et al., 2005, Min and Logan, 2004, Rabaey et al., 2003, Rabaey et al., 2006)

Although experiments done with single or simple substrates give important information about metabolic processes inside the reactor, it is not economically feasible to use them as power sources in industrial scale. Instead, more complex sources such as domestic wastewater, brewery wastewater or anaerobic wastewater plant effluents are preferred as cheaper alternatives. Use of wastewaters also serve one of the most important purposes of MFCs, that is treatment of waste while producing energy from it. However, amount of power produced from the wastewater was only a fraction of that produced from pure substrates. (Liu et al., 2004, Feng et al., 2008, Aelterman et al., 2006)

It was shown that wastewaters may also contain compounds with detrimental effects on the power generation. When acetate was added to wastewater as a readily available and easily biodegradable pure substrate, and it resulted in an increase in the performance. (Rabaey et al., 2005)

Structure and diversity of the microbial community in MFCs are expected to be affected by the substrates utilized. As the oxidation potential of the substrate increaes, amount of the energy accessible to the microbial community becomes larger. As a result, a higher potential for electricity production with more diverse populations can be expected as well. Nevertheless, there is no clear evidence for such a correlation.

2.6.1 Acetate

Acetate is the most common substrate in microbial fuel cells. It is a simple carbon source and it can induce exoelectrogens. (Bond et al., 2002)

In most metabolic pathways of higher hydrocarbons acetate is the end-product and it doesn't have any interactions with methanogenesis or fermentation pathways at ambient temperatures. (Aelterman, 2009) These characteristics make them a great choice of substrate when new MFC conditions, designs or parameters are tested.
According to Chae et. al who compared the power densities of some major substrates used in MFCs, acetate showed the highest performance when compared to glucose, butyrate and propionate. (Chae et al., 2009)

2.6.2 Glucose

Glucose is another substrate that is commonly encountered in MFCs. Maximum power density of 216W/m^3 was illustrated using glucose as a substrate in a fed-batch reactor which uses ferric cyanide as a catalyst. (Rabaey et al., 2003)

In another study power generation using glucose was compared with anaerobic sludge fed MFC. While anaerobic sludge could generate as few as 0.3mW/m^2 , same system had 161mW/m^2 with glucose. (Hu, 2008) However, in comparison with acetate, glucose was shown to exhibit much lower energy conversion efficiency (Acetate 42%, glucose 3%). (Lee et al., 2008)

2.6.3 Lignocellulose and derivatives

Lignocellulosic materials are another alternative for utilization as substrates in fuel cells because of their abundance as agricultural wastes. Their abundance also makes them inexpensive substrate for energy production. (Huang et al., 2008)

Only disadvantage of lignocellulosic compounds is that they can not be directly used for power generation. First they should be broken down to their building blocks, monosaccharides, to be utilized as substrates. Catal et al. (2008) demonstrated that every monosaccharides that is derived from lignocellulose is suitable as substrate for power generation in MFC. MFC with a modified air-cathode design and corn stover waste biomass as a substrate exhibited power densities up to 371mW/m^2 (Zuo et al., 2006). However, in a single chamber MFC, its comparison with glucose showed much lower power generation. (Wang et al., 2009)

2.6.4 Synthetic Wastewater

Synthetic wastewaters are another type of substrates used in MFCs with their defined content. Also pH, conductivity and loading strength are simple to manage. Rodrigo et al. studied to effect of wastewater content on MFC performance by using two different synthetic wastewaters with different ratios of glucose and peptone. Wastewater with higher glucose content which is readily biodegradable substrate showed lower power generation.

2.6.5 Brewery Wastewater

Brewery wastewater is another ideal choice of substrate for MFCs. Its characteristics of low strength, absence of inhibitory molecules and high carbohydrate content are main reasons for this choice. Concentration of wastewater changes from 3000 to 5000mg COD/L which is almost 10 fold concentrated than the average domestic wastewater.(Vijayaraghavan et al., 2006) In an air cathode MFC, beer brewary wastewater was treated with addition of 50Mm phosphate buffer and power density was monitored as high as 528mW/m^2. (Feng et al., 2008) However, when domestic wastewater was brought to same strength with brewary wastewater by dilution, it exhibited lower power density probably due to significant decrease in its conductivity.

2.6.6 Landfill leachates

Landfill leachates are composed of four major components which are dissolved organic matter, inorganic matter, heavy metals and xenobiotic compounds. (Kjeldsen et al., 2002) In recent studies electricity generation for short period of time using leachate was revealed. Maximum power optimized to unit volume was measured as 12.8 W/m^3. (Zhang et al., 2008) Current studies aims to generate electricity while leachate is treated. Indeed, MFCs fluidically connected in series was reported for performing both at the same time. (Galvez et al., 2009)

2.6.7 Dye Wastewater

In MFCs, different types of wastewater samples may be utilized as substrates. Textile dye process wastewaters that are produced in high amounts as effluents is one example to that. Thus they should be removed from the effluents because of their

harmful effects for the environment. (Pant et al., 2008) Some of these dyes are also known with their toxic natures. Keeping this in mind, recent studies aimed to use dye wastewaters as substrates for MFCs. Sun et al. (2009) decolorized a dye called active brilliant red X-3b when he used confectionary wastewater together with glucose. Even when glucose was used as single substrate, concentrations up to 1500mg/L was decolorized. However, at higher concentrations competition of azo dye and anode for the electrons decreased the power generated. For this purpose use of two different wastewaters with different natures, one with textile dye and other with biodegradable substrate, economically feasible method for decolorization can be achieved for future applications.

2.7 Microbial diversity of the MFC

In respiration, bacteria, especially anaerobes, transfer their electrons to different compounds as terminal electron acceptors. In most cases, these compounds are soluble like nitrate or sulfate that can diffuse through the cell membrane. However, as mentioned in previous sections some bacteria have unique ability to transfer their electrons outside their membranes. Exoelectrogens and their fascinating nature enable them to be major role players in MFCs.

Recent studies have pointed out two genera capable of metal reduction, Shewanella and Geobacter as exoelectrogens in MFCs. Study of these organisms also provided significant information for the understanding of the electrogenesis mechanism. (Heidelberg et al., 2002, Methe et al., 2003)

Characterization of biofilms that perform exoelectrogenic activity indicated a far diverse presence of exoelectrogens than these two bacteria.

Even though, it is not studied in detail, MFC design is expected to have an important influence on the community inside the reactor. When air-cathodes are used and oxygen has access to the reactor in a single chamber reactor with no membrane, interaction of bacteria with oxygen and alternative electron acceptors is inevitable. (Neilsen et al., 2002) This provides a more suitable environment for more diverse microbial communties.

2.7.1 Microbial diversity in air-cathode MFC

Brief analysis of studies done with different inocula and substrates in air-cathode MFCs demonstrate no apparent dominance of certain bacterial groups. Betaproteobacteria comprised the major group of the isolated clones in two-chamber MFCs, and inoculated with anaerobic sludge with starch as the carbon source. (Kim et al., 2004)

In the MFC that was inoculated with river sediment with glucose and glutamate as a substrates Alphaproteobacteria was the dominant group. (Phung et al. 2004) In another system containing acetate as substrate and inoculated with an activated sludge, almost equal distribution of Alpha, Gamma and Deltaproteobacteria was observed. (Lee et al., 2003)

Furthermore, in a study with two-chamber MFC that utilized ethanol as a substrate, microbial community exhibited 83% of 16S rRNA sequences, similarity with Betaproteobacteria group, and among these sequences only one was shown to have similarity with Geobacter or Schwanella. (Kim et al., 2007)

Inoculum	Substrate	Community
River sediment (*Phung et al.* 2004)	Glucose+ glutamic acid	65% = *Alpha*-(mainly *Actinobacteria*), 21% = *Beta*-, 3% = *Gammaproteobacteria*, 8% = Bacteroidetes, 3% = others.
River sediment (*Phung et al.* 2004)	River water	11% = *Alpha* -, 46% = *Beta*-(related to *Leptothrix* spp.), 13% = *Gamma*-, 13% = *Deltaproteobacteria*, 9% = *Bacteroidetes*, 8% = others.
Marine sediment (*Logan et al.* 2005)	Cysteine	*Gammaproteobacteria* (40% *Shewanella affinis* KMM), then *Vibrio* spp. and *Pseudoalteromonas* spp.
Wastewater (*Lee et al.* 2003)	Acetate	24% = *Alpha*-, 7% = *Beta*-, 21% = *Gamma*-, 21% = *Deltaproteobacteria;* 27% = others.
Wastewater (*Kim et al.* 2004; *Methe et al.* 2003)	Starch	36% = unidentified, 25% = *Beta*- and 20% = *Alphaproteobacteria*, and 19% = *Cytophaga* + *Flexibacter* + *Bacterioides*.

Figure 2.6: Microbial community analysis results of MFCs with various inocula and substrates. (Logan, 2008)

In the studies that were summarized in Figure 2.7, phylogenetic analysis of the sequences demonstrated presence of Actinabacteria, Leptothrix and Shewanella as major phylotypes. However, occurance of Geobacter is not monitored. The obligate anaerobe nature of this organism was suggested as the main reason for this absence. Indeed, it is not easy to define or compare certain factors which effect microbial diversity because of their assorted designs. Each factor should be experimented one by one to have a better understanding of factors effecting the diversity.

2.7.2 Study of power producing species in MFCs

Either it is a pure culture or mixed community of bacterial cells, amount of power generated is effected by electrode spacing, design and conductivity of the materials used. Thus in order to compare power producing species within each other and with mixed cultures, controlled experiments should be conducted. Furthermore, resistance should be kept at minimum values for accurate comparison. (Logan et al., 2006) For instance, recently it was shown that lower power densities demonstrated using Geobacter species were due to high internal resistance of dual-chamber systems. (Logan et al., 2006)

Development of single-chamber systems which minimized internal resistance was turning point for exploration of various factors on performance. (Liu et al., 2005) First Alphabacterium species was isolated from this type of reactor and it was demonstrated that it generated more power than mixed culture itself. Power densities up to 2.72 W/m2 were reported, compared to mixed inoculum from wastewater which produced 1.74 W/m2. (Xing et al., 2008)

In another research, pure culture of Geobacter sulfurreducens was shown to produce 22% less power compared to enriched mixed culture though this organism was present in the mixed culture. (Ishii et al., 2008) Nevertheless, smaller sized anode with ferricyanide as a catholyte boosted power production by the same organism up to 4 times that was reported previously. (Nevin et al., 2008)

Shewanella putrefaciensis is the first published bacterial species that can generate power when mediators are not present. (Kim et al., 1999) Shewenella species have two main characteristics for their exoelectrogenic activity. Firstly their outer membrane cytochromes enable them to transfer their electrons directly during contact. (Myers et al., 1992) Secondly they were shown to possess conductive nanowires for this purpose. (Gorby et al., 2006)

Briefly, in the context of mediatorless microbial fuel cells, following species are discovered as exoelectrogens. *Desulfuromonas acetoxidans* from sediment MFC consortium, *Geobacter metallireducens* from MFC wih poised electrode potential, *Aeromonas hydrophila*, *Desulfobulbus propionicus*, *Geopsychrobacter electrodiphilus*, *Desulfovibrio desulfuricans* are deltaproteobacteria, *Rhodoferax ferrireducens* from MFC fed with glucose is betaproteobacterium, *Pseudomonas*

aeruginosa G, *Shewanella oneidensis* DsP10, *Escherichia coli*, *Klebsiella pneumoniae* L17, *Ochrobactrum anthropi* YZ-1 are Gammaproteobacterium, *Acidiphilium* sp. is an Alphaproteobacterium, *Thermincola* sp. strain is a Firmicute, *Geothrix fermentans is an Acidobacteria and lastly Pichia anomala* is from the Kingdom Fungi. (Bond et al, 2002, Holmes et al, 2004, Holmes et al., 2009, Zhang et al., 2006, Zhao et al, 2008, Rabaey et al., 2004, Prasad et al., 2002, Borole et al., 2008, Wrighton et al., 2008)

2.7.3 Microbial diversity of the cathode

Microbial diversity on the cathode of a microbial fuel cell did not recieve much attenttion. In general, especially before the discovery of single chamber designs, only anode chamber was known to be biotic while cathode chamber was abiotic on which reduction of oxygen or some other molecules occured.

Idea of using biocathodes instead of abiotic cathodes which used platinium or other metals as catalysts emerged due to their several disadvantages compared to conventional alternatives. First and most importantly biocathodes are less expensive. Morever, they may provide longer sustainability. Third, complex metabolic pathways of microorganisms present on cathodes may help production of useful products and get rid of undesired ones. Finally cathode microorganisms can assist for nitrification or denitrification reactions of MFCs to be used in wastewater treatment.

In literature biocathodes are categorized as; aerobic or anaerobic. In aerobic biocathodes oxygen is the terminal electron acceptor as most commonly observed in MFC technology. Some transition metals are recognized to assist electron delivery to oxygen. Some researchers applied microorganisms to perform this reacton on cathode chambers. Manganese oxidizing bacteria Leptothrix discophara was utilized in this manner and a 40-fold increase in power output was observed compared to abiotic control. (Rhoads et al, 2005) In a similar study which used iron instead of manganese, Thiobacillus ferrooxidans was utilized for microbial oxidation in cathode compartment to increase power generation. (Nemati et al., 1998)

Other than the examples above, presence of biofilm on cathode material and microbial analysis of the biofilm is significant to demonstrate presence of microbial diversity on cathodes. Very few studies have illustrated presence of biofilm on cathode material and fewer of them phylogenetically investigated diversity.

