Influence of culture adaptation and nutrient supplement on microbial fuel cell performance and wastewater treatment

A dissertation thesis submitted to

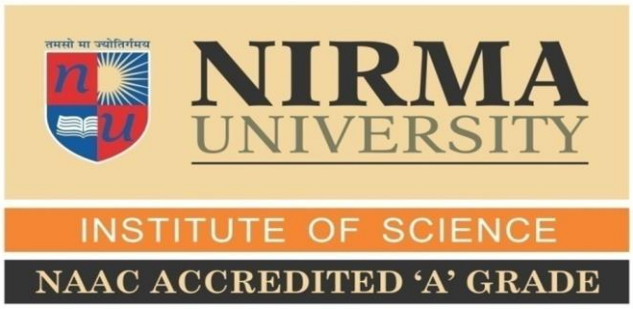
Nirma University

In Partial Fulfillent for the Degree of

Master of Science

In

Biochemistry & Biotechnology



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April, 2016

ACKNOWLEDGEMENTS

This thesis is the end of our journey in obtaining our Master's degree. Thesis has been kept on track and been seen through to completion with the support and encouragement of numerous people including our well-wishers, our friends, colleagues and various institutions.

We are extremely indebted to our Director Prof. Sarat Dalai, Director, Institute of Science, Nirma University, Ahmedabad; for providing necessary infrastructure and resources to accomplish our research work with great comfort.

At this moment of accomplishment, we pay homage to our guide, Dr. Nasreen S. Munshi. This work would not have been possible without her guidance, support and encouragement. Under her guidance we successfully overcame many difficulties and learned a lot. Her unflinching courage and conviction will always inspire us, and we hope to continue work with her noble thoughts. We can only say a proper thanks to her through our future work.

We also wish to express our gratitude to the other faculty members Dr. Shalini Rajkumar, Dr. Sonal Baksi, Dr. Amee Nair, Dr. Sriram Sheshadri, Dr. Vijay Kothari, Dr. Heena Dave and Dr. Mili Das who rendered their help during our dissertation period.

We are thankful to Ph.D students Ms. Purvi Zaveri and Ms. Rushika Patel for their great support during our experimentation in laboratory. We are thankful to graphicra for providing us graphitite.We would also like to thank our friends Dharvi Jhala, Tabassum Mansuri, Vipul Mori, Arti Patel, Harshadba Zala, Mehul Lakhani and Mamta, Ravi, Nisarg, Vaishwik, Monika, Surbhi for their help and support in our project work. We are also thankful to Mr. Hasit Trivedi, Mr. Sachin Prajapati, Ms. Sweta Patel and Mr. Rajendra Patel for their constant support and helping nature. We also thank to our librarian Mrs. Sveta Shukla.

Last but not the least, we convey our special thanks to God for giving us strength and moral support. We also wish to avail ourselves for this opportunity, express a sense of gratitude and would like to pay high regards to our parents and our family for their sincere encouragement and inspiration throughout our research work and lifting our uphill this phase of life. We owe everything to them. Besides this, thank to several people who have knowingly and unknowingly helped us in the successful completion of this project.

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Index

INTRODUCTION

1. INTRODUCTION

With increasing population, the global energy demand also has increased. Petroleum products demand increased day by day. This has put a pressure on finding new and renewable energy sources. Fuel cell is the alternative renewable energy source. Fuel cells also offer a possible solution to problem of treatment of wastewater containing hydrocarbons derived from petroleum (Bagotzky *et. al*., 2003).

In addition, due to global environmental concerns and energy insecurity there is emergent interest to find out sustainable and clean energy source with minimal or zero use of hydrocarbons. Microbial fuel cells, if used for wastewater treatment, are capable to provide clean energy, apart from effective treatment of wastewater. The enriched microbial culture in these MFCs have capabilities to use organic matter present in the wastewater as energy source and produce electrons and protons, through which electricity can be recovered (Ghangrekar *et. al.*, 2006).

Microbial fuel cell (MFC), provide the sustainable production of energy from biodegradable, reduced compounds. Microbial fuel cell function on assimiable carbon source as well as complex substrates present in waste water. The operational parameters of the MFC, uses different metabolic pathways used by bacteria. Microbial fuel cell converts the energy which is stored in chemical bonds of organic compound to electrical energy through the catalytic reaction of microorganisms.

1.1 OPERATING PRICIPLES OF MFC

Microbial fuel cell (MFC) is a device which converts chemical energy to electrical energy during substrate oxidation with the help of microorganisms (Allen and Bennetto, 1993; Kim *et. al.,* 1999; Park and Zeikus, 2000; Bond and Lovely, 2003; Gil *et. al.,* 2003; Liu *et. al.*, 2004).

Microbial fuel cell is made up of two compartments, anode and cathode, separated with proton/cation exchange membrane. Microorganisms oxidize the substrate and produce electrons and protons in the anode chamber of MFC. Electrons collected on the anode (negative terminal) are transported to cathode (positive terminal) by external circuit and protons are transferred through the cation exchange membrane or salt bridge internally. Positive current flows from the positive to negative terminal which is opposite to electron

flow. Thus, potential difference is produced between anode and cathode chamber due to dissimilar liquid solutions. Electrons and protons are consumed in the cathode compartment by utilizing oxygen (Ghangrekar *et. al.,* 2006). Microbes in the anodic chamber of an MFC oxidize added substrates and generate electrons and protons in the process. Electric current generation is made possible by keeping microbes separated from oxygen or any other end terminal acceptor other than the anode and this requires an anaerobic anodic chamber.

