MICROBIAL FUEL CELL: AN APPROACH TO GENERATE ELECTRICITY BY DEGRADING CAULIFLOWER LEAF WASTE

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RECOMMENDATION

This is to certify that **Mr. Rocky Maharjan** has successfully completed his thesis work entitled **"Microbial Fuel Cell : An Approach to Generate Electricity by Degrading Cauliflower Leaf Waste"** under my supervision.

This thesis work was performed for the partial fulfillment for award of Master of Science in Biotechnology under the course code BT 621. The result presented here is his original findings. I hereby recommend this thesis for final evaluation.

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Glossary Acronyms

CNT	Carbon nanotube
COD	Chemical Oxygen Demand
CTAB	Cetyl trimethylammonium bromide
DNA	Deoxyribonucleic acid
DNS	Di-nitro Salicylic Acid
DO	Dissolved oxygen
gm	gram
LB	Luria-Burtani
mA	Milli Ampere
MFC	Microbial Fuel Cell
PANI/ MWCNT	Polyaniline multiwalled carbon nanotubules
mA	Milliampere
mg	Milligrams
ml	Milliliter
mV	Millivolts
m^2	meter square
NA	Nutrient Agar
NMP	N-methyl -2 Pyrrolidone
nm	Nanometer
OCV	Open circuit voltage
PEM	Proton exchange membrane
ppm	Parts per million
RVC	Reticulated Vitreous Carbon
SDS	*S*odium Dodecyl Sulfate
SEM	Scanning Electron Microscopy
W	Watt
Ω	Ohm
^oC	Degree Celsius

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ABSTRACT

Today we are facing great problem in solid waste management along with global energy crisis due to increasing population. Microbial fuel cell (MFC) technology, which transforms chemical energy of organic compounds into electrical energy is considered a promising alternative. In this study, double chambered microbial fuel cell (MFC) was operated to evaluate the potential for degradation of cauliflower leaf waste under anaerobic microenvironment using mixed consortia as anodic biocatalyst. The potential for bioelectricity generation from the system was also evaluated. Degradation potential of cauliflower leaf waste was evaluated by observing chemical oxygen demand (COD), Ammoniacal-nitrogen, phosphorus and soluble reducing sugar removal. The fresh cauliflower leaf waste had COD contents of (143.48±5.60mg/g), Ammoniacal-nitrogen (0.90±0.4mg/g), phosphorous (67.82mg/g) and soluble reducing sugar (0.530mg/g). When multi-walled carbon nanotubes (MWCNTs) coated graphite electrode was used as anode with cauliflower waste paste as anolyte and Platinum wire was used as cathode along with phosphate buffer, reduction in COD by 24.73%, Ammoniacal-nitrogen by 76.9%, Phosphorous by 22.52% and soluble reducing sugar by 53.40% was achieved, after a week treatment of cauliflower leaf waste in MFC. The open circuit voltage (OCV) obtained in such condition was 426mV. A maximum power density of 7.2 W/m^3 was obtained when an external resistance of 1000 Ω was used. When Potassium ferricyanide was added as potential mediator in cathodic compartment, there was drastic increase in OCV by 681 mV. Concerning to the extracellular electron transfer mechanism(s), the biofilm formation on the anode was visualized by Scanning electron microscopy (SEM). SEM showed intensive adherence of microbes on anodic electrode enhancing the biodegradation. Molecular characterization of isolated microbial community based on 16S rRNA gene fragment sequence analysis confirmed *Bacillus spp* as predominant microbe in the culture. The result also indicated that the isolated *spp* were very effective for cellulose degradation and electron transfer. Overall, this study demonstrated the potential of using MFC for cauliflower leaf waste degradation and to achieve electricity as alternative.

Keywords: Cauliflower leaf waste, Chemical Oxygen Demand (COD), Microbial Fuel Cell (MFC), Multi-walled carbon nanotubules (MWCNTs), Potassium ferricyanide, Scanning electron microscopy (SEM)

CHAPTER ONE
INTRODUCTION

1.1 Vegetable waste

Vegetable waste includes the rotten, peels, shells and scraped portions of vegetables or slurries. Vegetable waste is generated in huge quantities and much of it is directly dumped on land to rot, which not only emits a foul odor, but also creates a big burden by attracting birds, rats, and pigs-vectors of various diseases. Vegetable waste is the major household wastes that cause environmental deterioration due to the lack of proper management. Apart from post-harvest losses due to lack of storage capacity, processing and packaging of vegetables according to customers' specifications play a major role in waste generation.

Vegetable waste is a special group of biomass that needs to be characterized to understand its nature for application as raw material and to propose the best methodology for its proper utilization. Waste composition also influences the overall yield and kinetics of the biological reaction during digestion (Afifi, 2011). Cauliflower is one of the most abundantly consumed vegetables in our country from which wastes are generated day by day. Due to this reason, in this work cauliflower leaves are used as a substrate for the electricity generation.