Biofilm formation on the cathode was first observed on marine sediment MFCs. As their name indicated, they have anode located inside the sediment, but their cathode is exposed to sea water environment which possess almost the largest global microbial diversity on Earth. In related studies, Hasvold et al. (1997) discovered that cathode biofilm certainly advances the rate of oxygen reduction. Also Bergel et al. (2005) found that fuel cell with cathode biofilm produced almost 100 fold more power density when compared to fuel cell with platinium catalyst. Lastly, in a molecular study which explored the abundant species on cathode biofilm showed Pseudomonas fluorescens as the most dominant (Reimers et al., 2006).

In one of the rare studies which investigated the microbial diversity of the cathode biofilm from a dual-chamber microbial fuel cell which was inoculated with mixed environmental samples on cathode department. Biofilm formation after about three months of operation was analyzed using 16S rRNA gene clone library analysis. Results indicated that abundant species of the biofilm were *Sphingobacterium*, *Acinetobacter*, and *Acidovorax* (Rabaey et al., 2008).

In conclusion, in contrast to studies which investigated microbial communities on anode departments very few are present for microbial diversity of the cathodes. Despite their high possibility of presence especially in single-chamber designs without exchange membranes, there was not any reported study which analyzed cathodes biofilms of such systems. The current study therefore aims to investigate the identity of the organisms in cathode biofilms of MFCs during degradation of different textile dyes.

3. MATERIALS AND METHODS

In the research, the primary target was the investigation of bacterial communities in a single-chamber air-cathode microbial fuel cells during deolorization of different textile dyes. Main focus was given to phylogenetic analysis of cathode biofilm which was observed in single-chamber design. Comparison of both archaeal and bacterial 16S rRNA gene DGGE profiles was performed after decolorization of each textile dye. Furthermore clone library analysis was done to assess changes in abundant bacterial species biofilm during the process. Operational parameters of MFC such as voltage and power output were also measured and calculated as an indicator of proper MFC functioning.

3.1 Materials and Equipment

List of all equipments, chemicals and buffers and their suppliers are listed in Appendix A, Appendix B and Appendix C respectively.

3.2 Methods

3.2.1 Construction of MFCs

MFCs were designed as a modified version of a previous construction (Liu et al., 2005). A plexiglass cylindirical chamber of 12 ml of volume was used as reactor. Cathode and anode materials were made of carbon fiber and they were placed on the opposite sides of the MFC chamber. Area of anode and cathode was identical and was 7 cm^2. Anode and cathode were prepared according to procedures described elsewhere (Liu et al., 2005). Four identical microbial fuel cells were used for the experiments (Figure 3.1).

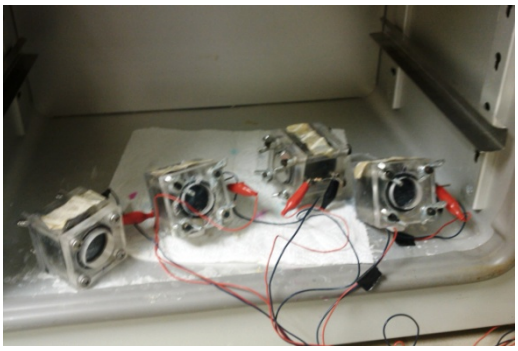


Figure 3.1: MFCs used in the study.

3.2.2 Measurement of MFC electricity yield

Voltage generated by microbial fuel cells were measured using a multimeter with a data aquisition system (Figure 3.2. Keithley 2700, Cleveland, OH, USA). In order to calculate power densities, P=VI/A equation was used (V = voltage, I= current, and A is the anode or cathode surface area.

Figure 3.2: Multimeter with a data aquisition system (Keithly 2700, Cleveland, OH, USA).

3.2.3 Operation of MFC

Four identical MFCs were inoculated with a mixed culture kindly provided bythe wastewater treatment plant of a baker's yeast production company.(Pakmaya İzmit, Turkey). Initially, production wastes obtained from the company that is rich in molasses and some other carbohydrate derivatives was used as medium. This complex substrate was replaced each week until the MFCs started to produce electricity, as an indication of bacterial biofilm settling on the electrodes. After the power generation was stabilized, the complex medium was replaced gradually with the defined medium containing only sodium acetate as the carbon source (Liu and Logan, 2004). The medium solution contained per liter: Sodium acetate (2 g) , NH_4Cl (310 mg), KCl (130 mg), $NaH_2PO_4.H_2O$ (4.97 g), $Na_2HPO_4.H_2O$ (2.75 g),

mineral solution (12.5mL) and vitamin just (12.5 mL) (Lovley and Philips, 1988) Every other day, the medium solution was replaced with fresh one. After reaching a steaady power generation, five different reactive textile dyes of 40 and 80 mg/L concentrations; Remazol Brillilant Blue BB Gran 133 (Dyestar GA10503), Remazol Black RL (Dyestar C19143), Remazol Turquoise Blue G 133 (Dyestar FA09807), Reactive Red 195 (Setaş Kimya 5017), Reactive Yellow 145 (Setaş Kimya 5030), were added each week and decolorization was monitored spectrophotometrically.

3.2.4 Sample collection and storage

After decolorization of two different concentrations of the same dye was accomplished, biofilm samples were collected from cathode surface of each MFC where a thick bacterial biofim formation was observed. Samples were also collected from the MFC medium preceeding the replacement with the fresh one. There were seven batch cycles for potential generation between each sampling time. Both biofilm and MFC solution samples were stored at -20 ºC for DNA extractions and further analysis.

3.2.5 DNA extraction

Total genomic DNA of biofilm, and MFC solution samples were extracted using FastDNA Spin Kit for Soil (MP Bio, USA). MFC solutions were centrifuged at 14000rpm for 5 minutes and suspended solids were precipitated preceeding the DNA extraction.

Samples were analyzed on agarose gels. The gels were prepared using 1% (w/v) agarose in 1XTAE buffer containing 0.5 µg/mL ethidium bromide. 3µl of DNA samples were mixed with 2x gel loading buffer. Electrophoresis was performed at 10V/cm and the gel was visualized under UV using Gel Doc (BIORAD, US), gel imaging system. DNA concentrations were measured using fluorimetric assay on Qubit fluorimeter (Invitrogen, US) according to the procedure supplied by the manufacturer. DNA concentrations were used to determine dilution factors for further PCR and other downstream analyses.

3.2.6 Polymerase chain reaction (PCR)

PCR is an in vitro method for amplification of certain DNA fragments using specific complimentary oligonucleotide sequences . In this study, Bacterial and Archaeal PCRs targeting 16S rRNA gene fragments were performed for assessing microbial diversity of samples in DGGE, and identifying abundant species in clone library analysis.

3.2.6.1 Bacterial PCR

In order to investigate bacterial microbial diversity, primers targeting V3 region of bacterial 16S rRNA gene, named Vf-GC and Vr (Table 3.1), were utilized. Amplification reaction conditions were summarized in Table 3.2. PCR reaction consisted of 1µM from each primer, 0.2mM dNTP, 10x PCR buffer (300mM Tris-HCl, 300mM salt solution, 20mM Mg^{+}, enhancer) and 1U i-StarTaq^{TM} DNA polymerase (INTRON Biotechnology, Inc, USA). 1µL of DNA served as a template in a 40µL of total PCR volume. Reaction was performed using C1000^{TM} Thermal Cycler. (BIORAD, USA). PCR products were visualized by agarose (1% w/v) gel electrophoresis following the staining with ethidium bromide in 1XTAE buffer. Visualisation was done using GelDoc^{TM} (BIORAD, USA). All PCR products were stored at 4 ºC until DGGE analysis.

3.2.6.2 Archaeal PCR

For assessment of archaeal diversity in samples, nested PCR procedure was used to enhance sensitivity and specificity targeting 16S rRNA fragments. Primers for the first round PCR and nested PCR were Arch7f and Arch1384r and Arch344fgc and Univ522r, respectively (Table 3.1). PCR reaction consisted of 1µM from each primer, 0.2mM dNTP, 10x PCR buffer (300mM Tris-HCl, 300mM salt solution, 20mM Mg^{+}, enhancer) and 1U i-StarTaq^{TM} DNA polymerase (INTRON Biotechnology, Inc, USA). 1µL of DNA served as a template in a 25µL of total PCR volume in the first round reaction and 1µL from the PCR product of first round reaction served as a template for the nested PCR procedure. Total volume for the second round amplification was 40µL. Amplification cycles were listed in Table 3.2 in detail. PCR products were visualized using the procedures described above.

PCR products from the first round reaction was used for clone library analysis of Archaeal abundance in the sample. DGGEwas carried out using nested PCR products.

3.2.6.3 PCR for clone library analysis

In order to determine Bacterial species in biofilm and MFC solution samples, clone libraries were constructed by amplifying a certain region of genomic DNA specific to Bacterial domain. 16S rRNA gene serves for this purpose because of its characteristic of being a molecular clock. Specifically; primers used to target the longest fragment of this gene named pA and pH (Table 3.1) were used for PCR prior to clone library analysis. Composition of PCR reaction was identical with that described in previous sections. 1µL of genomic DNA was added as template in a total volume of 50µL. Following the cloning reactions, analysis of the transformants was accomplished via PCR using M13 reverse and T7 primers supplied by the manufacturer (Invitrogen, Carlsbad, USA). (Table 3.2). Gel visualization was performed using agarose gel electrophoresis as described in previous sections.

3.2.6.4 Sequencing PCR

For phylogenetic analysis of biofilm and MFC solution samples, sequence data is necessary to idenify affiliated phylotypes. PCR procedure was applied for this purpose, as suggested in the Sequencing kit manual. T7 (Table 3.1) was used as a primer in the amplification reaction. PCR reaction mix was composed of 2µL of 5X Big Dye Terminatior Buffer, 1µL of 10ng/µL primer and 2µL of Big Dye Terminator in a total volume of 10µL. Conditions of PCR reaction was applied according to kit manual. Sequence PCR products were purified using Sodium acetate buffer (3M, pH=5.5) and ethanol. In the final step the products were dissolved in formamide and were analyzed in a DNA sequence analyzer (ABI , USA)

Table 3.1: List of primers used in the study

Primer name	Annealing site*	Annealing Temperature (ºC)	Sequence 5'-3'	Reference
VfGC	341-357	55	GC**-GGC CTA GGG GAG GCA GCA	Muyzer et al.,1993
Vr	518-534	55	ATT ACC GCG GCT GCT GG	Muyzer et al., 1993
Arch7f	7-24	57	TTCYGGTTGAT CCYGCC	Lueders et al., 2004
Arch1384r	1384-1402	57	CGGTGTGTGCA AGGAGCA	Lueders et al., 2004
Arch 344fGC	344-358	62.5	GC**-GAC GGG GHG CAG CAG GCG CGA	Raskin et. al, 1994
Univ 522r	504-522	62.5	GWA TTA CCG CGG GKG CTG	Raskin et. al, 1994
pA	20-40	55	AGA GTT TGA TCC TGG CTC AG	Edwards et al., 1989
pH	1533-1553	55	AGG GAG GTG ATC CAG CCG CA	Edwards et al., 1989
M13 Reverse	pCR®4-TOPO®vector***	55	CAGGAAACAGC TATGAC	Invitrogen
T7	pCR®4-TOPO®vector***	55	TAATACGACTC ACTATAGGG	Invitrogen
T3	pCR®4-TOPO®vector***	55	ATTAACCCTCA CTAAAGGGA	Invitrogen

*E.coli numbering according to Brosius et. al (1978)

** GC-clamp: CGC CCG CCG CGC GCG GGC GGG GCG GGG GCA CGG GGG

*** Plasmid vector of TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, USA)

Table 3.2: Details of PCR amplification reaction conditions

Primer Set	VfGC/ Vr	Cycles	Arch7f/Arch1384r	Cycles	Arch344fGC/ Univ522r	Cycles
Initial Denaturation	95 ºC - 4'	1	95 ºC - 4'	1	95 ºC - 4'	1
Denaturation	95 ºC - 30''		95 ºC - 40''		95 ºC - 30''	
Annealing	55 ºC - 30''	35	57 ºC - 45''	35	55 ºC - 45''	35
Elongation	72 ºC - 45''		72 ºC - 50''		72 ºC - 1'	
Final Elongation	72 ºC - 10'	1	72 ºC - 10'	1	72 ºC - 10'	1

Table 3.3: Details of PCR amplification reaction conditions in the study of pA and T7 primers

Primer Set	pA/pH	Cycles	T7	Cycles
Initial Denaturation	95 ºC - 5'	1	95 ºC - 5'	1
Denaturation	95 ºC - 30''		**3.1.1.1** 95 ºC - 1'	
Annealing	55 ºC - 30''	35	50 ºC - 30''	30
Elongation	72 ºC - 45''		60 ºC - 4'	
Final Elongation	72 ºC - 10'	1	-	

3.2.7 DGGE analyses

DGGE is used to separate DNA fragments of same length with different nucleotide content, depending on its Tm. Partial separation of fragments occur at sequence specific melting temperature (Tm). At their specific Tm temperatures, migration of fragments on the gel retard. Different DNA fragments move in different paces on polyacrylamide gels containing urea and formamide as denaturing agents. This indicates microbial complexity and diversity of a given sample.

Microbial diversity of Archaeal and Bacterial domains on cathode biofilm and MFC solution samples were studied via DGGE analysis. Standard gel profiles were used to standardize each gel, andcomputer programs were utilized as well. For standard profiles seven random colonies were picked from bacterial clone library of cathode biofilm samples and run separately on the gel. Upon confirrmation of the evenly distributed bands, they were selected as suitable standards..