Typical electrode reactions are shown below using acetate as an example substrate.

Anodic reaction:

Cathodic reaction:

1.2 COMPONENTS OF MICROBIAL FUEL CELL (MFC)

1. Anolyte
2. Catholyte
3. Electrodes
4. Separator- Salt bridge OR Proton exchange membrane
5. Copper wire
6. Multimeter

1.2.1 ANOLYTE

a) Substrate

Basically the substrates have work to provide energy to the bacterial cells to grow in the MFCs, which improves the overall performance such as power density and columbian efficiency of MFCs. The bacteria uses supplement for its growth. Substrates like carbohydrates (glucose, sucrose, cellulose, starch), volatile fatty acids (formate, acetate, butyrate), alcohols (ethanol, methanol), amino acids, proteins and even inorganic components such as sulfides or acid mine drainages can be used as substrate (Mali *et. al.,* 2012). Other than these substrates wastewater can also be used as

substrates. Synthetic wastewater, actual waste wastewater from places like animal wastewater, brewery wastewater, rice mill, raw sludge and from primary effluents etc, have been used as substrate in MFC (Tenca *et. al*., 2013).

b) Organisms

Microorganism generally acts as a catalyst in MFCs and activates the catalytic reaction to convert the energy preserved in the chemical bonds between the organic molecules into electrical energy. It has been reported that mixed culture show higher power output (Zain *et. al.,* 2011).

c) Mediator

Mediators are mainly used for the transfer of electrons from organism to the anode. Several mediators used are as follows (Ieropoulos *et. al.,* 2005)

- Methylene blue
- Neutral red
- Thionine
- Meldola's blue
- Humic acid
- 2-ydroxy-1,4-naphthoquinone

d) Oxygen scavenger

Cysteine is used as oxygen scavenger to maintain the anaerobic condition in anodic chamber.

1.2.2 CATHOLYTE

Many different types of buffers have been used in MFCs to maintain suitable pH conditions and increase the solution conductivity, including phosphate, bicarbonate, and zwitterionic i.e. buffers. Phosphate buffer solution (PBS) has been commonly used in MFC studies to maintain a suitable pH for electricity generating bacteria and/or to increase the solution conductivity. Increasing the buffer concentration can improve power within certain concentration ranges. The use of high concentrations of phosphate in practice is not feasible due to its high cost, and the need to avoid phosphate releases into the environment. Bicarbonate buffers are more useful, but they can enhance the growth of methanogens. Zwitterionic

buffers can be toxic to bacteria as metals tend to remain more bio-available than with phosphate buffers (Yongtae *et. al.*, 2013).

1.2.3 ELECTRODES

Various electrode materials like copper, zinc, aluminum, carbon, stainless steel, mild steel, graphite etc. has been used in MFCs. The electrodes used in the construction of microbial fuel cells should have a good electrical conductivity, more surface area, less resistance and should be non-corrosive, biocompatible, chemically and mechanically stable to obtain a reproducible result. The anode materials such as graphite rod, graphite fiber brush, carbon cloth, carbon paper, carbon felt have been used in the MFCs. The distance between the electrode is also plays an important role on the performance of the microbial fuel cell so the distance should be as close as possible to overcome the electrical leakage and to have a more reduced internal resistance (Hadagali *et. al.*, 2012).

1.2.4 SEPERATORS

SALT BRIDGE & PROTON EXCHANGE MEMBRANE

A typical MFC has two chambers, cathodic and anodic and is generally separated by salt bridge or proton exchange membrane, to allow protons to move across to the cathode while blocking the diffusion of oxygen into the anode. Proton exchange system can affect an MFC system's internal resistance and concentration polarization loss and they in turn influence the power output of the MFC. Nafion is most popular because of its highly selective permeability of protons. The ratio of PEM surface area to system volume is important for the power output. The PEM surface area has a large impact on maximum power output if the power output is below a critical threshold. The MFC internal resistance decreases with the increase of PEM surface area over a relatively large range. Another method to study the proton transfer is by using a salt bridge which is made from mixture of agar and sodium chloride (Surajnhan *et. al.*, 2012).

1.2.5 COPPER WIRE

Externally the circuit is connected via a copper wire.

1.2.6 MULTIMETER

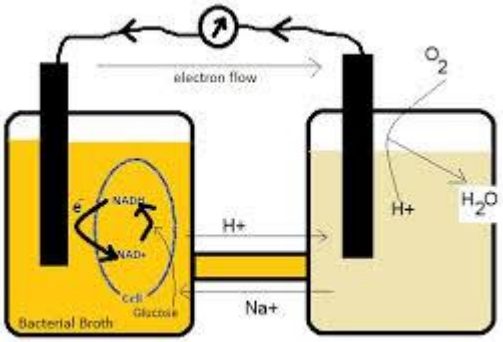
To measure the internal resistance and voltage, multimeter is used.

1.3 TYPES OF MFC

1.3.1 Two compartment MFC

Two-compartment MFCs are typically run in batch mode often with a chemically defined medium, glucose or acetate used as carbon source in solution to generate energy. They are currently used only in laboratories. A typical two-compartment MFC has an anodic chamber and a cathodic chamber connected by a PEM, or sometimes a salt bridge, to allow protons to move across to the cathode while blocking the diffusion of oxygen into the anode. As shown in Figure 1, the two chambers are connected by a salt bridge and circuit is completed by copper wire externally (Mahendra *et. al.,* 2008).