Table 1: The characteristics of cauliflower leaves:

Characteristics	Cauliflower leaves
pH	7
Ash content (%)	10.5263
Bulk density (g/ml)	0.2887
Iodine Index (mg/g)	368.9081

(Gupta *et al*., 2018)

1.2 Conventional disposal for Vegetable waste

1.2.1 Landfill

Landfill is the most popularly used method of waste disposal in many countries. This process focuses on burying the waste in the land. Number of issue arises due to landfills such as infrastructure disruption, pollution of local roads, pollution of the local environment such as contamination of groundwater or aquifers or soil contamination may occur. Extensive efforts are made to capture and treat leachate from landfills before it reaches groundwater aquifers, but engineered liners always have a lifespan,

though it may be 100 years or more. Eventually, every liner will leak, allowing the leachate to contaminate the groundwater (Nabavi-Pelesaraei *et al*., 2017).

Methane is naturally generated by decaying organic wastes in a landfill. It is a potent greenhouse gas and can itself be a danger because it is flammable and potentially explosive however gas is collected and utilized in properly managed landfills. This could range from simple flaring to landfill gas utilization. As well, poorly run landfills may become nuisances because of vectors such as rats and flies which can cause infectious diseases.

1.2.2 Incineration

Incineration is a method in which municipal solid wastes are burned at high temperatures so as to convert them into residue and gaseous product (Nabavi-Pelesaraei *et al*., 2017). The advantage of this method is that it can reduce the volume of solid waste to 20-30% of the original volume, which decreases the space that the solid waste taking up and reduce the stress on landfill. This process is also called as thermal treatment where solid waste materials are converted by incinerators into heat, gas, steam and ash.

1.2.3 Composting

Composting is an easy and natural bio-degradation process that converts organic wastes i.e. remains of plants and kitchen waste, into nutrient-rich food for the plants. Composting, normally used for organic farming, occurs by allowing organic materials in one place for months until microbes decompose. It is a good method of waste disposal because it can convert unsafe organic products into safe compost however; it takes time and occupies a lot of space (Rawoteea *et al*., 2017).

1.2.4 Anaerobic digestion

Anaerobic digestion is a collection of processes by which microorganisms break down biodegradable material in the absence of oxygen. This process is used for industrial or domestic purposes to manage wastes and/or to produce fuels. Anaerobic digestion is used industrially to produce food and drink products, and also in home fermentation (Pang *et al*., 2014).

The digestion process begins with bacterial hydrolysis of the input materials. Insoluble organic polymers such as carbohydrates are broken down to soluble derivatives that become available for other bacteria. Acidogenic bacteria then convert the sugars and amino acids into carbon dioxide, hydrogen, ammonia, and organic acids. These bacteria convert these resulting organic acids into acetic acids, along with additional ammonia, hydrogen and carbon dioxide. Finally, methanogens convert these products to methane

and carbon dioxide. The methanogenic archaea populations play an indispensable role in wastewater treatments.

It is used as part of the process to treat biodegradable waste and sewage sludge. As part of an integrated waste management system, anaerobic digestion reduces the emission of landfill gas into atmosphere. Anaerobic digesters can also be fed with purpose-grown energy crops, such as maize.

Anaerobic digestion is widely used as a source of renewable energy. The process produces a biogas, consisting of methane, carbon dioxide and traces of other 'contaminant' gases. This biogas can be used directly as fuel, in combined heat and power gas engines or upgraded to natural gas-quality biomethane. The nutrient-rich digestate also produced can be used as fertilizer (Pang *et al*., 2015).

1.2.5 Recovery and recycling

Resource recovery is the process of taking useful discarded items for a specific use. These discarded items are then processed to extract or recover materials and resources or convert them to energy in the form of useable heat, electricity or fuel. Recycling is the process of converting waste products into new products to prevent energy usage and consumption of fresh raw materials. Recycling is the third component of reduce, reuse and recycle waste hierarchy. The idea behind recycling is to reduce energy usage, volume of landfills, air and water pollution, greenhouse gas emissions and to preserve natural resources for future use (Du *et al*., 2015).

1.2.6 Waste to energy

Waste to energy involves converting of non-recyclable waste items into useable heat, electricity, or fuel through a variety of processes. This type of energy source is a renewable energy source because non-recyclable waste can be used over and over again to create energy. It can also help to reduce carbon emissions by offsetting the need for energy from fossil sources. Waste to energy is the generation of energy in the form of heat or electricity from waste (Du *et al*., 2015).

1.3 Microbial fuel cell

Our planet is undergoing the challenges bought in by the non-renewable energy scarce and environmental pollution, it is urgent for us to explore some sustainable technologies to deal with this situation. Microbial fuel cell (MFC), which is capable of directly generating electricity from wastewater/organic waste without much environment footprints, offers a possible solution. The core of MFC is the variation of redox gradient during metabolism of microorganisms when digesting substrates. In spite

of the electrophysiology discovered by Galvani (1791) when dissecting a frog, bioelectricity produced by microbial activity was not recognized until the beginning of 20th century (Potter, 1911). The possibility of utilizing the bioelectricity in a fuel cell expanded after the works of Cohen (1931) and Davis *et al*., (1962). In terms of the development in the field of MFC during the last century, several intermittent and significant breakthroughs were discovered. These include: 1) the MFC configuration from using mediator (Bennetto *et al*., 1983; Thurston *et al*., 1985; Allen and Bennetto, 1993) to mediator less MFC (Joo *et al*., 1999; Chaudhuri and Lovley, 2003; Liu et al., 2005; Scholz and Schroder, 2003); 2) the MFC with the presence or absence of separators (Liu and Logan, 2004; Jang *et al*., 2004) and 3) the explorations underlying mechanisms of exoelectrogenic bacteria which aimed to improve the performance and practical application of MFC in commercial ways (Logan, 2009; Logan, 2010; Kim *et al*., 2011; Chen *et al*., 2012; Ahn *et al*., 2014; Ma *et al*., 2014).