3.2.7.1 Casting of the DGGE Gel

In DGGE methodology, formation of denaturing gradient gel with anundisrupted linear concentration gradient is crucial to eliminate errors. Gels with linear gradients were poured into the glass plates separated with 1.0 mm-thick separators. Desired gradient concentrations were prepared by mixing 0% and 100% gradient polyacrylamide solutions. (Appendix C).

3.2.7.2 DGGE of the Archaeal and Bacterial PCR Products

DGGE procedure was applied using Bio-Rad Dcode System (Bio-Rad, Hercules, CA, USA). The PCR products were loaded onto 10% (w/v) polyacrylamide (37:5:1 acrylamide: bisacrylamide) gels containing 35-60% linear denaturing gradient for Bacterial PCR products and 30-70% for Archaeal nested PCR products in 1XTAE buffer. Both Archaeal and Bacterial products were run on gels at 60ºC and 180V for 330 minutes. Gels were photographed under UV on gel documentation system immediately after staining processes for further image processing.

3.2.7.3 Fingerprinting Analysis

In diverse microbial environments like activated sludge, sediments, soils and biofilm samples, interpretation of complex DGGE band profiles is difficult. Therefore, densitometric curves are determined using computer based programs for analysis. Number of peaks and their intensity on these curves indicate the presence of different

16S rRNA gene sequences. Thus, their interpretation using related software aids in analytical comparison of microbial populations. On each gel, three standard gel profiles were loaded for normalization purposes and statistical analysis methods were applied on normalized gel photos.

Distance methods, maximum parsimony and maximum likelihood are the most commonly used methods for phylogenic analysis of DGGE patterns. Choice of the most appropriate method depends on the aim of the experiment and complexity of the data set.

Unweighted Pair-Group Method with Arithmetic Mean (UPGMA) and Neighbor Joining are most widely used algorithms for phylogenetic tree construction among distance methods. Main principle of both methods is to calculate, for each pair of taxa or operational taxonomic units, the distances between them. (Fitch, 1967) Distances are used to build distance and similarity matrices.

Furthermore, especially for bacterial assessment of microbial diversity, DGGE gives important information on approximately how many different taxa are present on each sample. This information is also a validation of clone library analysis where phylogenetic analysis of each taxa is investigated.

3.2.8 Clone Library Analysis of the Samples

In order to determine Bacterial and Archaeal species found in cathode biofilm samples, clone libraries were built. For bacterial cloning and Archaeal cloning, PCR amplicons obtained using pA/pH primer pair and PCR amplicons of the first round Archaeal PCR reaction were used, respectively. TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, USA) was used according to the instructions provided by the manufacturer. Fifty random colonies were selected for each sample and after overnight inoculation, plasmids were extracted using QIAGEN MiniPrep Plasmid isolation kit. (Qiagen, Inc., Valencia, CA) PCR reactionsmentioned in previous chapters were used to analyze transformants that contained the inserted PCR product. Figure 3.3 shows plasmid vector and its primer annealing sites. Size of the amplicon after the PCR reaction using M13 reverse and T7 primers determined proper insertion of desired 16S rRNA gene fragments. Prepared plasmids possessing the transformants were used as templates for sequencing PCR reactions.

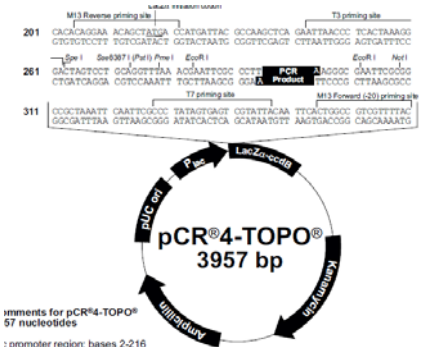


Figure 3.3: Plasmid vector with primer annealing and PCR product insertion sites. (Invitrogen, Carlsbad, USA)

3.2.9 Phylogenetic Analysis

Sequencing data were analysed and homology searches were made using Basic Local Alignment Search Tool (BLAST) server of the National Centre for Biotechnology Information using the BLAST algorithm. Nucleotide sequences were queried against nucleotide sequence database (blastn).

Once BLAST finds similar sequences with query sequence, it is important to understand whether this demonstrates a biological relation or a coincidental similarity. BLAST algorithm applies statistical theory to calculate bit score and expect value (E-value) for each alignment pair. Bit score shows the quality of the alignment. Higher bit scores mean better alignment. E-value indicates the statistical importance of the alignment. The lower E-values implies more significant query hit. For example if E-value for an alignment is 0.05, tells occurance of this similarity is by chance alone in 1 to 20 probability. Although this value may seem statistically significant, biologically it may be otherwise.

During the study, sequences were retrieved from six different clone libraries representing liquid culture samples and cathode biofilm samples collected after decolorization experiments. Overall, 37 sequences were retrieved from inoculum samples and a total of 210 sequences were retrieved and analyzed from cathode biofilm sample libraries. However, relatively low microbial diversity was found amongthe Archaeal sequences, providing only 19 different sequences.

After determining the closest GENBANK matches, Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 4 (Tamura, Dudley, Nei,

and Kumar 2007). A phylogenetic tree was constructed using Weighbor analysis available on Ribosomal Database Project. RDP classifier was used to obtain cultured isolates that were affiliated with sequences available. (Wang et al.,2007, Cole et al., 2009)

3.2.10 Fluorescence *In Situ* Hybridization (FISH)

Whole cell FISH is a methodology that provides better quantitative active cell information about a microbiota. Fluorescent dye conjugated probes enable direct detection of single cells in environmental samples using fluorescence or confocal laser microscopy techniques. Whole cell FISH technique not only provides information about active cells inside a sample but also morphology and spatial distribution of these cells in the assessed microbial community.

FISH methodology can be described as follows; sampling of the part which will be further analyzed, sample fixation with paraformaldehyde, hybridization of fluorescent labeled probes, washing of excess probes and microscopy either with fluorescent of confocal scanning laser microscopes.

Fixation step usually includes permeabilization of the cells as well. Cells to be analyzed are fixed to protect their morphological integrities and this process also makes them permeable to short oligonucleotides. Common fixatives are alcohols that help precipitation of proteins and aldehydes that cross-link the cell walls. In most studies 4% paraformaldehyde (an aldehyde) is used to fix and permeabilize the cells.

In FISH methodology, hybridization occurs *in situ* between fluorescent labeled probe with a rRNA molecule in a fixed cell. Hybridization depends on three factors: presence of target molecule, sequence homology between probe and target and most importantly, the stringency of the conditions for binding. Salt concentrations, temperature, denaturant concentrations (formamide) and washing steps have important impact on optimum stringent conditions. Different conditions can be maintained depending on the target sample. defines The optimum stringent conditions is defined as the conditions for which the target organism shows a good signal and there are no other signals. For each hybridization, appropriate controls were included to avoid misinterpretation of the results. Negative control was included to detect possible autofluorescence, another negative control that does not

hybridize to any target molecule was included to visualize non-specific binding, and finally, a positive control was included.

Hybridization and wash buffers were prepared according to previous methods(Amann et. al. Appendix C). EUB 338, Non EUB and Arch 915 probes were selected for this study (Table 3.3) and optimum hybridization conditions were applied according to Alm et al. (1996)

In this study, FISH was performed to detect the active cells and their abundance in the cathode biofilm. CSLM was used to scan three dimensional biofilm structure on the electrode.

Table 3.3: Probes, binding sites, sequences and hybridization conditions for the oligonucleotide probes used.

Oligonucleotide	Probe Sequence 5'-3'*	Binding Site	Hybridization conditions (Temperature (ºC) / Formamide Concentration)	Reference
EUB 338	GCT GCC TCC CGT AGG AGT	Bacteria (338-355)	37/30%	Amann et al., 1990
Non EUB	ACT CCT ACG GGA GGC AGC	None	46/40%	Manz et al., 1994
Arch 915	GTG CTC CCC CGC CAATTCCT	Archaea (915-935)	46/35%	Stahl et al., 1991

*E.coli numbering according to Brosius et al., 1978.

4. RESULTS AND DISCUSSION

4.1 Establishment of Microbial Community in MFC

All four MFCs were inoculated with a mixed culture obtained from the yeast production wastewater, and then acclimatized to defined medium using acetate as carbon source. Stable voltage generation using ane external resistance of 1 kΩ was reached within 8 weeks. In parallel with MFC activity, a thick cellular mat was formed on cathode. Figure 4.1 is a cartoon demonstrating the MFC design and sampling points for microbial community analysis of Archaeal and Bacterial domains. Sampling was performed after microbial community establishment upon addition of the textile dyes into the regular medium. The time period between each medium replacement was considered as one batch of MFC run. All the sampling were done at the end of each batch.

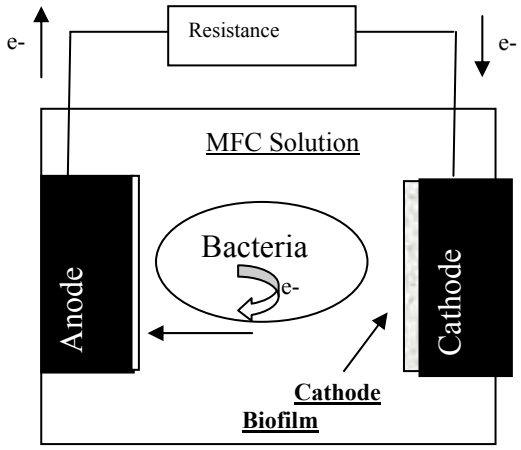


Figure 4.1: Schematic diagram of MFC used in the study. Arrows denote the sampling points. Five samples were collected from each sampling point during decolorization of five different reactive textile dyes.

4.2 Performance and Sampling

Sampling was started to be performed after 2 months of acclimitization . Thereafter five different textile dyes were added successively and sampling has been done after decolorization of each dye.. During decolorization and sampling period Stable voltage values of 0.4-0.45V were observed. Maximum power density reached was 325.6 mW/m^2 anode surface area, which is consistent with those obtained in previous works When acetate is used as the carbon source, and xenobiotics are not included, power densities of up to 506 mW/m^2 were reported (Liu et. al., 2005). Lower values in power density in our study might be attributed to the influence of the toxic dyes and thick biofilm formation on the cathode.

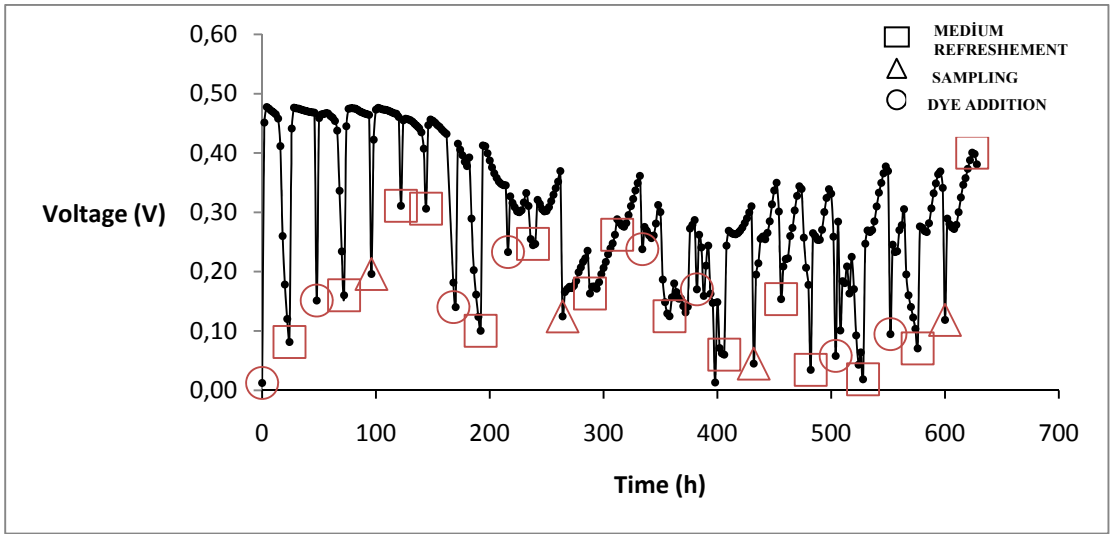
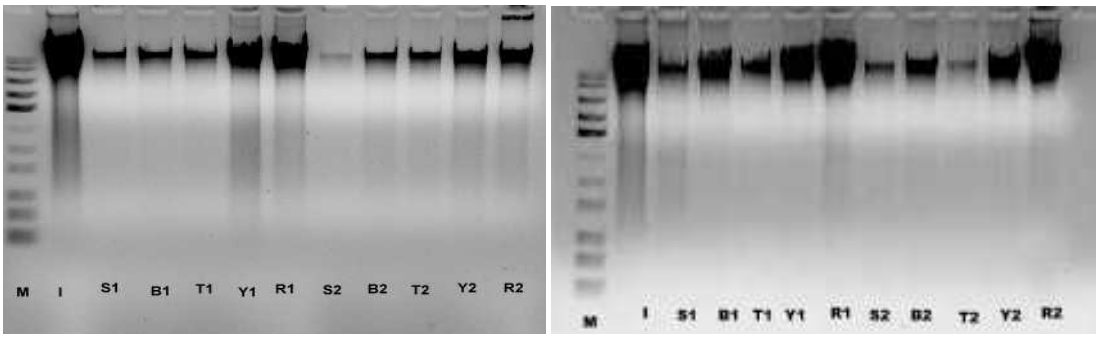


Figure 4.2: Measurement of electricity production in MFCs.