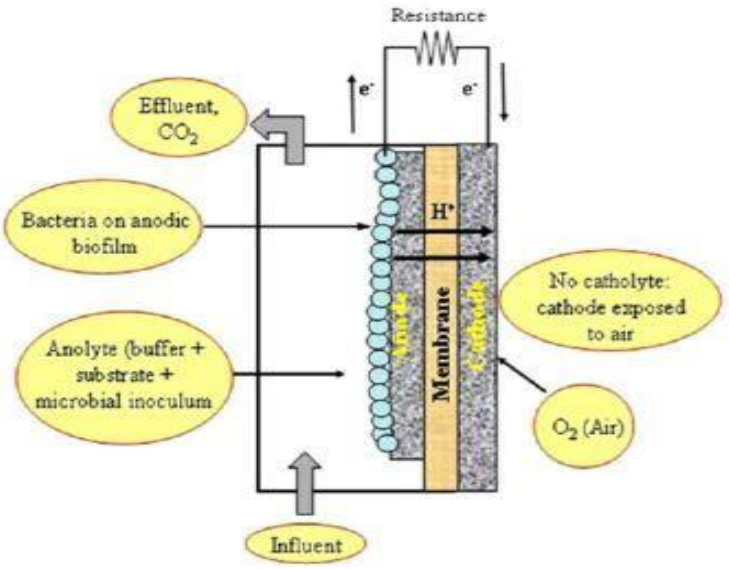
Figure 1: Schematic diagram of MFC run



1.3.2 SINGLE CHAMBERED MFC

This mostly possess only anodic chamber without the requirement of aeration in a cathodic chamber. One compartment MFC consist of an anode in a rectangular anode chamber coupled with a porous air-cathode that is exposed directly to the air. The MFC consisted of an anode and cathode placed on opposite sides in a plastic cylindrical chamber. Electrons pass from the bacteria to the electrode (anode) in the same chamber and then via a circuit to the cathode where they combine with protons and oxygen to form water (Lui *el. al.,* 2004). Figure 2, represents schematic diagram of the single chambered MFC set up separated by proton exchange membrane internally and externally connected by copper wire to multimeter (Santimoy *et. al.,* 2013).

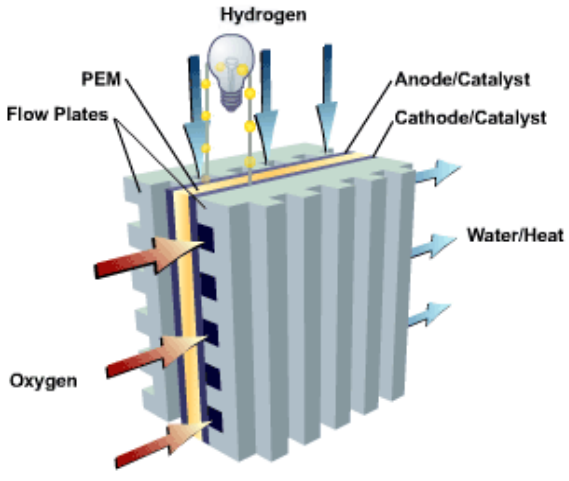
Figure 2: Single chambered MFC



1.3.3 STACKED MFC

Several MFCs are connected in series and in parallel. The use of series or parallel stacked MFCs are essential to increase the voltages and currents produced by MFCs. Any desired current or voltage could be obtained by combining the appropriate number of series and parallel connected fuel cells or power sources. Figure 3 shows the stacked MFC (Zhuwei *et. al.*, 2007).

Figure 3: Stacked MFC



1.4 APPLICATIONS

- **Wastewater treatment (**Mathuriya *et. al.*, 2009)
- **Analytical application of Microbial fuel cell** (Ximena *et. al.*, 2015)
  - **As BOD sensors**
  - **As toxicity sensors**
- **As implanted medical devices** (Oji *et. al.*, 2012)
- **For hydrogen production (**Zhuwei *et. al.*, 2007)

Objectives

2. Objectives

Previous studies have indicated efficiency of organism used in MFC (Maximum of 0.7 V using textile industrial wastewater as substrate). Thus organism was required to be characterized for its efficiency of electron transfer out of the cell. In addition, each industry differs in the composition of their effluent. To increase horizon of usage of the system developed it was required to test the efficiency under varied anodic conditions. Industrial effluents are stressed environment and may need several cycles of adaptation for successful functioning of MFC.

- To determine electrogenicity of isolate
- To study influence of culture adaptation on MFC performance
- To study performance of MFC in fed batch system
- To study influence of supplement addition on MFC performance and actual wastewater treatment in MFC treatment

Materials and Methods

3. Materials and Methods

1. Preservation of culture

The culture (Electrogenic *E. coli*) was preserved at 4^{o}C on nutrient agar slants with periodic transfers. The culture was revived by streaking on nutrient agar plate and was incubated at 37^{o}C for 24 h. The purity of culture was confirmed by isolation in plate and performing gram staining test.

2. Culture activation and cell harvesting

Culture activation

Loop full of pure culture was inoculated in sterile Nutrient broth (250 ml) and was incubated on shaker at 80-90 rpm for 24 h at 37^{o}C.

Cell harvesting procedure

1. The optical density (OD) of activated culture was allowed to reach to ~0.8 at 560 nm.
2. Activated culture was centrifuged at 7500 rpm for 10 min in oak ridge tube (50 ml).
3. Supernatant was discarded. The pallet was re-suspended and washed twice with normal saline.
4. At the end, pellet was re-suspended in synthetic waste water to make up the 20% inoculum for set up (with final volume 1200ml).