MFC represents the novel approach for generating electricity from biomass using microorganisms. A typical MFC consists of anode and cathode chambers separated by a proton exchange membrane. A schematic representation of a MFC is illustrated in Figure 1.

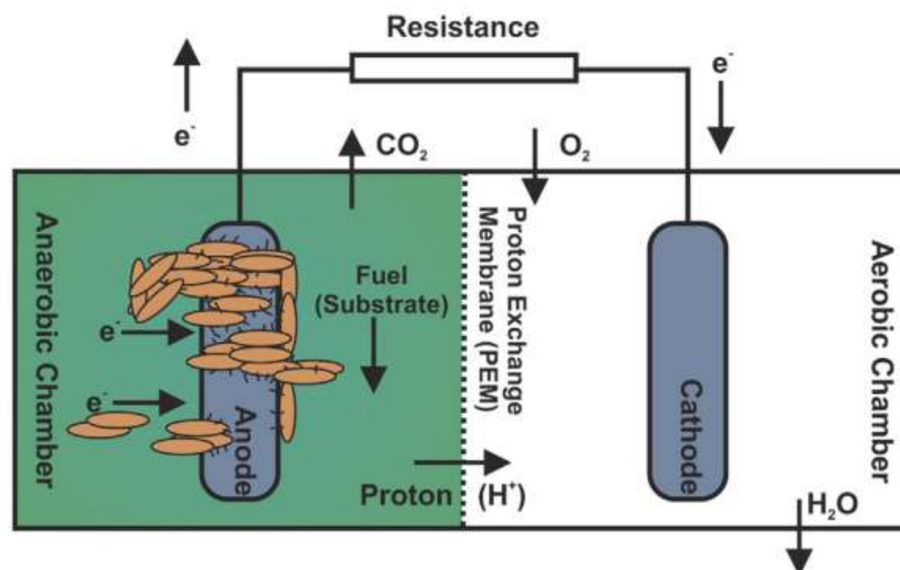


Figure 1: Schematic representation of Microbial fuel cell (Slate, 2019)

In the anodic chamber, organic matter is oxidized by microorganisms, generating electrons and protons. When microorganisms consume a substrate such as sugar in aerobic condition, they produce carbon dioxide and water. However, when oxygen is not present, they produce carbon dioxide, protons and electrons which can be utilized to generate electricity using a specialized device called Microbial Fuel Cell (MFC).

One of the most promising aspects of MFCs is the wide range of substrates that can be used to generate power by microorganisms. MFC can be fed with COD concentrations as low as 21.8 mg/L (Jang *et al*., 2006) to high as 127,500 mg/L (Zhang *et al*., 2009) with promising COD removal efficiencies.

1.4 Previous studies on the treatment of organic waste by microbial fuel cell

Energy in any form plays the most important role in this modern era. Also, reduction and recycling of waste are very serious problems all over the world due to the limitation of final disposal sites and decreasing environmental loads. From the characteristic analysis of the solid waste of many developing countries it is found that the major portion (more than 80%) of the total solid waste comprises of organic waste, that does not usually get much attention for recycling or resource recovery (Moqsud *et al*., 2008). Most of this waste is directly incinerated with other combustible waste, and the residual ash is disposed in landfill. However, incineration of this water-containing waste is energy-consuming.

Researches have been already carried out in MFC to generate electricity from organic wastes or wastewaters (Mosqud *et al*., 2013; Khalid *et al*., 2011; Daniel *et al*., 2009; Logan 2007). Composite vegetable waste was used as renewable resource in the MFC for bioelectricity generation (Venkata Mohan *et al*., 2010); the transformation characteristics of the organic matters in the food waste during the electricity generation from the food waste in the MFC was studied and the preferentially degradable fraction of the organic matters in the food waste was determined (Li *et al*., 2016); waste activated sludge and kitchen waste was anaerobically fermented together to produce short-chain fatty acids and then the fermentation liquid was used in the MFC for electricity generation (Chen *et al*., 2013); furthermore, the effect of algae species on the treatment of kitchen waste anaerobic digestion effluent in the MFC and the effect of MFC on the growth and metabolism of different algae were also reported (Hou *et al*., 2016). In this study cauliflower leaf waste was used as representative vegetable waste and waste degradation was analyzed in terms of COD removal. Also, soluble reducing sugar, Ammoniacal nitrogen and phosphorous content were obtained and analyzed. Additionally, electricity generation was measured to see the feasibility of MFC, using this cauliflower leaf waste as substrate.

1.5 Rationale

The demands of energy in the world continue to accelerate and which surely triggers the global energy crisis and environmental pollution. Recently, great attentions have been paid to MFCs due to their mild operating conditions and using variety of biodegradable substrates as fuel. It has got significant application in bioelectricity generation, used to produce biohydrogen, used in waste treatment and biosensors. Besides, the advantage of this technology, it still faces practical barriers such as low power and current density. To overcome this barrier, researchers have identified new class of nanomaterials to enhance the activities which makes the MFC more efficient and cost effective method for generation of electricity and organic waste degradation (Maski *et al*., 2018).