4.3 DNA Extraction Results

Total genomic DNA was extracted from cathode biofilm, MFC solution and inoculum samples. Prior to PCR-DGGE analysis of microbial diversity, nucleic acid concentrations were determined. Except for the S2 sample (cathode biofilm sampled after decolorization of S dye in MFC2), concentrations were measured above 10 µg/mL and diluted for subsequent PCR reactions. Agarose gel electrophoresis images of the genomic DNA showed that pure and clean DNA were extracted. (Figure 4.3) Sample legends used throughout the study is as follows: I, inoculums; S, B, T, Y and R are Remazol Black RL, Remazol Brillilant Blue BB Gran 133, , Remazol Turquoise Blue G 133, Reactive Yellow 145 and Reactive Red 195

respectively. Numbers following the letters indicate the numbering for four replicative MFCs.



(a) **(b)**

Figure 4.3: Extraction results of genomic DNA samples. (a) Cathode biofilm samples (b) MFC solution samples.

Table 4.1: DNA concentrations of the samples.

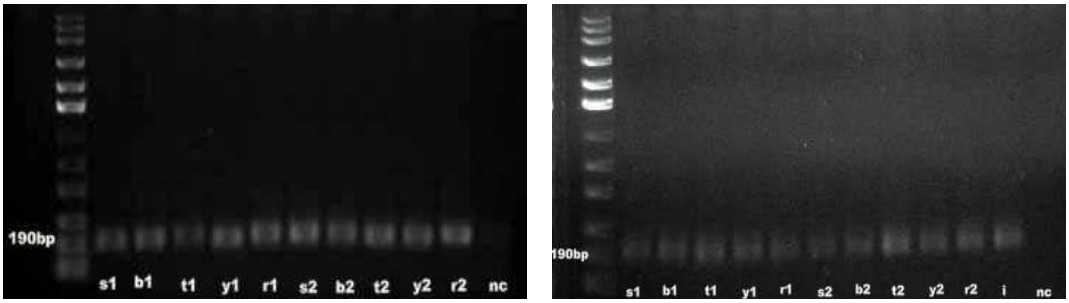
Cathode Biofilm				**MFC Solution**			
Sample	**Concentration (ug/mL)**	**Sample**	**Concentration (ug/mL)**	**Sample**	**Concentration (ug/mL)**	**Sample**	**Concentration (ug/mL)**
S1	31.6	**S3**	80.3	**S1**	44.5	**S3**	41.6
B1	29.6	**B3**	132	**B1**	58.2	**B3**	77.2
T1	34.5	**T3**	47.5	**T1**	34.4	**T3**	36.6
Y1	86.9	**Y3**	82	**Y1**	66.8	**Y3**	43
R1	107	**R3**	168	**R1**	92.1	**R3**	56.9
S2	6.45	**S4**	26.8	**S2**	21.4	**S4**	n.d
B2	43.1	**B4**	43.9	**B2**	64.7	**B4**	137
T2	39.9	**T4**	44.8	**T2**	35.5	**T4**	25.5
Y2	47.5	**Y4**	47.6	**Y2**	55.8	**Y4**	57.8
R2	72.5	**R4**	58.9	**R2**	116	**R4**	117
I	45.5						

4.4 PCR Results

Presence of archaeal and bacterial communities was detected using domain specific 16S rRNA gene primers. Amplicons obtained in PCR reactions served as a template for subsequent DGGE methodology to assess microbial diversity of each sample. Briefly, 20 cathode biofilm and inoculum samples were screened for both Archaeal and Bacterial diversity, and 18 MFC solution samples were screened for Bacterial diversity only. After the amplification, all of the PCR products were of the expected length.

4.4.1 Bacterial PCR Results

Bacterial 16S rRNA genes were successfully amplified although S2 sample from cathode biofilm was below desired concentration value (10 μg/mL). Total of 20 cathode biofilm samples and 18 MFC solution samples plus the inoculum sample were screened for presence of Bacteria (Figure 4.4). Amplicons were used for consequent DGGE analysis.

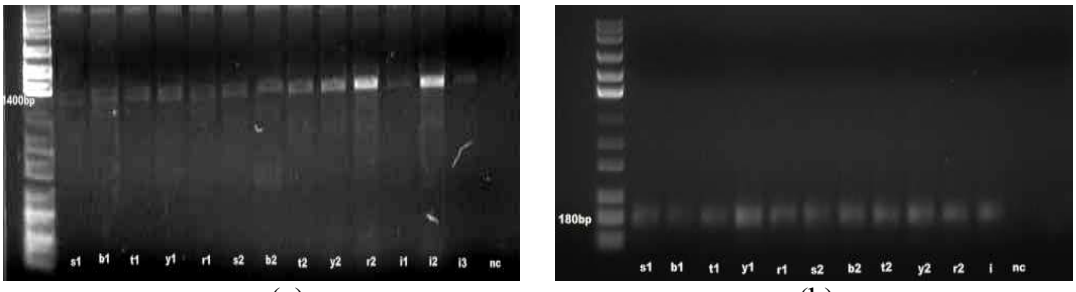


(a) (b)

Figure 4.4: PCR results of bacterial 16S rRNA gene amplification using VfGC/Vr primer. (a) MFC solution samples (b) Cathode biofilm samples including inoculum.

4.4.2 Archaeal PCR Results

Archaeal community 16S rRNA genes were amplified successfully in nested PCR reactions (Figure 4.5). Successful amplification indicated presence of Archaea in all of the samples. PCR amplicons were used as samples for the DGGE process to assess Archaeal diversity.



(a) (b)

Figure 4.5: PCR results of Archaeal 16S rRNA gene amplification using primers Arch7f/Arch1384r and Arch344fGC/Univ522r. (a) First round amplification using Arch7f/Arch1384r (b) Second round amplification using primers Arch344fGC/Univ522r.,

4.5 DGGE Results

Bacterial and Archaeal DGGE band patterns were successfully produced after targeting domain-specific 16S rRNA primers to check microbial diversity within each sample set. Also DGGE profiles of fuel cells indicated diversity within the four different MFCs with respect to each other.

4.5.1 Cathode Biofilm Samples

In order to assess Bacterial and Archaeal diversity in cathode biofilm samples, DGGE analysis was performed. Most importantly, DGGE band profiles of different samples within each MFC were used to study the effect of different textile dyes on microbial communities and also temporal change in microbial diversity.

Bacterial DGGE profiles of the samples from the first two MFCs demonstrated very diverse Bacterial microbial communities especially in cathode biofilms (Fig. 4.6). Number of detected taxa in biofilm samples was approximately 16-20 and the bands numbered 1-7 were determined as the major taxa. Each band, especially the bands numbered 1,3,5 and 6 had different intensities in different samples which may indicate varied quantities of related taxa among the samples.On the other hand, it is undeniable that DGGE is not an exact quantitative method.. Thus, calculations of this change were determined using similarity matrices of phylogenetic softwareprograms.

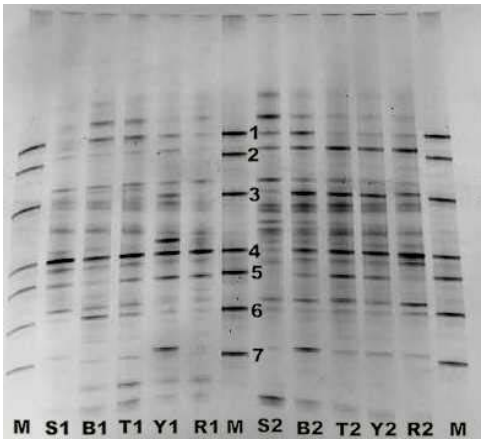


Figure 4.6: Bacterial 16S rRNA DGGE profiles of cathode biofilm samples. Bands numbered 1-7 were determined as major taxa.

Archaeal diversity was observed in the biofilm samples, yet, the number of taxa varied between 2 to 4 only. However, in the case of Archaeal 16S rRNA genes of cathode biofilm, emergence and disappearance of some DGGE bands were obvious.

Archaeal DGGE band profiles showed steady number of taxa regardless of different dyes added. However, DGGE band profiles within identical MFCs differed. Four different bands were detected in cathode archaeal community profiles (Figure 4.7). In MFC1, emergence of ARC4 as a new band in Y1 and R1 was observed while ARC1 and ARC2 that were detected in the previous samples disappeared. On the other hand, in MFC2, bands that disappear in MFC1 were conserved. Lastly, ARC3 band detected in inoculum community profile, which shows very little intensity among other bands, could not be observed in any of the other Archaeal profiles.

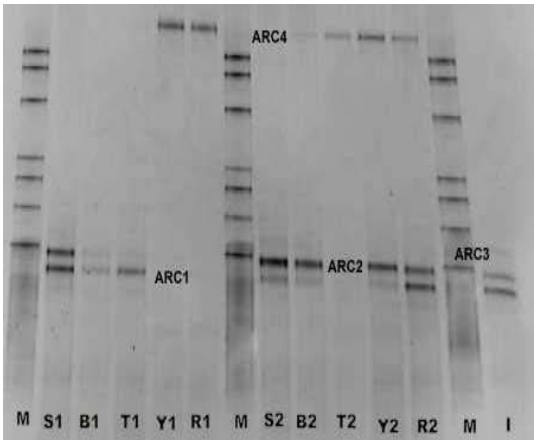


Figure 4.7: Cathode archaeal community profiles revealed by DGGE. M is the marker.

4.5.2 MFC Solution Samples

The number of taxa detected in MFC solution samples, varied between 3 to 6. Since MFC reactors were operated under batch conditions in which only medium was replaced, less number of taxa in MFC solution samples compared to biofilm samples is an expected outcome. . Occurrence of similar bands which were denoted as major taxa (1-7) in bacterial cathode biofilm profiles demonstrated presence of similar taxonomic units in MFC solution though less diverse. Thus bacteria that were observed in MFC solutions were most probably the ones detached from electrode biofilm during batch runs. As seen in the microbial diversity of MFC solution samples from two MFC reactors, some bands appeared in R1 and not in S1 sample (Fig. 4.8). In the light of this information, it can be interpreted that microbial diversity of MFC solution samples increases, as new textile dyes are introduced to reactors.

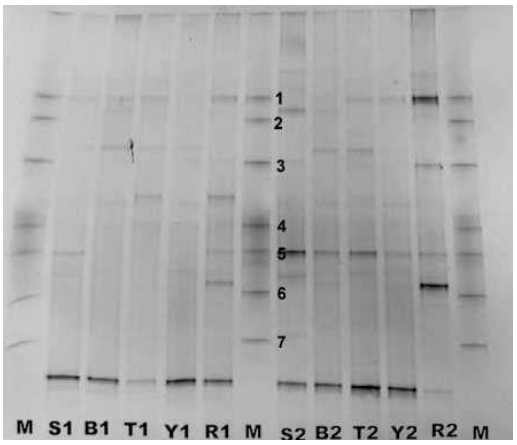


Figure 4.8: Bacterial community profiles in MFC solution revealed by DGGE.

4.5.3 Inoculum

Bacterial and Archaeal communities in inoculum sample was also studied via DGGE (Figure 4.7). Archaeal banding pattern of inoculum sample were highly conserved throughout the study. Yet, Bacterial profile of inoculum should be compared with bacterial profiles from both cathode and MFC solution samples using software programs because of their higher complexity.

4.6 Effect of Different Textile Dyes on Microbial Diversity

In order to investigate the effect of different textile dyes on microbial diversity of the MFC reactor, DGGE images were processed and normalized on Bionumerics Software (Applied Maths, Belgium). According to similarity matrices generated in the software, similarities of band patterns with respect to Day 0 (Inoculum sample) and previous days were plotted and analyzed.

4.6.1 Effect on Cathode Biofilm Diversity

DGGE band patterns of cathode biofilm were used to compare microbial diversities after different textile dyes were added. Archaeal and Bacterial DGGE band patterns of the inoculum were used as reference point for this comparison and it was referred as the sample of Day 0. Comparison was performed using Pearson product moment correlation coefficients (densitometric curve based) and UPGMA method.

4.6.1.1 Bacterial Cathode Biofilm Communities

In cathode biofilm bacterial DGGE band patterns, it was found that samples clustered for each MFC reactor except for the samples S3 and S4 (Fig. 4.9).