3. Biochemical characterization of culture

Following biochemical tests were performed for characterizing the MFC culture:

1. Carbohydrates fermentation test (Glucose, Mannitol, Sucrose, Lactose and Maltose)
2. Methyl red (M-R) test
3. Voges - Proskaur (V-P) test
4. Citrate utilization test
5. Triple sugar ion agar test
6. Oxidase test
7. Catalase test
8. Eosin Methylene blue agar plate (EMB)

9. Mac Conkey's agar test
10. Oxidation-Fermentation test (Hugh and Leifson Test) (Patel, 2008)

4. Construction of Microbial fuel cell

Double chambered MFCs was constructed from two polyvinyl bottles of 1.2 liter capacity (diameter 10 cm and transparent).

3.4.1 Anodic chamber

Anolyte

The anodic chamber consisted of synthetic waste water (Ganghraker and Shinde, 2009), 1% Methylene blue, 1% sucrose, 0.5% cysteine and 20% (v/v) electrogenic culture. In this cysteine was used as oxygen scavenger. Methylene blue was used as mediator. Electrogenic culture that was lab isolate gram negative bacteria used in MFC run.

3.4.2 Cathodic chamber

Catholyte

The cathodic chamber consisted of PBS buffer (Logan *et. al.,* 2005). Phosphate buffer saline was used to maintain the pH for electricity generation.

3.4.3 Electrode

Graphite electrode (14.9 cm x 3.1 cm x 0.5 cm) was used. It was activated at 80^{o}C in hot air oven for overnight and then used in the study figure 4.

Figure 4: Graphite electrode

3.4.4 Salt bridge

The salt bridge was made as described by (Khan *et. al.*, 2012). Figure 5 represents the photograph of salt bridge used in this dual chambered MFC operation.

Figure 5: Salt Bridge

5. Operation

The anodic chamber was filled with 20% v/v activated electrogenic bacterial culture (*E. coli*), Synthetic waste water, 1% methylene blue, 1% sucrose and 0.5% cysteine. The cathodic chamber was filled with 1200 ml PBS. Activated graphite electrodes were fixed in both the chamber. The anodic chamber was tightly sealed to make anaerobic condition. The two chambers were connected by salt bridge internally and externally by copper wire to multimeter as shown in figure 6.

Figure 6: Operating MFC system

6. Determination of reducing capacity of Methylene blue (MB)

The MFC culture was grown in liquid medium (LB broth). The samples were taken at different time intervals. The standard optical density of culture was adjusted and CFU titer was determined. For determination of discoloration time 1ml of 0.01% MB solution was added to the sample (5 ml). Then the discoloration time was determined under vigorous shaking. The optical density was measured at 620 nm then serial dilution of sample of sample was prepared. 100 ul was spread on nutrient rich agar plate and average CFU was calculated using the following formula: (Voeikova *et. al.*, 2012).

CFU = (number of colonies × dilution factor)/ volume plated in mL

Dilution factor = volume of sample/ total volume

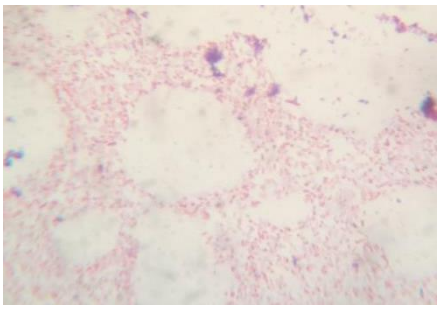
RESULT AND DISCUSSION

4. RESULT AND DISCUSSION

4.1 Gram staining of organism

Before running fuel cell set primary characterization of strain was conducted. The organism was revived from refrigerated nutrient agar slants and was purified thrice on Luria Bertani (LB) agar. Figure 7, shows microscopic image of electrogenic culture used in MFC.

Figure 7: Gram staining of electrogenic organism.

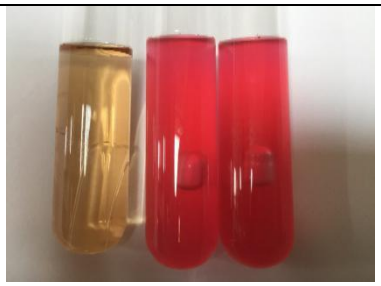


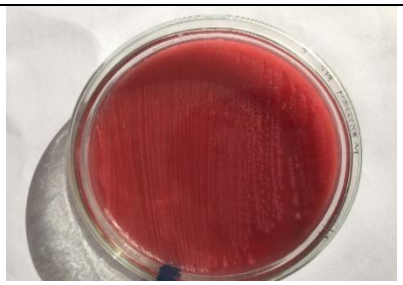
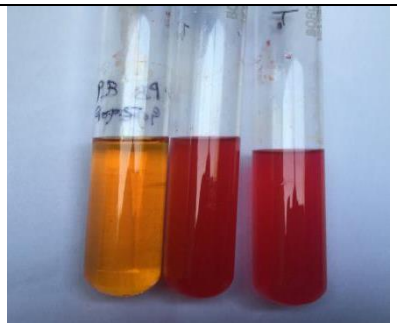
Gram staining of pure culture showed gram negative reaction. They were observed as single and clusters. The characterization was then followed by biochemical tests.

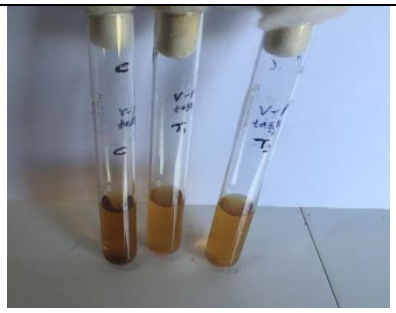
4.2 Biochemical tests

The Table 1 describes the result of biochemical test. Specific set of biochemical tests used for gram negative culture like, Sugar utilization, Citrate utilization, growth on EMB, TSI agar etc. Were performed and results were compared with reference standards.