1.6 Hypothesis

1.6.1 Null Hypothesis (H0)

The cauliflower wastes cannot be used by microbes for electricity generation in MFC.

1.6.2 Alternative hypothesis (H1)

The cauliflower wastes can be used by microbes for electricity generation in MFC.

1.7 Objectives

1.7.1 General Objectives

To degrade cauliflower leaf waste by mixed culture of microbes and study the generation of electricity in microbial fuel cell

1.7.2 Specific Objectives

1. Estimation of COD, Phosphorus, Ammoniacal nitrogen and Soluble Reducing sugar in cauliflower wastes.
2. Use of cauliflower leaf waste in MFC for component degradation and electricity generation.
3. Optimization of MFC in terms of its efficiency.
4. Molecular characterization of microbial isolates from MFC aliquote.

CHAPTER TWO
LITERATURE REVIEW

2.1 Role of MFC in solid waste treatment

Vegetable-based waste generated from vegetable markets is one of the potential substrates to generate energy due to higher biodegradable organic fraction. The carbohydrate rich vegetable waste, generated in large quantities across the globe, can be beneficially used for retrieving the energy through MFCs. Venkata Mohan and his co-workers reported the use of vegetable waste as such and its fermented effluents (from hydrogen production process) in MFCs (Venkata Mohan *et al.*, 2010). Vegetable waste showed a power output of 57 mW/m^2, while its pre-fermentation doubled the power output in MFC (111 mW/m^2). The treatment efficiency also showed good increment with pre-fermentation (62 to 80) %, due to the hydrolysis of complex polysaccharides and proteins into their respective monomers during fermentation. Clauwaert *et al.*, 2008, achieved a maximum current density of 193 A/m^3 and a maximum power density of 70 W/m^3 using clover sap as the substrate of MFC. Another study by Zhang *et al*. 2013, found that corn stover could be used to remove sulfide and generate electricity in MFC, with the maximum power density reaching 744 mW/m^3, the maximum sulfide removal of 91% and the maximum COD removal of 52%.

Agricultural residues like corn stover, cattle manure, wheat straw, etc. have been tried out as substrate in Bioelectrochemical systems (BES). On the other hand, highly biodegradable food wastes in the form of vegetable waste, canteen based waste, yogurt waste are available. These can be readily used to trigger biochemical energy due to their rich organic content (Srikanth *et al.*, 2016). Haixia Du *et al.*, 2017 performed an experiment using MFC in order to treat potato waste with total COD removal of about 84% and current density being observed at 208mA/m^2. Municipal solid waste (MSW) is another major aspect of solid waste which is accumulating in huge quantities everyday and has a possibility to use as fuel in Bioelectrochemical systems (BES). Among the solid wastes studied, food industry based wastes are more studied. Chandradekhar and his co-workers studied the degradation of canteen based food waste (chemical oxygen demand (COD), 380 g/L) in solid state fermentation mode using a cylindrical single chambered MFC with air cathode (Chandrasekhar *et al.*, 2015). Anaerobic mixed culture was used as inoculums and food waste collected from canteen was used as substrate. The oil content (38 g/L) was removed by gravimetric separation prior to feeding into reactor and added with 10% tap water to maintain moisture content. Maximum power density of 164 mW/m^2 was observed along with a hydrogen production rate of 21.9

ml/h followed by ethanol production at 4.85% (w/v). As the waste contained huge biodegradable organic content, there is possibility of 3 different energy productions along with a substrate removal of 72% in terms of COD. This shows the strong potential of BES in energy generation as well as conservation into fuels/chemicals. In another study by Kook and his co-workers, using liquid fraction of municipal solid waste as substrate, about 94% COD removal was observed in association with a current density of 152-218 mA/m^2 (Koók *et al*., 2015).

2.2 Working Principle of MFCs

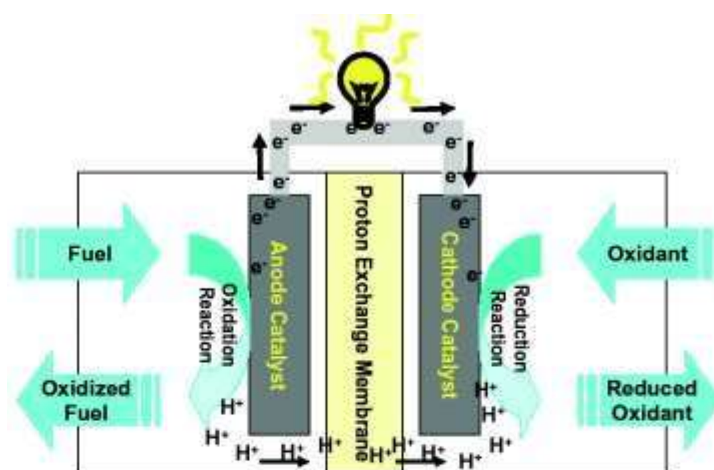
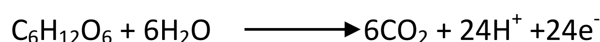


Figure 2: Microbial electricity Generation (Nealson, 2006)