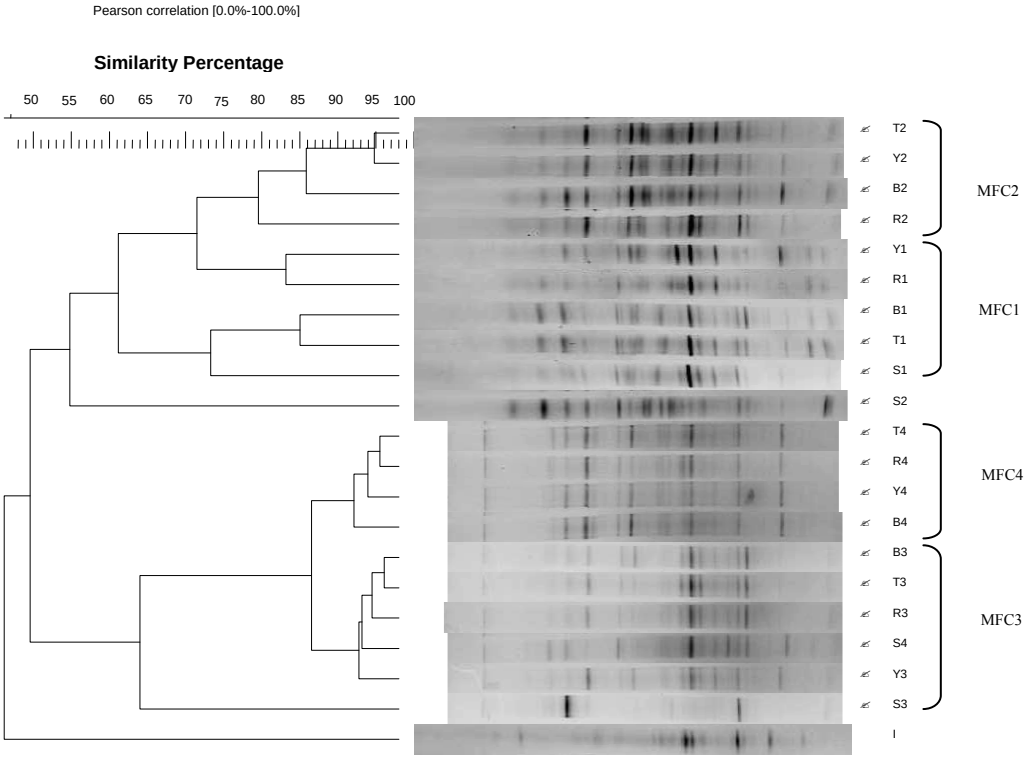
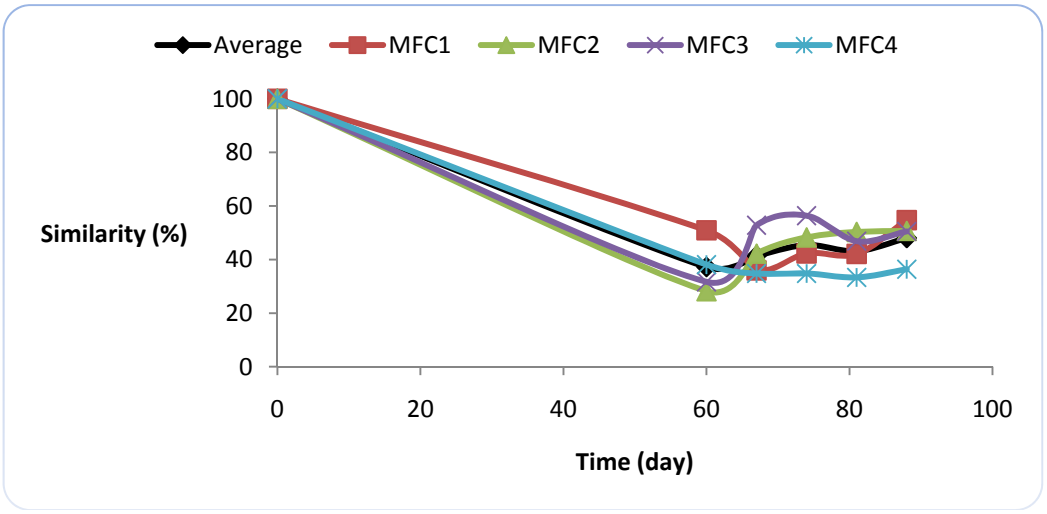


Figure 4.9: Similarity (Pearson correlation coefficient, UPGMA) of the DGGE banding patterns for the bacterial communities for four identical MFC reactors containing different textile dyes. The similarity values were clustered using the coefficient and found to cluster by reactor.

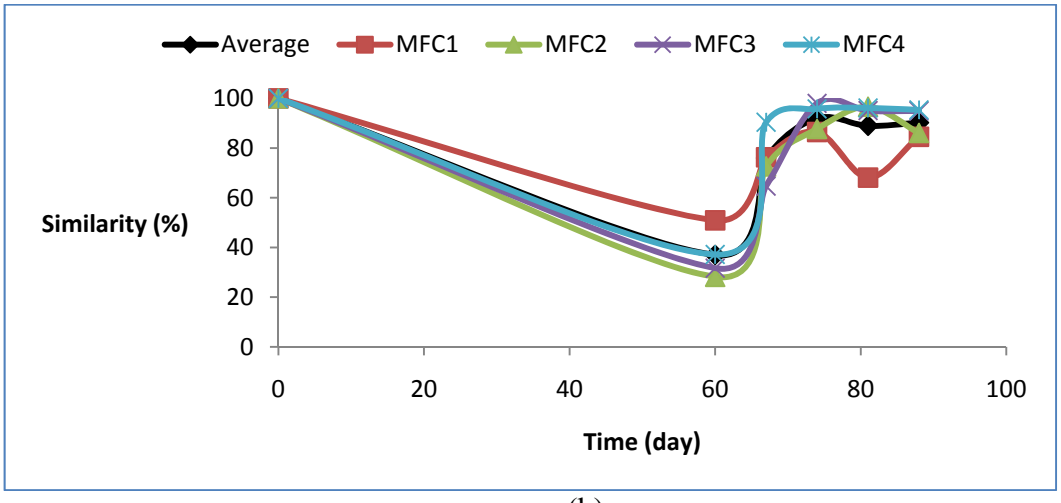
Clustering of MFC reactor profiles among themselves necessitated pairwise comparisons within each MFC. Pairwise comparison of patterns showed a mean value of 37.2% of similarity when MFC samples collected after S decolorization were compared to initial inoculum. This result indicated high dissimilarity in biofilm microbial community compared to complex inoculum at the beginning. Introduction of initial textile dye (S) other than acetate to the system as a major distruption could be the major cause of high amount of dissimilarity. When new dyes are introduced to the system higher similarity values are calculated when compared to initial inoculum sample. At this stage, microbial communities in the biofilm recover and adapts to reactor conditions with subsequent textile dye as a potential substrate. Thus on day

88, after red dye was decolorized, similarity value increases to a mean value of 48.06%.

Each sample was also compared to its previous sample instead of inoculum to calculate the effect of different dye addition on the previous microbial community in the biofilm,. (Figure 4.10a) In this case, after a sharp decrease was observed with black samples and inoculum, almost similar microbial composition was observed with addition of turquoise, yellow and red dyes as a new textile dye. Mean values of 92.0%, 89.0% and 90.2% similarities were calculated when microbial community profiles were compared with previous samples. (Figure 4.10b)



(a)



(b)

Figure 4.10: Similarity of the DGGE banding patterns for the bacterial cathode biofilm communities at different sampling times. Day 0 sample is inoculum. Day 60, 67,74, 81 and 88 samples are samples collected after decolorization of S, B, T, Y and R respectively. (a)Similarity with Day 0 (Inoculum) (b)Similarity with previous sample.

4.6.1.2 Archaeal Cathode Biofilm Communities

Archaeal microbial communities in the biofilm were also compared with respect to Archaeal communities in inoculum sample. Figure 4.11 shows distribution of Archaeal communities in each sample. Each MFC sample group cluster according to MFCs they belong to.

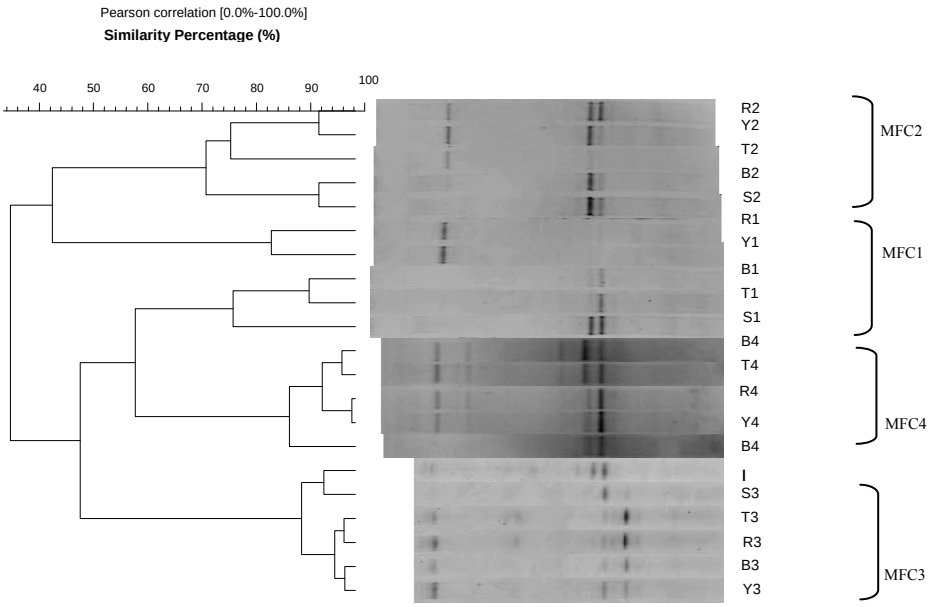
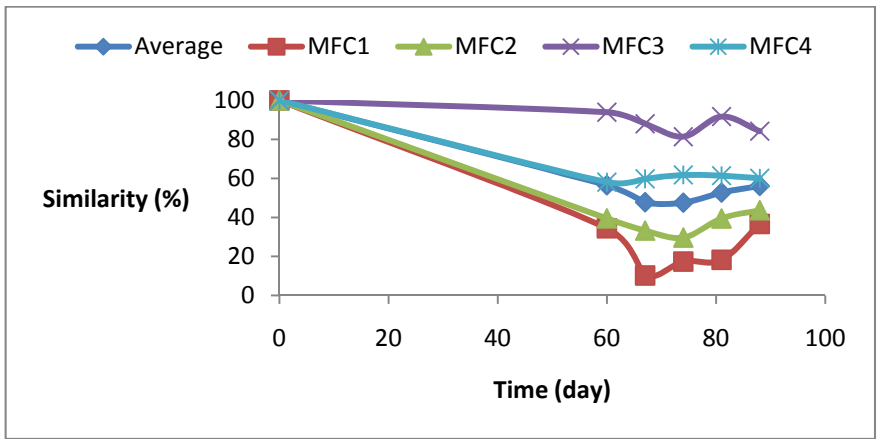


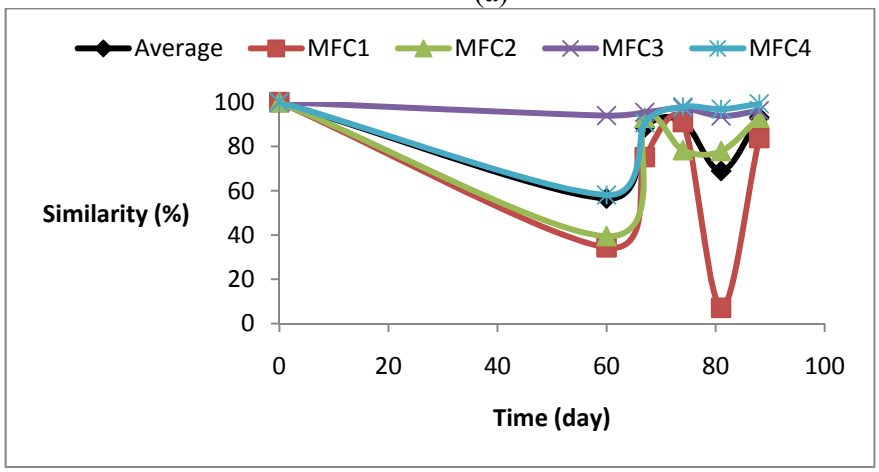
Figure 4.11: Similarity (Pearson correlation coefficient, UPGMA) of the DGGE banding patterns for the Archaeal cathode biofilm communities for four identical MFC reactors run with different textile dyes temporally. The similarity values were clustered using the coefficient and found to cluster by reactor.

Although similar clustering of the MFC samples was observed with Bacterial profiles, in Archeal communities, pairwise comparisons showed inconsistent similarity values. While in MFC1 and MFC2 similarity of S1 and S2 samples were calculated as low as 34.40% and 39.40% respectively similarity of S3 sample to initial inoculum was as high as 93.9%. Considering this information, high average deviation of similarities, 19.50%, makes it hard to interpret general trend for all MFC reactors in terms of Archaeal diversity. Few number of taxa that were detected in inoculum sample may be the major cause of this situation. However, when MFCs were interpreted individually, similarity with inoculum decreased as new textile dyes were introduced. Then, as microbial communities adapted to new environment, initial similarity values were recovered (Figure 4.12). On the other hand; when

comparisons with previous samples were considered, higher similarity values were encountered. Mean values of similarities with previous samples were not calculated below 88.50%, except for the dramatic decrease in the similarity between T1 and Y1 samples up to 7.13%. If detection limitations of DGGE methodology are neglected, addition of Y as a new compound caused a significant change in Archaeal abundant species which will be further evaluated in phylogenetic analysis of the samples.



(a)



(b)

Figure 4.12: Similarity of the DGGE banding patterns for the archaeal cathode biofilm communities at different sampling times. Day 0 is the sample from inoculum. Day 60, 67,74, 81 and 88 samples are samples collected after decolorization of S, B, T, Y and R respectively. (a) Similarity with Day 0 (Inoculum) (b) Similarity with the previous sample.