Table 1: Biochemical test result

Sr. No.	Tests	Figures	MFC culture result	*E.coli* (MTCC 40)
1	Sugar utilization test		+	+

2	EMB		Colony with green metallic sheen were observed	Colony with green metallic sheen were observed
3	MacConkey's agar		Pink colony were observed	Pink colony were observed
4	Citrate utilization test		-	-
5	Triple sugar agar test		-	-
6	Methyl red test		+	+

7	V-P test		-	-
8	Catalase test		-	-

The culture shows biochemical characteristics similar to *E. coli*. The change in media color and gas production for sugar utilization shows that it could utilize all the sugars. Greenish metallic sheen was observed on EMB agar and pink colored colonies were observed on MacConkey's agar. Moreover the culture gave positive result for methyl red test. Whereas the organism showed negative result for citrate utilization, catalase production and triple sugar agar test.

4.3 Growth curve of *E. Coli*

To study the decolorization efficiency of the organism, harvesting of cells from various growth phases of culture was required. Viable count of cells were determined and plotted against incubation time until considerable decline was observed.

Figure 8: Growth curve of *E. coli*

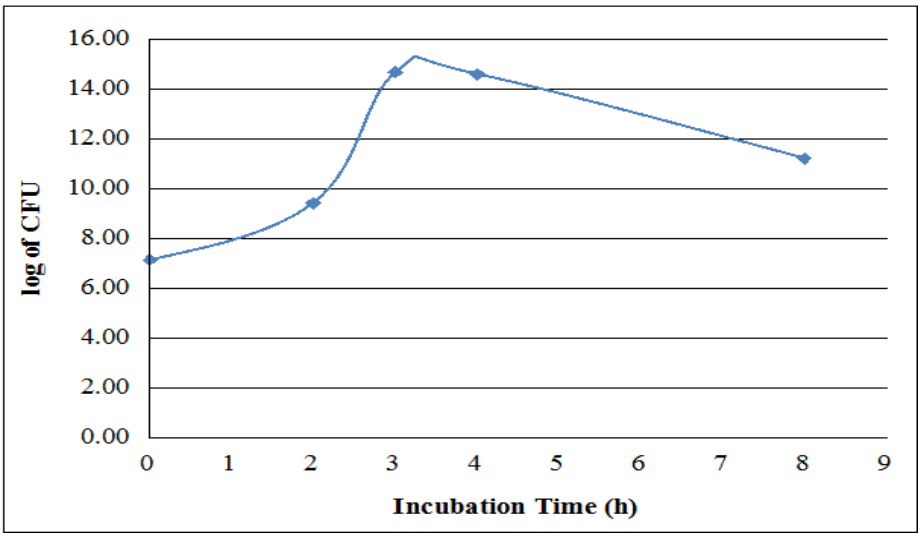


Figure 8 shows the lag, log and decline phase of growth of *E. coli*. From the growth curve it was estimated that exponential increase in CFU/ml was observed during 2nd and 3rd hours. Cells harvested from log phase were used for further analysis.

4.4 Discoloration time of methylene blue

Electrogenicity of the organism is due to organism's efficiency of transporting electron out of the cellular membrane. Electrons donated by the organism reduced methylene blue to leuco compound and time required was analyzed (Figure 9,10).

Figure 9: Time of methylene blue discoloration depending upon the concentration of *E.coli*

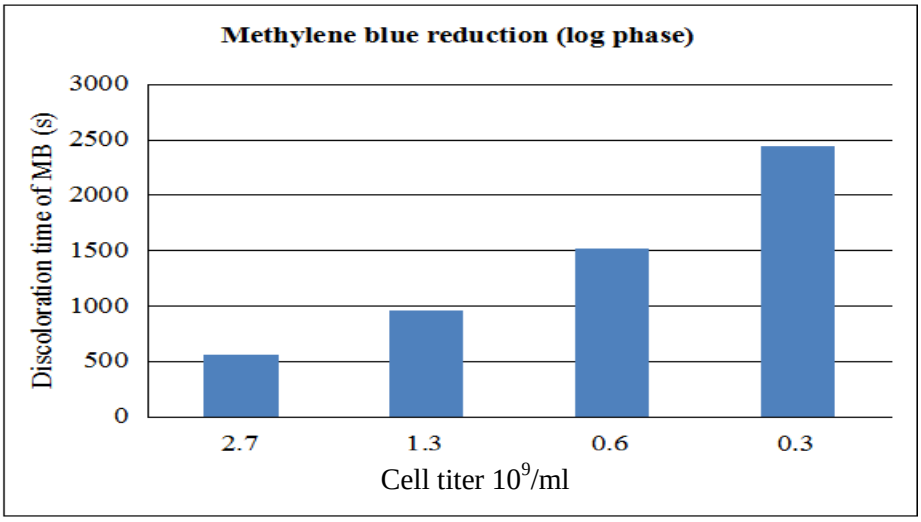


Figure 9 shows the discoloration (reduction) time of methylene blue during the log phase with increase in cell concentration the discoloration time decreases. The higher the cell titer the less time required for its discoloration. Undiluted cells of early log phase (2.7×10^9) required the least time (500 s) .