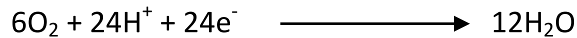
A basic MFC contains a biological, anaerobic anode and an oxidizing cathode which separates by a proton exchange membrane. Microorganisms in the anode act as a biological catalyst. In the absence of oxygen they catabolize the organic substrate and produce CO_2, H^+ and electrons (Bennetto, 1990). The simplified representation of the oxidation of glucose in anode by a whole microbial cell would be as follows:



$$C_6H_{12}O_6 + 6H_2O \longrightarrow 6CO_2 + 24H^+ + 24e^-$$

The electrons are stored in intermediates which become reduced. These are used to fuel the reactions that provide the living cell with energy for growth and maintenance via biosynthetic reactions (Bennetto, 1990). In absence of oxygen in anode chamber, electrons may be diverted from the respiratory chain and transferred to the electrode (which becomes a negatively charged electrode). These absorbed electrons flow through external circuit to cathode. The H^+ ions (protons) produced by the oxidation of

glucose get transported to cathode chamber via proton exchange membrane. At the cathode, electron, H^+ ions and oxygen gets reacted and produce water.



$$6O_2 + 24H^+ + 24e^- \longrightarrow 12H_2O$$

The positively charged cathode (acts as electron sink) is equivalent of the oxygen sink at the end of the electron transport chain, external to biological cell. The oxidizing material can be oxygen or chemical oxidizer such as potassium ferricyanide. Even the chemical oxidizers normally give high power densities, these cannot be renewably sourced.

2.3 Components of MFCs

The electrodes used in the construction of MFCs should have a good electrical conductivity, more surface area, less resistance and should be non-corrosive, bio-compatible, chemically and mechanically stable to obtain a reproducible result (Jang, 2004).

The distance between the electrodes also plays an important role on the performance of the MFC so the distance should be as close as possible to overcome the electrical leakage and to have a more internal resistance (Jang, 2004). One of the critical challenges in MFC is selecting proper electrodes (cathode and anode) which affect the power output (Logan *et al*., 2006).

The basic components of MFC include anode, cathode, proton/ ion exchange membrane, substrate and electrode catalyst (Das and Mangwani, 2010) :

Anode: Anodic materials must be conductive, biocompatible and chemically stable in the reactor solution. The most versatile electrode material is carbon, available as compact graphite plates, rods or granules, as fibrous material (felt, cloth, paper, fibers and foam) and as glassy carbon. The simplest materials for anode electrodes are graphite plates or rods as they are relatively inexpensive, easy to handle and have unambiguous surface area. Much larger surface areas are achieved with graphite felt electrodes which can have high surface areas. The main disadvantage of the material is that it is quite brittle. It has been shown that current increases with overall internal surface area in the order carbon felt > carbon foam > graphite (Chaudhuri and Lovley, 2003).

Cathode: Due to its good performance, ferricyanide ($K_3[Fe(CN)_6]$) is very popular as an experimental electron acceptor in microbial fuel cells (Park and Zeikus, 2003). The greatest advantage of ferricyanide is the low over-potential using a plain carbon cathode, resulting in a cathode working potential close to its open circuit potential. The greatest disadvantage, however, is the insufficient reoxidation by oxygen, which

requires the catholyte to be regularly replaced. The choice of the cathode material greatly affects performance and is varied based on application (Rhoades *et al*., 2005).

Membrane: The majority of MFC designs require the separation of the anode and the cathode compartments by a Proton Exchange Membrane (PEM). The most commonly used PEM is Nafion. When a PEM is used in an MFC, it is important to recognize that it may be permeable to chemicals such as oxygen, ferricyanide, other ions, or organic matter used as the substrate (You *et al*., 2009).

Substrate: It is the substance contained in the anode chamber that is to be oxidized. In a microbial fuel cell the substrate used can be any form of organic matter. Microbial fuel cells have been successfully operated on chocolate, wine, wastewater, acetate, glucose and more. Most frequently glucose, wastewater and acetate are used in experiments with the highest results being obtained with acetate (Logan, 2006).

Catalysts/catholytes: The cathode chamber is where protons and electrons recombine and reduce an electron acceptor. Oxygen is the most suitable electron acceptor for a MFC due to its high oxidation potential, availability, low cost, sustainability and the lack of a chemical waste product (water is formed as the only end product). However, when oxygen is used, the reaction is very slow therefore; the need for a catalyst arises. Most MFCs use platinum as the catalyst however, this is extremely expensive. Chemicals such as ferricyanide and potassium permanganate have been used successfully with results comparable to those achieved with platinum. These chemicals are far less expensive than platinum however, the disadvantage is that they are consumed in the reaction and must be replaced (He and Angenent, 2006).