4.6.2 Bacterial MFC Solution Communities

Like all other sample groups, MFC solution samples from different reactors clustered into four distinct groups. However, MFC3 and MFC4 sample groups were more dissimilar to inoculum sample, when compared to MFC1 and MFC2 samples. (Figure 4.13)

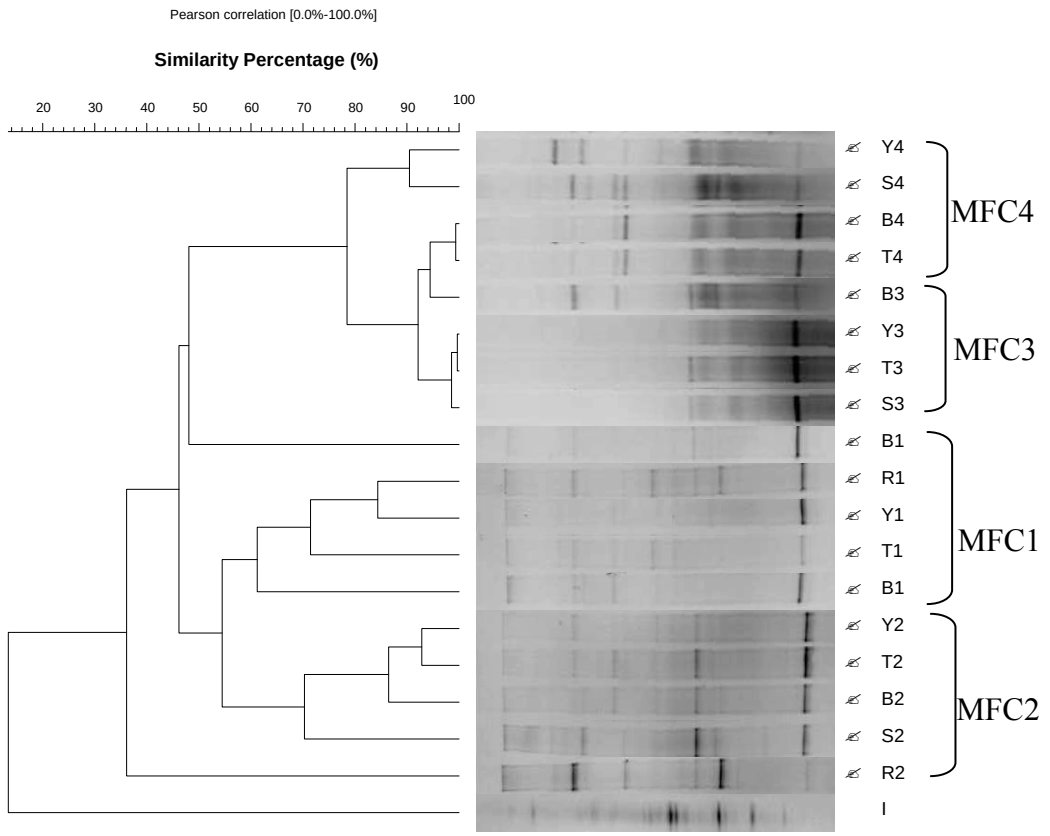
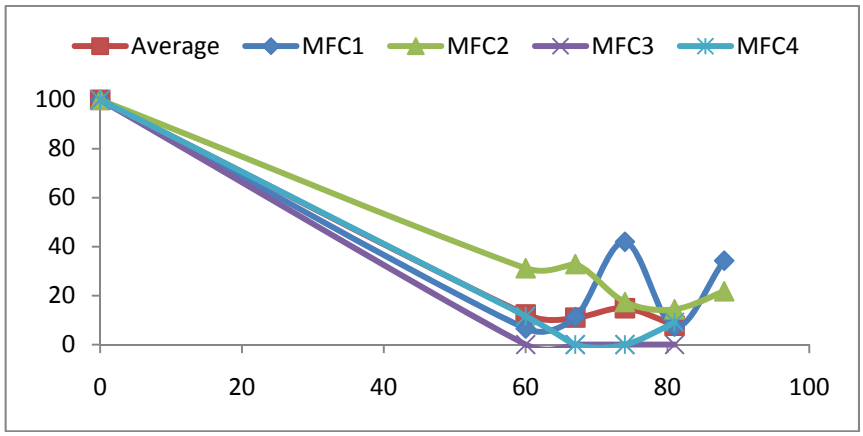
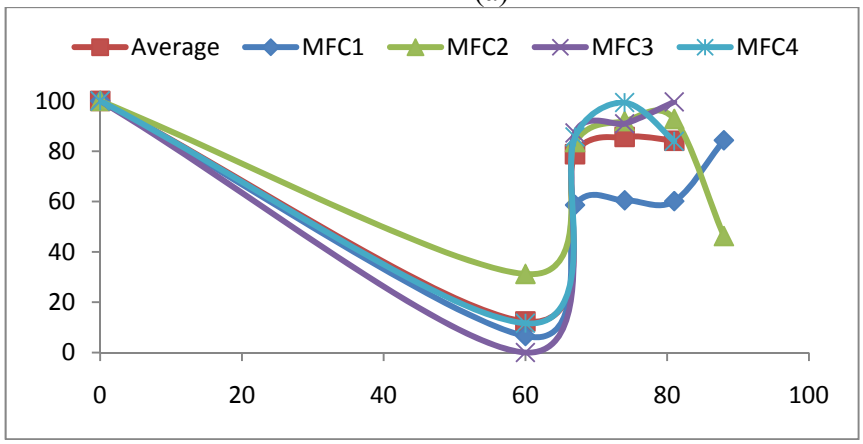


Figure 4.13: Similarity (Pearson correlation coefficient, UPGMA) of the DGGE banding patterns for the Bacterial MFC solution communities for four identical MFC reactors run with different textile dyes temporally. The similarity values were clustered using the coefficient and found to cluster by reactor.

Pairwise comparison of solution samples after decolorization of each dye within same MFC reactor showed similar trends. According to the Figure 4.14 Black samples were 12.3% similar compared to inoculum. Especially in S3 sample 0% similarity was calculated. However, in latter samples, for example in R5 sample, similarity increases up to 34,30% with higher number of bands detected.



(a)



(b)

Figure 4.14: Similarity of the DGGE banding patterns for the bacterial MFC solution communities at different sampling times. Day 0 is the inoculum sample. Day 60, 67,74, 81 and 88 samples are samples collected after decolorization of S, B, T, Y and R respectively. Red samples for MFC3 and MFC4 were not determined. (a)Similarity with Day 0 (Inoculum) (b) Similarity with previous sample.

4.7 Changes in Microbial communities versus MFC Performance

Together with microbial communities, MFC performance varied throughout decolorization of different dyes. Results indicated that changes in microbial communities in separate reactors correlated with means of maximum closed circuit potentials generated. Table 4.2 lists similarities of both Bacterial and Archaeal DGGE profiles to previous samples and Table 4.3 lists means of maximum potentials maintained during decolorization of different dyes. According to Table 4.3, peak potentials of MFC1 are not stable during utilization of different dyes. Also same stability problems could be observed in Bacterial and Archaeal DGGE profile

similarities in the same reactor. However, in MFC3 and MFC4, after black dye was introduced, peak values remained stable during addition of four other dyes. Similarly, microbial communities in MFC3 and MFC4, after a relatively higher similarity changes from samples collected after decolorization of S to B, similarities above 95%. This relation can be observed in both Bacterial and Archaeal communities.

Table 4.2: Similarity values of DGGE band profiles for Archaeal and Bacterial communities. Similarity values are given for previous samples.

Bacteria	MFC1 (%)	MFC2 (%)	MFC3 (%)	MFC4 (%)	Archaea	MFC1 (%)	MFC2 (%)	MFC3 (%)	MFC4 (%)
S	100	100	100	100		100	100	100	100
B	76.28	72.63	64.67	90.4		75.18	92.94	95.41	90.72
T	86.48	87.71	98	95.78		91.02	78.29	97.27	97.39
Y	68.13	96.62	95.08	96.14		7.13	77.91	93.77	96.86
R	84.54	86.09	94.9	95.38		83.76	92.93	96.19	99.27

Table 4.3: Means of highest circuit potentials reached during batch cycles of five different dyes in four MFCs.

	MFC1 (V)	MFC2 (V)	MFC3 (V)	MFC4 (V)
S	0.474742	0.466302	0.388715	0.390397
B	0.357829	0.492701	0.584563	0.450644
T	0.308916	0.485173	0.591952	0.436274
Y	0.366513	0.473793	0.592694	0.453436
R	0.334289	0.455652	0.594374	0.450727

4.8 Phylogenetic Analysis

Major bands of DGGE profiles were also subject to phylogenetic analysis. Bands numbered 1-7 were found to be affiliated with several different Bacterial classes including Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria from the diverse Proteobacteria phylum and also from Actinobacteria affiliated OTUs. (Table 4.4)

Table 4.4: Phylogenetic affiliations and closest matches of sequences retrieved from seven major bands of Bacterial DGGE profles.

	Class	Order	Closest GenBank Affiliate	Similarity (%)
Band1	Betaproteobacteria	Rhodocyclales	Thauera sp. (AY838760)	98
Band2	Betaproteobacteria	Burkholderiales	Hydrogenophaga sp. (AB166889)	99
Band3	Gammaproteobacteria	Pseudomonadales	Pseudomonas sp. (AF482684)	99
Band4	Alphaproteobacteria	Rhizobiales	Chelatococcus sp. (FJ529045)	97
Band5	Actinobacteria	Actinomycetales	Streptomyces sp. (AY299619)	99
Band6	Alphaproteobacteria	Rhodobacterales	Paracoccus sp. (AJ012068)	97
Band7	Alphaproteobacteria	Rhizobiales	Aquamicrobium sp. (GQ254284)	99

Further phylogenetic analysis of inoculum and five other biofilm samples collected after decolorization of five different dyes yielded significant temporal changes in abundant phylotypes. (Table 4.5, Table 4.6, Table 4.7, Table 4.8).

Table 4.5: Phylogenetic affiliations, closest matches and number of clones from inoculum sample.

Sample Group	Phylum	Order	Sample	Accession Number/ Closest GenBank Affiliate		Clone Sayısı
INOCULUM (40)	ACTINOBACTERIA	n.d.	I14	FN554394	Uncultured	1
	ALPHA PROTEOBACTERIA	Rhodospirillales (1)	I6	DQ401091	Pelagibius litoralis	1
		n.d.	I9	DQ186614	Micavibrio sp.	1
		Rhodobacterales (1)	I11	FJ997595	Rhodobacter sp.	1
	BETA PROTEOBACTERIA	Burkholderiales (10)	I13	AM084007	Acidovorax caeni	3
			I8	AY512827	Acidovorax avenae subsp.	3
			I20	AM084011	Acidovorax caeni	2
			I10	AB015048	Leptothrix sp.	2
		Rhodocyclales (14)	I15	AJ315678	Thauera phenylacetica	2
			I1	CP001281	Thauera sp.	6
			I4	Y17591	Thauera selenatis	3
			I16	EU652481	Thauera sp	2
			I5	AM084104	Thauera sp.	1
		n.d.	I2	AB180657	Uncultured	1
	GAMMA PROTEOBACTERIA	Chromatiales (1)	I21	EU908046	Ectothiorhodospira sp.	1
	BACTEROIDETES	Bacteroidetes (1)	I19	FN554384	Bacteroidetes	1
	FIRMICUTES	Clostridia (4)	I3	FJ577254	Peptoniphilus sp.	2
			I22	X96959	Acetobacterium fimetarium	1
			I7	AB490809	Catabacter sp.	1
	UNCULTURED	n.d.	I12	DQ836758.1	uncultured	5

Majority of the clones (%40) analyzed in the inoculum sample belonged to Rhodocyclales order of Proteobacterium group. All of these clones were closely related to (more than 95%) *Thauera spp.* which is detected in many wastewater treatment plants. That species is well known for its ability to break down many different organic compounds. (Valle et al., 2004) Thauera was shown to degrade many aromatic compounds including phenol, polyphenol, toluene. (Schuhle and Fuchs, 2004) (Dibenedetto et al.,2006), (Biegert et al., 1996; Shinoda et al., 2004). MFC inoculum was obtained from yeast production wastewater treatment facility . Thus in many industrial treatment systems, they are important role players for degradation of organic material.

Table 4.6: Phylogenetic affiliations, closest matches and number of clones from cathode biofilm sample after decolorization of S dye.

Sample Group	Phylum	Order	Sample	Accession Number/ Closest GenBank Affiliate		Clones
S	ALPHA PROTEOBACTERIA	Rhizobiales (29)	S3	DQ305289	Mesorhizobium sp.	23
			S9	AM403218	Ochrobactrum sp.	4
			S5	AM236092	Caedibacter macronucleorum	1
			S8	AB238789	Shinella zoogloeoides	1
		Rhodobacterales (1)	S4	AB195172	Paracoccus sp.	1
	BETA PROTEOBACTERIA	Rhodocyclales (8)	S10	AF229881	Thauera aromatica	2
			S12	AB021377	Thauera butanivorans	6
	LENTISPHAERAE	Lentisphaeraceae (3)	S1	FJ394915	Victivallaceae bacterium	3

After acclimation with acetate; initial microbial populations in MFCs changed significantly after first dye addition. At the end of approximately 200 days of operation and about 80 batch cycles, only 20% of the analyzed clones were affiliated with *Thauera spp.* After decolorization of black dye, microbial community shifted towards Rhizobiales order of Alphaproteobacteria and 72% of the analyzed clones belonged to this group of organisms. However, it is not clear whether this change was a result of natural acetate fed environment of single chamber MFC or the effect of additional black dye to the system.

Table 4.7: Phylogenetic affiliations, closest matches and number of clones from cathode biofilm sample after decolorization of B dye.

Sample Group	Phylum	Order	Sample	Accession Number/ Closest GenBank Affiliate		Clones
B	ACTINOBACTERIA	Actinomycetales (7)	B16	DQ119293	Microbacterium sp.	4
			B16	EU244645	Gordonia cholesterolivorans	1
			B2	EU167912	Actinobacteria	2
	ALPHA PROTEOBACTERIA	Rhizobiales (10)	B6	DQ305289	Mesorhizobium sp.	9
			B10	EU703136	Mesorhizobium sp.	1
		Rhodobacterales (12)	B12	EU726271	Stappia sp.	2
			B13	Y16930	Paracoccus denitrificans	7
			B9	AB195172	Paracoccus sp.	3
	BETA PROTEOBACTERIA	Burkholderiales (2)	B15	AM084024	Comamonas sp.	1
			B3	GU184188	Acidovorax sp.	1
		Rhodocyclales (1)	B7	AB021377	Thauera butanivorans	1
	GAMMA PROTEOBACTERIA	Pseudomonadales (1)	B5	Y18006	Pseudomonas stutzeri	1
		Xanthomonadales (3)	B11	EU305571	uncultured Lysobacter sp.	3
	BACTEROIDETES	Bacteroidales (2)	B8	AY554420	Bacteroides sp	2

Microbial community after decolorization of B dye mainly consisted of Actinobacteria, Alphaproteobacteria, Beteproteobacteria, Gammaproteobacteria and Bacteroidetes. Alphaproteobacteria. *Thauera spp.* which comprised 20% of the analyzed clones in previous sample, occupied only 4% of the analyzed clones. As number of unique affiliates increased, diversity in the sample was greater than the sample taken from the MFC after decolorization of the S dye.