Figure 10: Time of methylene blue discoloration depending on concentration of *E. coli*

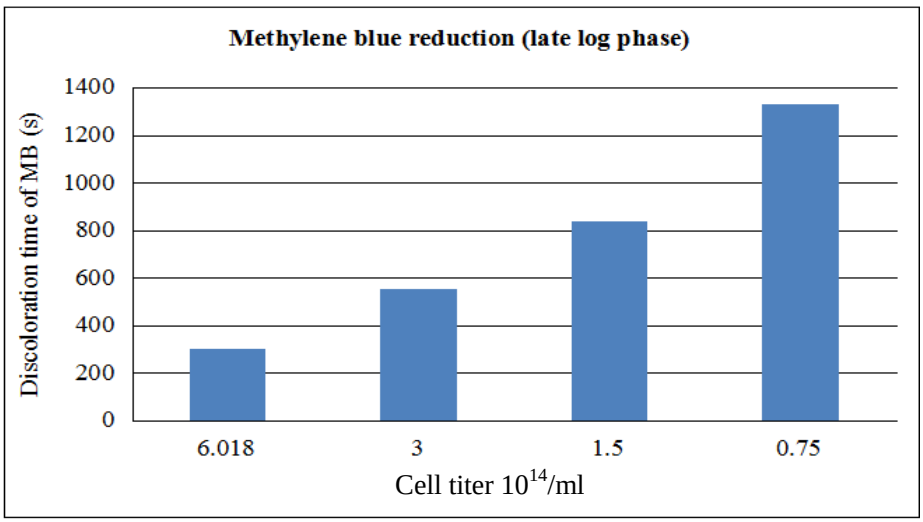


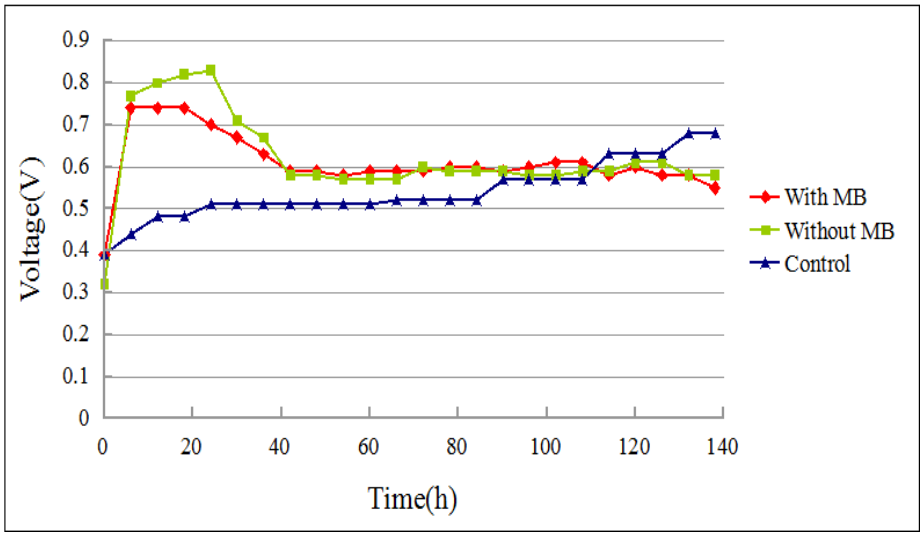
Figure 10 shows the discoloration time of methylene blue during the late lag phase of the growth curve. It was observed that MB discoloration time depends significantly on the cell concentration (Fig 9 and Fig 10). When the titer decreased to two and four folds results in

doubling of time and in the six fold diluted culture the discoloration time increased significantly. Correlation was observed between the 2- and 3-h cultures grown in liquid media. Longer cultivation time resulted in higher optical density and decreases in MB reduction time. MB discoloration time depended on the physiological state, cultivation conditions, duration of storage, and the pre-inoculation treatment of the culture. The rate of MB discoloration depends on dehydrogenase activity and the intracellular content of reduced NAD(P)H equivalents and their derivatives (Voeikova *et. al.*, 2012).

4.5 MFC with reduced concentration of sucrose with and without mediator

Microbial fuel was constructed using synthetic wastewater as anolyte and PBS as catholyte as described before. Figure 11 shows the MFC run with methylene blue and without methylene blue as mediator and mediator-less respectively.

Figure 11: MFC run with reduced sugar concentration (with and without mediator).

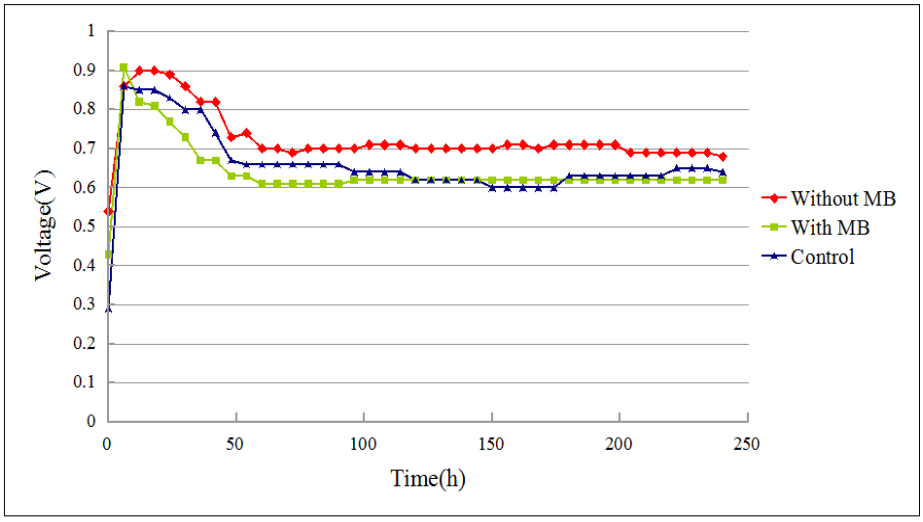


The highest voltage procured was 0.81 and was stable for a day using 1% sucrose. With the increase in time the voltage decreases was found to be stable. The results were compared to higher sucrose concentration (12%). The stable voltage was between 0.5V - 0.6V up to 140 h of incubation.