Table 2: Basic components of MFC

Items	Materials
Anode	Graphite, graphite felt, carbon paper, carbon-cloth, Pt, Pt black, reticulated vitreous carbon (RVC)
Cathode	Graphite, Graphite felt, carbon paper, carbon-cloth, Pt, Pt black, RVC
Anodic Chamber	Glass, polycarbonate, Plexi glass
Cathodic Chamber	Glass, polycarbonate, Plexi glass
Proton Exchange Membrane (PEM)	Nafion, Ultrex, polyethylene, salt bridge, porcelain septum or solely electrolyte
Electrode catalyst	Pt, Pt black, MnO_2, Fe^{3+}, polyaniline, electron mediator immobilized on anode

(Du *et al*., 2007)

2.3.1 Microbes used in Microbial fuel cells

Electrochemically active communities are readily developed from a diversity of environmental sources and maintained with a remarkable range of pure and complex substrates. The microorganisms with the capability of oxidizing organic compounds to CO_2 while transferring electrons to electrodes with high efficiencies are called "Electricigens" having a meaning of electricity generating microorganisms (Lovely, 2006) and some other authors (Logan, 2006) used to call these microorganisms as "Exoelectricigens" since it gives more descriptive meaning of exocellular electron transfer along with electricity producing ability. Such microorganisms have been identified in several studies. Only these exoelectricigens are viable for MFC since the problems associated with exogenous mediators make MFC technology a non-feasible option for renewable energy generation.

Naval Research Laboratories in Washington D.C. and Oregon State University in Corvallis have developed a system where a slab of granite buried in anaerobic marine sediment act as anode and another piece of graphite that suspended in aerobic water act as cathode produces electricity. Investigations have found that the sediment is enriched with microorganisms in the family *Geobacteraceae* (Lovley, 2006). The specific type of *Geobacteraceae* prevalent in the sediment depends on the environment. *Desulfuromonas* species predominate in marine sediment and *Geobacter* favor fresh water.

Korean Institute of Science and Technology has observed a fuel cell containing *Shewanella* species which could produce electricity without the addition of mediators.

Swades Chaudri has found that *Rhodoferax ferrireducens* can completely oxidize sugars with electron transfer to electrode (Lovley, 2006). Daniel Bond has found *Geothrix fermentans* can oxidize acetate with the production of electricity. This organism has found in fresh water sediment at a lower level than *Geobacter* (Lovley, 2006). Electrodes can be colonized by microorganisms in the family *Desulfobulbaceae* in marine sediments with high concentration of sulfide, according to a study of Dawn Holmes (Lovley, 2006).

2.3.2 Substrates tested on MFCs

Substrate is one of the most important biological factors that affect generation of electricity in MFC. Various substances can be used in MFC which range from pure compounds to complex mixture of organic matter that is present in wastewater. Most widely known substrates used for MFC experiments are pure substrates like 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 (Logan, 2008).

Acetate is a simple substrate and mostly used to benchmark new MFC components, reactor designs or operational conditions. It is also inert to alternative microbial conversions like fermentation and methanogenesis at room temperature. Due to this, acetate fed MFCs showed highest coulombic efficiency compared to other pure substrate (Pant *et al*., 2009). Glucose is another commonly used pure substrate for MFCs. However, this results in low coulombic efficiency due to the consumption by diverse competing metabolisms such as fermentation and methanogenesis that cannot produce electricity.

Lignocellulosic biomass is widely available as well as they are renewable. However these cannot be directly utilized by microorganisms in MFCs for electricity generation without converting to low molecular weight compounds. Waste water from breweries has been used as a substrate as well for MFCs, due to its low strength, food derived nature of organic matter with high carbohydrate content and lack of high concentrations of inhibitory substances. Azo dyes are present in effluent from dye manufacturing industries and textile industries. The removal of color from the effluent is very important since it minimizes the adverse environmental effects by obstruction of light into the water bodies. It was also reported that decolorization of wastewater containing brilliant red in a MFC where glucose and confectionary wastewater used as co-substrates (Pant *et al*., 2009). Therefore, simultaneous treatment of azo dye containing waste water and biodegradable organic matter containing wastewater could be achieved by mixing two kinds of wastewater in the MFCs with the advantage of saving both cost and energy.

Landfill leachates consisting complex compositions of organic and inorganic pollutants and heavy metals has also been tested in a MFC. It was demonstrated that it is possible to generate electricity while treating landfill leachate. MFC have been developed in rice paddy fields to produce electricity by rhizosphere populations oxidizing organic carbon delivered to the rhizosphere (Pant *et al.*, 2009). Different sizes of chitin particles have been tested as the substrate for MFC. The maximum power density has obtained for the smallest particle and the power production is limited due to the low rate of hydrolysis of particulate material. Electricity production with paper recycling water has also tested in MFC (Pant *et al.*, 2009). However, with unamended waste water (without the addition of phosphate buffer), the power output was low due in large part to the low solution conductivity.

There are some other possible substrates used for MFCs. Some of them are waste and wastewater from livestock industry, effluent from cane molasses based distilleries that are produced in large quantities as well as containing higher organic load. Whey is the major waste in dairy industry and wastewater from fruits and vegetable processing are also possible organic waste substrate for MFCs (Logan, 2008).

2.3.3 Electrode materials

In order to optimize power output from MFCs, one area can be potentially be explored is electrode materials. Ideally, the electrode materials should be economical, exhibit favorable electron transfer, mechanically stable, large surface area, giving rise to large current densities (Slate *et al.*, 2019). Carbon materials and non-corrosive metals like Platinum, steel are currently the most widely used electrode materials in MFC configurations. Potassium ferricyanide ($K_3[Fe(CN)_6]$) is frequently used as an electron acceptor in the MFCs due to its good performance and low overpotential (Logan and Regan, 2006). Biocathodes increases the power by decreasing the overpotential (Huang *et al.*, 2011). Alternately, the cathode can contain oxygen and is preferred because it simplifies the operation of the cell and is the most commonly used electron acceptor in MFC (Singh, 2012).