Table 4.8: Phylogenetic affiliations, closest matches and number of clones from cathode biofilm sample after decolorization of T and Y dyes.

Sample Group	Phylum	Order	Sample	Accession Number Closest GenBank Affiliate		Clones
T	ACTINO BACTERIA	Actinomycetales (3)	T3	AY114177	Rhodococcus ruber	1
			T18	AF233375	Streptomyces sp.	1
			T2	EU167912	Actinobacteria	1
	ALPHA PROTEOBACTERIA	Rhizobiales (27)	T4	DQ305289	Mesorhizobium sp.	17
			T5	GQ254286	Aquamicrobium sp.	4
			T12	AY332178	Mesorhizobium sp.	1
			T13	FJ907162	Aminobacter sp.	1
			T7	GU199003	Pseudaminobacter sp.	1
			T15	EF584507	Chelatococcus daeguensis	2

Table 4.8: Phylogenetic affiliations, closest matches and number of clones from cathode biofilm sample after decolorization of T and Y dyes. (continued)

Sample Group	Phylum	Order	Sample	Accession Number Closest GenBank Affiliate		Clones
T			T16	AM286397	Nitrobacter sp.	1
		Rhodobacterales (3)	T8	FM956479	Rhodobacter sp.	1
			T10	EU726271	Stappia sp.	1
			T6	Y16930	Paracoccus denitrificans	1
		Rhodospirillales (1)	T17	AF521650	Azospirillum sp.	1
	BETA PROTEOBACTERIA	n.d.	T19	AF011343	Azoarcus communis	1
	GAMMA PROTEOBACTERIA	Xanthomonadales (3)	T9	EU305571	uncultured Lysobacter	2
Y	ALPHA PROTEOBACTERIA	Rhizobiales (21)	Y3	DQ305289	Mesorhizobium sp.	2
			Y6	AM041247	Ochrobactrum oryzae	1
			Y18	EU305598	Uncultured Aquamicrobium sp.	12
			Y13	FJ529045	Chelatococcus sp.	5
			Y9	DQ337576	Pseudaminobacter sp.	1
		Rhodobacterales (2)	Y7	EU726271	Stappia sp.	1
			Y2	AY332116	Mesorhizobium sp.	1
	BETA PROTEOBACTERIA	Hydrogenophilales (5)	Y5	AM403494	Thiobacillus sp.	5
		Burkholderiales (11)	Y20	AM398226	Achromobacter sp.	7
			Y19	BX640430	Bordetella parapertussis	2
			Y12	AF233879	Comamonas denitrificans	1
			Y15	EU851054	Castellaniella sp.	1
	GAMMA PROTEOBACTERIA	Pseudomonadales (1)	Y1	AJ288151	Pseudomonas stutzeri	1
	DELTA PROTEOBACTERIA	Desulfuromonadales (1)	Y4	AF404348	Geobacter sp.	1
	FIRMICUTES	Bacillales (2)	Y16	EU375313	Bacillus sp.	1
			Y17	DQ852633	Bacillus sp.	1
	SPIROCHAETES	Spirochaetales (1)	Y8	AY800103	Spirochaeta sp.	1

Other than black dye sample in which sequences were related to only eight different phylotypes (Table 4.9,), number of different phylotypes that were found as closest GENBANK matches for the available sequences was in concordance with DGGE results. In DGGE profiles there were 12 to 19 OTUs detected for MFC1 samples(Table 4.9). For Black and Brilliant Blue samples number of detected unique affiliates were lower than number of OTUs. However, in all the other samples affiliated phylotypeswere greater than or equal to number of OTUs. These results

indicate that two methods validated each other with different detection limits and biases. On the other hand; DGGE profile results and phylogenetic analysis of the clone library had shown different results in detection of the most dominant species. Band 4 on the Bacterial DGGE profiles emerges as the most intense band on DGGE image. (Figure 4.6) According to sequence analysis of Band 4, highest affiliation was detected with Chelatococcus sp. (FJ529045) from the Order Rhizobiales, but clone library analysis shows the most dominant species in those samples as Mesorhizobium sp. (DQ305289) from the same order. However, close affiliation of two species and their similar metabolic characteristics makes this contradiction neglectable. Also DGGE is rather a semi-quantitative methodology, so intensity of the bands doesn't always reflect abundance accurately.

Table 4.9: Number of OTUs detected in Bacterial DGGE profiles according to Figure 4.6 and numbers of unique affiliates from different samples.

	S	B	T	Y	R
Number of OTUs detected	13	19	18	16	12
Number of unique affiliate detected	8	14	18	17	16

Table 4.10: Phylogenetic affiliations, closest matches and number of clones from cathode biofilm sample after decolorization of R dye.

Sample Group	Phylum	Order	Sample	Accession Number/ Closest GenBank Affiliate		Clones
R	ALPHA PROTEOBACTERIA	Rhizobiales (41)	R4	DQ305289	Mesorhizobium sp.	26
			R1	GQ254286	Aquamicrobium sp.	2
			R2	GU199003	Pseudaminobacter sp.	2
			R9	AF229877	Mesorhizobium sp.	1
			R13	EF584507	Chelatococcus daeguensis	1
			R19	AM041247	Ochrobactrum oryzae	1
			R7	AF229877	Mesorhizobium sp.	1
			R8	FJ529045	Chelatococcus sp.	4
			R12	CP001196	Oligotropha carboxidovorans	1
			R14	EU928869	Phyllobacterium sp.	1
			R17	AM286397	Nitrobacter sp.	1
		Rhodobacterales (2)	R16	AY332116	Mesorhizobium sp.	1
			R10	Y16930	Paracoccus denitrificans	1
		n.d.	R20	AY728070	uncultured	1

Table 4.10: Phylogenetic affiliations, closest matches and number of clones from cathode biofilm sample after decolorization of R dye. (continued)

Sample Group	Phylum	Order	Sample	Accession Number/ Closest GenBank Affiliate		Clones
R	BETA PROTEOBACTERIA	Rhodocyclales (2)	R5	AB021377	Thauera butanivorans	2
	BACTEROIDETES	Bacteroidales (1)	R11	EU136680	Bacteroides uniformis	1

Phylogenetic analysis of Archaeal 16S rRNA gene libraries yielded less diverse archaeal microbial community which consisted of sequences related to only 5 different phylotypes, one of which is a very distant relative. These results also correlated with four different OTUs that were marked on Archaeal DGGE profiles. Except for one of the clones analyzed (BandArc4) which did not have any significant similarity with any of the GENBANK affiliates in BLAST alignment.Remaining four Archaeal clones belonged to methane producing microorganism groups. BandArc1 was affiliated with *Methanosarcina mazei*, which is one of the most active methane producing, cyst forming and aggregating Archaea. They were shown to produce methane from acetate which was used as carbon source in this study *Methanosarcina mazei* found widely on anaerobic wastewater treatment systems (Mah, 1980). However, Methanomethylovorans affiliated BandArc2 are also methylotrophic and they can utilize methanol and methyl derivatives for methane production. (Yu et al., 2005).Thus, these two organisms may alternate in environments to become predominant with changing acetate concentrations.

Table 4.11: Phylogenetic affiliations of Archaeal clones and their closest GENBANK matches

	Phylum	Class	Order	Closest GenBank Affiliate/ Similarity (%)
BandArc1	Euryarchaeota	Methano microbia	Methano sarcinales	Methanosarcina mazei (AB065295)/ 93%
BandArc2	Euryarchaeota	Methano bacteria	Methano bacteriales	uncultured Methanobrevibacter (FJ919272) / 95%
BandArc3	Euryarchaeota	Methano microbia	Methano microbiales	Methanocorpusculum bavaricum (AY196676)/ 96%
BandArc4	Euryarchaeota	Thermococci	Thermo coccales	Thermococcales archaeon (AJ585956) n.d.
Arc5	Euryarchaeota	Methano microbia	Methano sarcinales	uncultured Methanomethylovorans sp (DQ631887)/ 90%

The cathode biofilm sample which was collected after decolorization of red textile dye was shown to be dominated by Rhizobiales order in Alphaproteobacteria. 87% of the clones analyzed was shown to be affiliated with this group. (Table 4.10) In other studies, Rhizobiales were commonly found as emerging bacterial group in membrane biofilm communities. Rhizobiales are reported to be metabolically versatile under aerobic conditions. Especially, in biofilm environments where some of the substrate may become limiting, they can adapt to different substrates more easily to avoid competition with other populations. (Pang and Liu, 2007).

Mesorhizobium spp. which comprised 59,5% of the clones analyzed were also identified formerly in microbial communities of a single chamber air-cathode MFC. According to this study, DGGE profiles showed that microbial communities from three different compartments (cathode, anode, mfc solution) were almost the same. However, each compartment had different OTUs as more abundant species. Yet, this data was not supported with clone library analysis. (Sukkasem et al., 2008)

Mesorhizobium bacteria are known as aerobic, and during respiration they use oxygen as the terminal electron acceptor. Meso implies their growth rate which is between fast growing *Rhizobium* or *Sinorhizobium* genera and slow growing *Bradyrhizobium*. They are chemoorganotrophic microorganisms and they can use various carbohydrates and salts of organic acids as carbon sources. As nitrogen source, they can metabolize ammonium salts, nitrates and urea along with many amino acids. Widely studied genus of *Mesorhizobium* was shown to produce extracellular polysaccharides which supports their abundance in biofilm.

Finally, phylogenetic trees shown in Figure 4.15 and Figure 4.16 demonstrates phylogenetic affiliations of major OTUs found in biofilm samples.

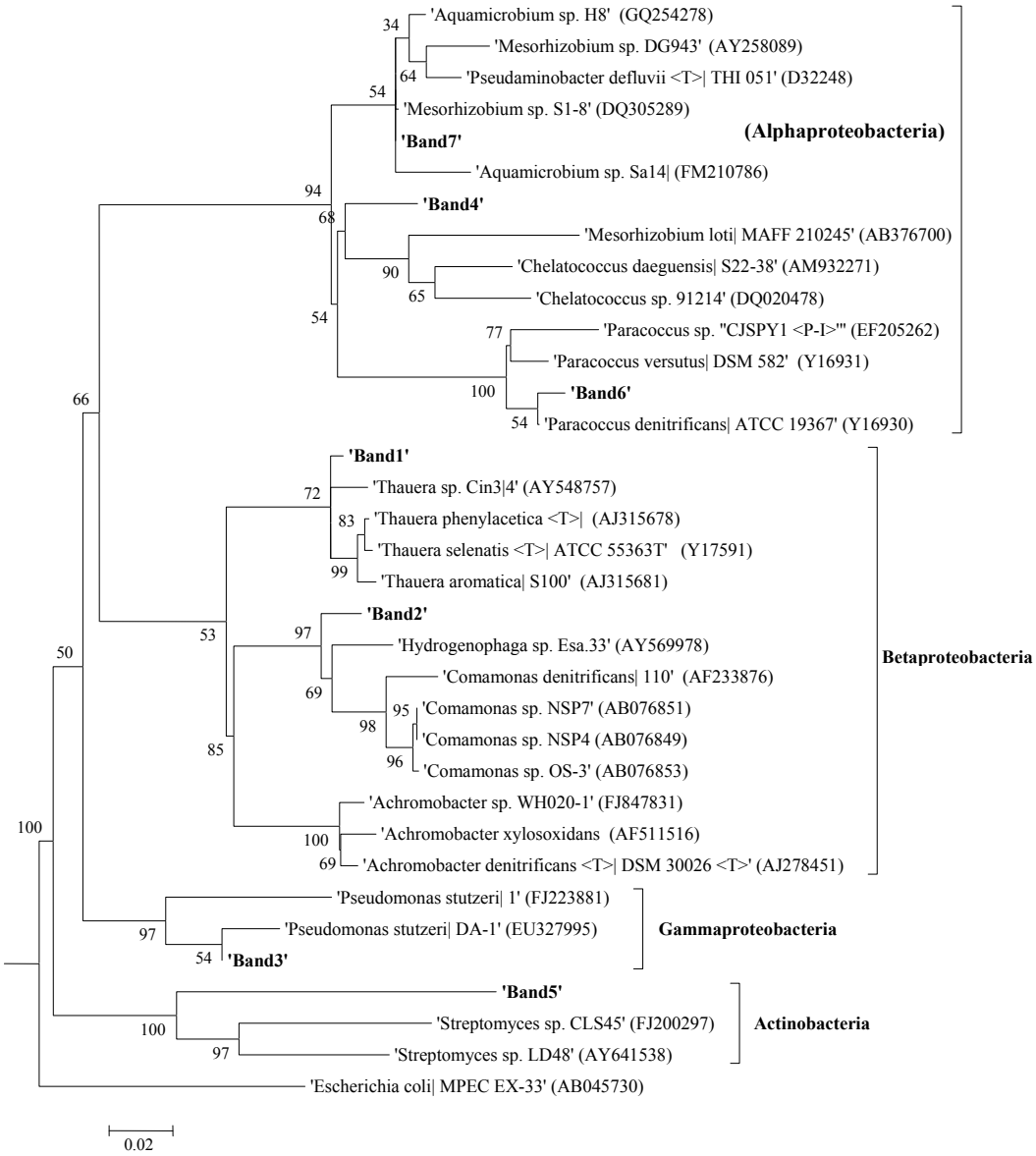


Figure 4.15: Phylogenetic tree illustrating the relationship between the closest relatives in the RDP and GenBank databases and bacterial 16S rRNA gene sequences retrieved from the cathode biofilm. The tree was constructed by the Weighbor-joining matrix from weighted pairwise comparisons made by the Jukes and Cantor (1969) algorithm in RDP Tree Builder. E.coli was selected as the outgroup. Bootstrap values over 70, from 100 replicate trees, were shown at the nodes. The scale bar represents 2% difference in nucleotide sequence.