4.6 MFC using increased sucrose concentration (12%) a mediator and without a mediator

Industrial wastewater like beverage industry, sugar industry has been found to contain varying concentrations of carbon (Nelson *et. al.*, 2010; Chaudhary *et. al.*, 2013). In some cases saturation concentrations are also reported. Thus to verify the efficiency at higher sucrose concentrations MFC run was conducted using 12% sucrose. The voltage profile was recorded and was analyzed (Figure 12).

Figure 12: Voltage changes in MFC containing 12 % sucrose concentration, with and without mediator

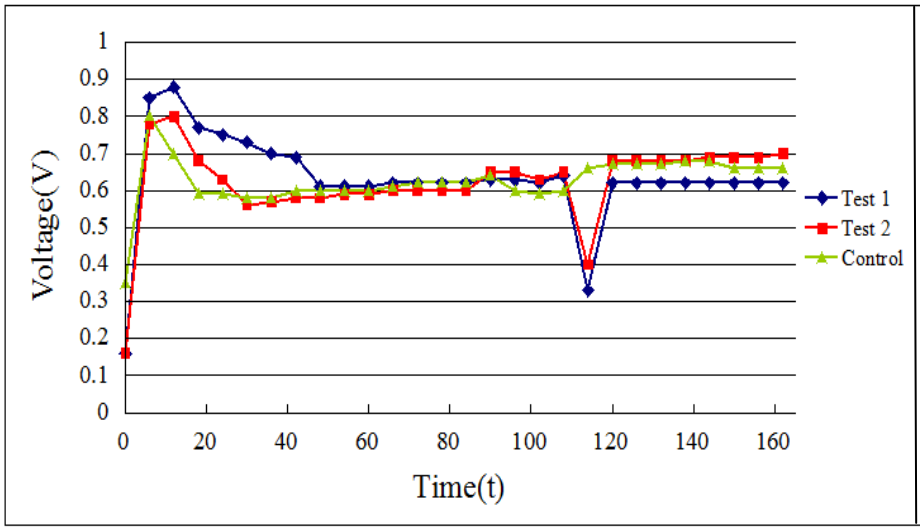


The highest voltage procured was upto 0.91V and was stable for 1 day. The result were compared with lower sucrose concentration (1%). As the time increased the voltage started to reduce and was stable between 0.6V-0.8V upto 240 hours of incubation. Increased sucrose concentration from 1 to 12% gave considerable difference in voltage generated. Due to the less sucrose concentration the voltage drop is also faster than observed with 12% sucrose.

4.7 MFC with fed batch old set:

The first set of MFC fed batch was run for 7 days and from that 200ml of inoculum was taken for second cycle and the first cycle was allowed to run for another 160 h.

Figure 13: MFC run with fed batch



The highest voltage procured was 0.89V and was stable for a day. As the time increased the voltage decreased, after a week in the when cells were added to new system, as media was fresh media was gradual increase in voltage. The voltage went on increasing for few days and then was stable between 0.7V - 0.8V. This indicates addition of fresh media led to increase in voltage. The observation also indicated stability of voltage up to next 160 h of incubation.

4.8 MFC in second cycle of of batch operation

Adaptation of culture helps in sustainance and survival of strain under various environmental conditions. For microbial fuel cell and treatment of wastewater it becomes of utmost important to adapt and acquire to the conditions prevailing in the chamber. Thus inoculum was developed for 7 days in anodic conditions and then was transferred to another set. Adapted culture from previous set was used as an inoculum this batch of MFC. Results of the same are depicted in Figure 14.

Figure 14: MFC run with changing the system

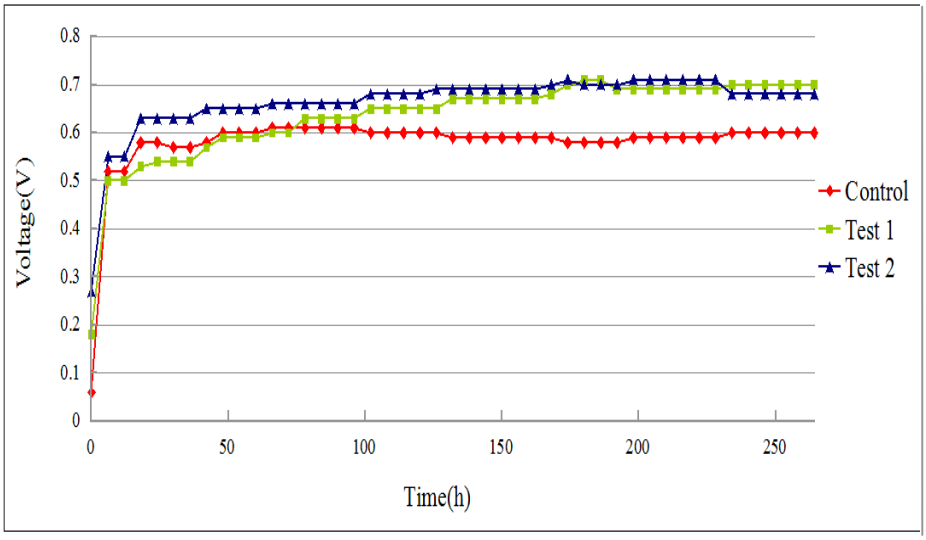


Figure 14 shows voltage obtain during MFC run by taking the media from the old system and then by adding fresh media to it. Here the highest voltage procured was 0.72V. Here with the increase in time the voltage gradually started to increase. The stable voltage was 0.6V- 0 .7V.