2.3.4 Proton Exchange Membrane

Membranes are primarily used in two chambered MFCs as a method for keeping anodic and cathodic liquids separate. However, the membrane should be selectively permeable and should allow protons produce at the anode to migrate to cathode.

The most widely used membrane is Nafion membrane. Nafion is a sulfonated tetraflurethylene copolymer. Nafion's unique ionic properties are a result of incorporating perfluorovinyl ether groups terminated with sulfonate groups on to a

tetrafluoroethylene backbone. Protons on the SO_3H (sulfonic acid) groups hop from one acid site to another. Pores allow movement of cations but the membranes do not conduct anions or electrons. A cation exchange membrane (CEM) made by Membrane International Inc. has also been used in many MFC studies especially with ferricyanide as catholytes. This membrane is much thicker and stiffer than Nafion and the cost is also lesser than Nafion (Ashrafi, 2017).

To support microbial catabolism, MFC employ a number of chemicals in addition to the fuel. These chemicals dissociate in the aqueous phase generating cations such as K^+, Na^+, NH_4^+, Mg^{2+}, and Ca^{2+}. The concentration of these ions is typically 10^5 times higher than that of protons at neutral pH. These cations can interface with proton transfer through the membrane. Another constraint for proton transfer in MFC is the use of an aqueous solution as the electrolyte. According to the studies done using Nafion 117 membrane, cations other than protons transferred from anode compartment to cathode compartment were same as the electrons transferred through the circuit. Analysis of the membrane also showed that K^+ and Na^+ occupied about 74% of the sulfonate residues of the membrane (Kim, 2007). It is evident that cation transfer was not driven by the concentration gradient but was an electrodialysis process. It is further reported that electroneutrality was mainly sustained by transport of the cations and not by proton transport, which leads to a number of electrochemical and microbiological problems. Since H^+ gets accumulated in anode compartment, it becomes acidified raising the anode potential and producing adverse conditions for microorganisms catalyzing the anode reaction, while the cathode compartment is alkalized lowering the cathode potential.

2.4 Electron Transfer in MFC

2.4.1 Direct Electron Transfer

Direct electron transfer means that the bacteria arrange their outer membranes to be adjacent and physically connected to the anode electrode (Schröder, 2007). The fundamental assumption is that the closeness of the bacteria to the electrode is necessary for electron transfer. Some have suggested that this mechanism is impractical for describing all anode reactions because MFC biofilms were shown to sustain bacteria more than 10µm from the electrode and that this extracellular electron transfer model could not kinetically account for the high current densities (i.e. 10 A/m^2) reported by the literature(Torres *et al*., 2010).

2.4.2 Mediated Electron Transfer

Mediated electron transfer, occurs when bacteria use chemical redox mediators to transfer electrons. In short, bacteria would reduce a mediator (i.e. thionine, methyl viologen, methyl blue, humic acid, neutral red) (Sanchez, 2013) which diffuses to the anode, is oxidized, and then diffuses back to the cell to be reduced. Newman and Kolter demonstrated this for the pure culture of *Shewanella oneidensis* (Newman, 2000). Another study suggested that some bacteria are able to use mediators produced by foreign bacteria to transfer electrons (Boon *et al*., 2008). The key to this mechanism is that you have a soluble mediator diffusing back and forth between bacteria and electrode. Given this external electron transfer (EET) model, the main limitations for electricity production would be concentration of the mediators and the mass transport resistance that would be experienced at high current densities.

2.4.3 Conduction-based Electron Transfer by microbes

Conduction-based electron transfer implies that bacteria are able to conduct electrons to the anode via biofilm matrix (Marcus *et al*., 2007). A cursory review suggests that bacteria are able to do this via a network of bionanowires (El-Naggar *et al*., 2008) and sequestered riboflavins (Marsili *et al*., 2008). Technically, conduction based transfer could use the same mechanisms used in mediated transfer however; the mediators would be fixed and conjugated in the biofilm. Richter *et al.* demonstrated that *Geobacter sulfurreducens* used a conductive network of non-diffusing intermediates to transfer electrons to the anode (Richter *et al*., 2009). Research is currently addressing how complex bacterial networks are able to transfer electrons in biofilms that can be as thick as 80μm (Torres *et al*., 2010) but the more important aspect is that the conduction-based electron transfer model has thus far been the basis for understanding the biofilm-anode and that it kinetically accounts for the highest current densities reported in the literature (Torres *et al*., 2010).

2.4.4 Mixed community microbial fuel cells

The different mechanisms in which bacteria can be used for the electron transfer has been considered. Interaction between bacteria in MFC biofilms and other bacteria contribute indirectly to the production of electricity. For example – *Brevibacillus spp*, co-cultured with *Pseudomonas spp*, there was a marked increase in electricity generation (Franks, 2010).

Pyocyanin, has shown greater antimicrobial activity against aerobic bacterial strains (Baron, 1981). The addition of pyocyanin to non-pyocyanin producing biofilms could be

used to increase power outputs. This approach could be applied in the degradation of waste/toxic matter in order to efficiently convert organic matter to electricity in a MFC setup (Wu *et al*., 2014).