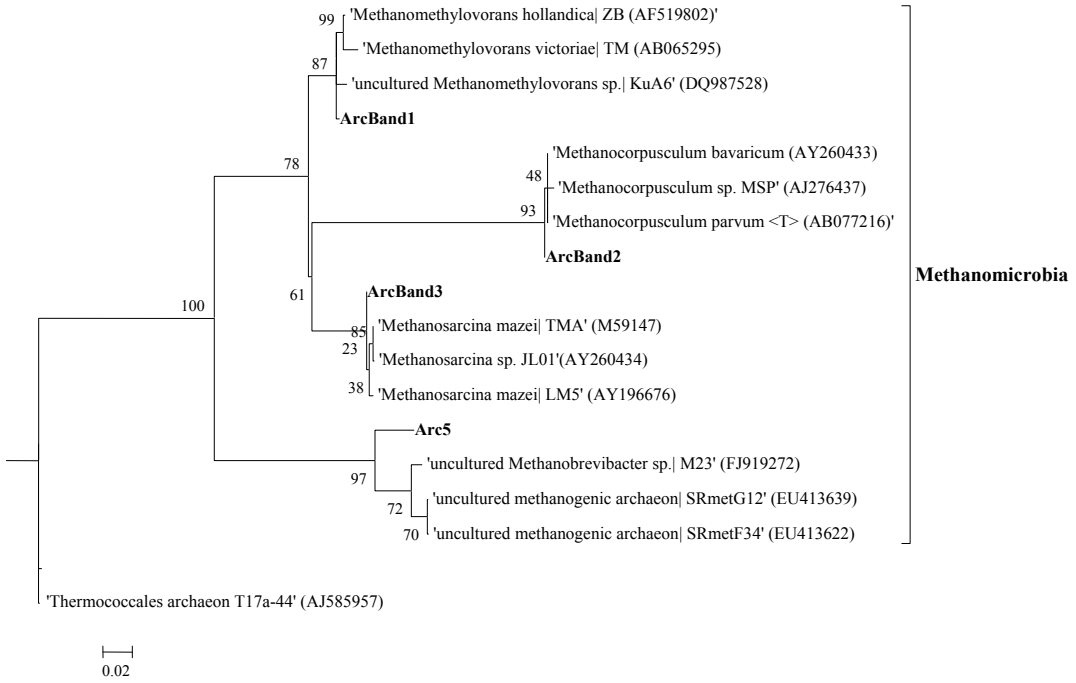


Figure 4.16: Phylogenetic tree illustrating the relationship between the closest relatives in the RDP and GenBank databases and Archaeal 16S rRNA gene sequences retrieved from the cathode biofilm. The tree was constructed by the Weighbor-joining matrix from weighted pairwise comparisons made by the Jukes and Cantor (1969) algorithm in RDP Tree Builder. Thermococcales archeon was selected as the outgroup. Bootstrap values over 70, from 100 replicate trees, were shown at the nodes. The scale bar represents 2% difference in nucleotide sequence. ArcBand4 was not included in tree because it aligned poorly with other sequences.

In this study, cathode biofilm was analyzed as a totally different approach than previous studies. To the knowledge, this study is the first to phylogenetically analyze cathode biofilm of an air-cathode single-cell mediatorless and membraneless MFC. According to previous studies it is known that traditional dual chamber design is disadvantegous because of the high internal resistances and requirement for aeration in cathode chamber. In order to reduce cost of aeration, air-cathodes were developed. Single chamber designs were also used to overcome limitations due to high internal resistances. Proton exchange membranes (PEM) which separated the chambers were a subject of debate since they were important factors towards cost-effective designs. Results showed removing PEM from MFC design yields increased maximum power outputs; but coulombic efficiencies decrease by almost 3-fold. Increase in power

outputs were correlated to increased cathode potentials which results in higher voltage values (Liu and Logan, 2004). Cathode design is the most difficult element of MFC technology towards system scale up. The reaction at the cathode is very complicated to optimize since oxygen, protons and electrons require a catalyst to produce water. Catalyst should be a conductive material but it must be in contact with both liquid and air phases so that oxygen from the air, protons from liquid and electrons from solid phase react. Engineering wise optimization of this type of reaction is very difficult. In the absence of PEMs, movement of protons toward cathode get easier, but still some catalyst binders like Nafion are used to bind catalyst to cathode surface. Nafion has high proton conductivity and permeable to oxygen, so it is commonly used as a binder.

Effect of cathode biofilm on MFC performance was investigated only in a very few studies. It was shown that with the formation of biofilm, power outputs of MFC increased in the beginning, but decreased later (Yang et al., 2008). In the same study, it was shown that oxidation-reduction potentials decreased first and then it reached stable values. Normally when PEM is absent, oxygen has access to cathode and this may lead to aerobic degradation of substrates present. This would have a negative effect on Coulombic efficiency of the system. In this manner, formation of thick biofilm on the cathode can restrict oxygen access to anode electrode. Yet, in this study, it was shown that aerobic organisms are abundant in cathode biofilm which may use this oxygen in their respiratory metabolism. Utilization of carbon source by cathode biofilm organisms may decrease coulombic efficiencies and thus effect MFC performance in a negative manner. Thus, optimization of cathode biofilm with certain strains may lead to better performance.

4.9 FISH Results

Bacterial biofilm samples on cathode were viewed with Leica Confocal Microscope using 63x oil immersion to be able to visualize viable cells. Numerous active cells were observed when samples hybridized with EUB338 probe (Figure 4.17). However, background fluorescence was high. FISH results indicated presence of metabolically active Bacteria in cathode biofilm which were morphologically uniform in shape.

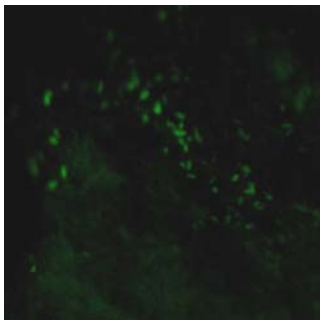


Figure 4.17: CSLM image of bacterial cells in cathode biofilm, 63x magnification.

5. CONCLUSION

Effect of cathode microbial communities was detected in four identical single-chamber air-cathode mediator-less MFCs. Results indicated continuous change in microbial biofilm communities temporally after several batches, and abundance of major phylotypes changed significantly compared to initial bacterial consortia according to 16S rRNA gene dependent phylogenetic analysis. Furthermore; regardless of the similar starting inocula, differences in microbial communities in different MFCs was observed.

PCR-DGGE analysis of microbial communities demonstrated a major change in microbial communities with introduction of textile dyes to the reactors. This change was followed by minor changes in microbial communities when the applied dye was replaced by another one. For instance, in one of the reactors, the organisms in samples collected after decolorization of blue dye was only 64.7% similar to those in sample collected after black dye decolorization. However, when turquoise, yellow and red dyes were added sequentially to cells, similarity values compared to previous samples were 98%, 95.1% and 94.9% respectively. Additionally, microbial community similarities among different MFC reactors clustered in similarity dendograms indicated that there is no significant similarity between MFCs..

Phylogenetic analysis of one of the reactors using 16S rRNA gene dependent clone library analysis demonstrated significant change in abundant species in cathode biofilm. In inoculum sample majority (%40) of the analyzed clones belonged to Rhodocyclales order of Proteobacterium group. All of these clones were closely related to (higher than 95%) Thauera spp. The cathode biofilm sample, which was collected after decolorization of five different dyes together with acetate as a substrate, was shown to be dominated by Rhizobiales order in Alphaproteobacteria. 87% of the clones analyzed was shown to be affiliated with this group.

Mesorhizobium spp. which comprised 59,5% of the Rhizobiales clones, emerged as the most abundant bacteria. This agrees with their ability to use oxygen as their terminal electron acceptor during aerobic respiration. Besides, ability of these organisms for extracellular polysaccharide production supports their major presence in biofilm community. In comparison to Bacterial diversity, few number of Archaeal taxa identified in cathode biofilm structure belonged to Methanobacteria and Methanomicrobia classes. Their occurrence suggests probability of acetoclastic methane production via readily available acetate. Finally, viability of bacterial groups inside the biofilm was verified using fluorescently labeled oligonucleotide probes.

Development of a thick biofilm comprised of aerobic and methanogenic bacteria on cathode certainly affects performance of the MFC. Further studies would improve our understanding of the relationship between biological processes of the microbial communities and performance for better efficient energy yields.

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APPENDICES

APPENDIX A : Laboratory Equipment
APPENDIX B : Chemicals
APPENDIX C : Solutions and Buffers

APPENDIX A

Laboratory equipment

Autoclave	TOMY SX-700E
Balances	Precisa BJ610, OHAUS pioneer
Centrifuges	Sigma 1-14
DGGE system	BIORAD DCODE universal mutation system
DRY heating thermostat block	BIO TDB 100, BIOSAN
Electrophoresis system	BIORAD mini sub cell GT
Fluorometer	Invitrogen, Qubit
Gel documentation system	BIORAD GELDOC
Incubators	Nüve EN120
Labwater	
Laminar flow	Faster BH-EN 2003
Magnetic stirrer, heater	Heidolph MR hei-standard
Microwave oven	Vestel MD17
Multimeter with data acquisition	Keithley 2700
PCR Thermocycler	BIORAD C1000 thermal cycler
pH meter	Mettler Toledo MP220
Pipettes	Eppendorf 2.5 µl, 10 µl, 20 µl, 100 µl, 1000 µl
Power supply	BIORAD power pac 300
Pure water systems	USF Elga UHQ-PS-MK3, Elga
Refrigerators	Whirlpool +4^{o}C, -20^{o}C, Vestel -20^{o}C; Haier -80^{o}C
The FastPrep instrument	Q-BIOgene, FP220A
Vortex	Heidolph reax top
Water Bath	Memmert

APPENDIX B

Chemicals

Ammonium Peroxosulfate	Sigma-Aldrich
Cloning Kit For Sequencing	Invitrogen
EDTA molecular biology reagent	Sigma-Aldrich
Ethyl alcohol absolute	Sigma-Aldrich
Formamide deionized solution	Sigma-Aldrich
Hot start *taq* polymerase	Qiagen
LB base	Invitrogen
LB Broth powder	Sigma-Aldrich
NaOH	Reidel-de Haën
Pottassium Hydrogen phosphate	J.T. Baker
Primers	Iontek
SOC Broth BioChemika, for microbiology	Fluka
Sodium Acetate Anhydrous	Sigma-Aldrich
Sodium Phosphate, Monobasic	Sigma-Aldrich
taq polymerase	INTRON
TEMED	Biorad
TRIS-HCl	Sigma-Aldrich
UREA	Fluka
Sequencing Kit	ABI
Polyvinyl pyrrolidone	Sigma-Aldrich
Phosphate buffered saline	Sigma-Aldrich
Bovine serum albumin	Sigma-Aldrich
Denhardt's solution	Sigma-Aldrich
40%ACRYLAMIDE/BIS SOLUTION 37.5:1	BIORAD
Acetic acid extra pure %99.5	Sigma-Aldrich

APPENDIX C

Solutions and buffers

Acetate Media Solution

Table C.1 Media solution (pH 7.0) used in Hong's lab (modified GM media)

	g/L
Sodium acetate	1 g or 20 mM
NH4Cl	0.31
KCl	0.13
NaH2PO4.H2O	5.84
Na2HPO4.7H2O	15.47
minerals solution	12.5ml
vitamins solution	1.25 for concentrated solution (x10)
Distilled Water	Up to 1 l

Table C.2 Composition of minerals and vitamins solutions (Lovley and Philips, 1988)

Vitamins (mg/l)		**Minerals (g/l)**	
biotin	2.0	NTA	1.5
folic acid	2.0	$MgSO_4$	3.0
pyridoxine HCl	10.0	$MnSO_4 \bullet H_2O$	0.5
riboflavin	5.0	NaCl	1.0
thiamin	5.0	$FeSO_4 \bullet 7H_2O$	0.1
nicotinic acid	5.0	$CaCl_2 \bullet 2H_2O$	0.1
pantothenic acid	5.0	$CoCl_2 \bullet 6H_2O$	0.1
B-12	0.1	$ZnCl_2$	0.13
p-aminobenzoic acid	5.0	$CuSO_4 \bullet 5H_2O$	0.01
thioctic acid	5.0	$AlK(SO_4)_2 \bullet 12H_2O$	0.01
		H_3BO_3	0.01
		Na_2MoO_4	0.025
		$NiCl_2 \bullet 6H_2O$	0.024
		$Na_2WO_4 \bullet 2H_2O$	0.025

Table C.3 FISH Hybridization Buffer

	30% Formamide
4.5 M NaCl	0.2 ml
Sterile water	0.1 ml
250 mM NaH_2PO_4, pH (7.0)	0.1 ml
Denhardt's solution (× 50)	0.2 ml
0.5 M EDTA	10 ml
10% SDS	10 ml
Deionised formamide (FA)	0.3 ml

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