Out of all set ups run for MFC it was observed that set inoculated with adapted culture (as fed batch, twice) mode did not show any considerable fall in voltage even after incubation of 240 h. This observation also gives important insight of usage of such system for further field application.

The electricity generation in the batch mode included three phases: ascending phase, stationary phase and declining phase. In order to make the MFC more applicable, the stationary phase should be as long as possible which can be achieved by reducing the internal resistance of the MFC. The fed batch mode MFC demonstrated very good performance in terms of electricity harvesting and organic matter removal (Jianna *et. al.*, 2013).

Summary

Summary

Before the MFC run the electrogenecity of the organism was checked by the methylene blue discoloration test and it was concluded that the methylene blue discoloration time depends on concentration of the cell. Hence it can be said that the organism can transport electrons out of the cell and is electrogenic.

MFC run was conducted using synthetic wastewater using different concentration of sucrose and also with and without a mediator. For the investigation of supplement addition on MFC one set was run with 1% sucrose and other with 12% sucrose. Both the results were compared and it was observed that the 12% sucrose concentration set gave voltage as high as 0.91V and was stable for a day and it sowed more voltage generation than 1% sucorse and the voltage drop was also comparatively less. So the substrate addition plays important role on voltage generation.

The second set was of MFC run was by fed batch mode in this during the second cycle there was no considerable drop in voltage even after incubation of 240 h hence it can be said that the culture got adapted and could give a stable voltage.

Table 2: Summary of out from all MFC run

Sr No	MFC set up with	Maximum voltage (V)	Stable Voltage (V)	MFC system sustainability (h)
1	1% Sucrose	0.81	0.5- 0.6	140
2	12% Sucrose	0.91	0.6- 0.8	240
3	Fed batch 1	0.89	0.7-0.8	260
4	Second cycle of batch operation	0.82	0.6-0.7	260

Appendix

Appendix

Synthetic wastewater (Ghangrekar and Shinde, 2009).

Component	Concentration(g/l)
$NaHCO_3$	0.1
K_2HPO_4	0.1
KH_2PO_4	0.1
$CaCl_2.H_2O$	0.1
$FeSO_4.7H_2O$	0.01
$NiSO_4.6H_2O$	0.1
$MnSO_4.7H_2O$	0.1
$ZnSO_4.7H_2O$	0.1
H_3BO_3	0.1
$CoCl_2.6H_2O$	0.1
$CuSO_4.5H_2O$	0.1

Normal Saline: 0.85 g NaCl was dissolved in 100 ml of distilled water.

Phosphate buffer saline

Composition	Concentration(g/l)
K_2HPO_4	1.8
KH_2PO_4	1.2
NaCl	10
pH	7

1% Sucrose: 1 g sucrose dissolved in 100 ml of distilled water. Autoclave at 10 psi (substrate).

1% Methylene Blue (MB): 1 g methylene blue dissolved in 100 ml distilled water (used as mediator).

0.5% Cysteine: 6 g of cysteine for 1200 ml of synthetic waste water.

Gulcose phosphate broth

Components	Concentration
Glucose	5 g
K_2HPO_4	5 g
Peptone	5 g
D/W	1000 ml
pH	6.9-7.0

Simmon's citrate agar

Components	Concentration
Sodium citrate	2.0 g
Magnesium sulphate	0.2 g
Sodium chloride	5.0 g
Ammonium dihydrogen phosphate	1.0 g
Di potassium phosphate	1.0 g
Bromothymol blue	0.08 g
Agar	20.0 g
D/W	1000 ml
pH	6.9

Triple sugar iron Agar

Components	Concentration
Meat extract	3 g
Yeast extract	3 g
Peptone	15 g
Proteose peptone	5 g
Lactose	10 g
Glucose	1 g
Sucrose	10 g
Ferrous sulphate	0.2 g
Sodium thiosulphate	0.3 g
Sodium chloride	5 g
Agar	20 g
Phenol red	0.24 g
D/W	1000 ml
pH	7.4

Eosin methylene blue agar (EMB)

Components	Concentration
Peptone	10 g
Lactose	5 g
K_2HPO_4	2 g
Eosin Y	0.4 g
Methylene blue	0.065 g
D/W	1000 ml
pH	7.2

Macconkey's Agar

Components	Concentration
Peptone	20 g
Lactose	10 g
Sodium chloride	5 g
Bile salt	3-5 g
Neutral red	30 g
Crystal violet	10 g
D/W	1000 ml
Agar	30 g
pH	7.4

Hugh and Leifson's / Oxidation – Fermentative Medium

Components	Concentration
Peptone	2 g
NaCl	5 g
K_2HPO_4	0.3 g
Agar	3 g
D/W	1000 ml
pH	7.1

Reagents:

Methyl red indicator

Components	Concentration
Methyl red	0.1 g
95% Ethanol	300 ml

Crystal violet:

Crystal violet	2.0 g
95% Ethanol	20.0 ml
Ammonium oxalate	0.8 g
Distilled water	80.0 ml

Gram's Iodine:

Potassium iodide	2.0 g
Iodine crystals	1.0 g
Distilled water	300.0 ml

Safranine:

Safranine	0.25 g
95% Ethanol	10.0 ml
Distilled water	100.0 ml

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