The interactions of mixed community biofilms is complex and is yet to be fully understood. However, the use of mixed community biofilms, both inter-bacterial and other microorganisms for MFCs, has vast potential (Slate *et al.*, 2019). Clearly, mixed biofilm communities, that have the ability to generate electricity by more than one mechanism, will play a pivotal role in the improvement of MFCs (Li *et al*., 2016).

2.5 Biofilm-anode

In an MFC the biofilm-anode provides the framework for conversion of a chemical substrate to electricity. The biofilm metabolizes the substrate and transfers the resulting electrons to the anode via a series of protein-based redox reactions. The amount of electricity actually generated from these reactions depends on the efficiency with which a bacterium's outer membrane proteins or cytochromes transfer electrons to the anode. This efficiency is affected by 1) the metabolic efficiency of the bacteria metabolism and its electron transport chain and 2) the activation resistance between the electron transport chain and the anode. Although genetic engineering may improve the metabolic efficiency of the electron transport chain, it cannot optimize the anode, the terminal electron acceptor within the biofilm-anode. However, the activation resistance experienced in the terminal electron transfer could be improved by engineering a better connection between the electron transport chain and the anode through the use of a catalyst or a modified electrode. This is supported by the fact that protein-protein interactions have evolved to be efficient but protein-electrode interactions continue to be understood and developed (Alwarappan *et al*., 2010; Gerasimov *et al*., 2010; Odenthal *et al*., 2007).

2.6 MFC designs

2.6.1 Two compartment Microbial fuel cells

This is the most widely used design consists of anode and cathode compartment separated by a Proton exchange membrane or salt bridge. Anode chamber contains microbes, media (Glucose, acetate etc.) and electrode whereas cathode chamber includes electrode, fresh water, and oxygen supply. As electrode materials - copper, stainless steel mesh, graphite, carbon paper, graphite fiber brush and carbon cloth are commonly used (Logan, 2008). Logan *et al* on 2005 designed the basic H type MFC,

which was constructed using two borosilicate glass bottles of capacity 300 ml. The carbon paper was used as an electrode for both anode and cathode. However, cathode was soaked with platinum catalyst. As an inoculum, the sediment was used from the lake. The microorganisms were grown in mineral salts medium. The maximum power density was 19 mW/m^2 and was increased to 39 mW/m^2 by increasing the concentration of cysteine (0.770 g/L) (Logan *et al*., 2005).

2.6.2 Single compartment Microbial fuel cells

Single chambered MFC, only anode compartment is present whereas cathode is exposed to the atmosphere. Supply of O_2 in cathode chamber is not necessary as it is directly exposed to air. Since the design is simple, the scale-up process is very simple and cost of the design decreases. This type of microbial fuel cells can be operated in both batch and continuous method (Park *et al*., 2003). Min and Logan on 2004 designed a simple flat plate MFC (Min, 2004). The nonconductive polycarbonate plate was used to construct the MFC where serpentine path was created for waste water retention with the total surface area of about 55 cm^2 and volume of about 22 cm^3. Screw and bolt system were used to seal the plate. Porous carbon paper was used as an anode, whereas carbon cloth with the platinum catalyst was used as cathode. Nafion membrane was used as proton exchange membrane and copper wire was used to connect electrodes with external circuit (Min, 2004).

2.6.3 Up-flow Microbial fuel cells

In Up-flow MFC design, the wastewater is pumped into the system from the bottom and the effluent flow out of the system from the top and it operates in continuous mode (Du *et al*., 2007). Jang *et al* on 2004 designed typical up-flow MFC without proton exchange membrane (Jang *et al*., 2004). The MFC was tubular in shape with the total height of 100 cm and diameter 10 cm and was made with polyacrylic plastic. Graphite felt was used as an anode and the same material was used as the cathode. Layers of glass bead and glass wool were used in between anode and cathode series and the sample ports were situated throughout the length of the reactor. The fuel (artificial wastewater containing glucose and glutamate at the rate of 0.28 mL/min was supplied from the bottom of the reactor and the effluent was taken out from the top. The aerators were used to aerate the cathode layer, and the platinum wire resistance 10 Ω was used to connect the electrodes to an external circuit. The main advantage of this design is the absence of proton exchange membrane and can also be operated in continuous mode which reduces the cost. The main disadvantage is energy utilized to pump the wastewater is high when compared to energy generated from it. Hence, this design can be used to treat wastewater where electricity generation is not a first priority (Jang *et al*., 2004).

2.6.4 Stacked Microbial fuel cells

Many MFC is stacked together either in series or parallel connection. Since many MFC are connected together high output or current generation can be obtained. Aelterman *et al* on 2006 designed stacked MFC which consisted of six individual continuous microbial fuel cells stacked together (Aelterman *et al*., 2006). Graphite granules were used as anode and cathode. Graphite granules provide a maximum surface area for microbes to transfer electrons and graphite rod was used to connect external circuit. The proton exchange membrane used was Ultrex CMI7000. By observation, parallel connection of cells has a better performance than series connection due to high efficiency and higher chemical oxygen demand (COD) removal (Aelterman *et al*., 2006).

2.6.5 Paper Microbial fuel cells

Paper was used for fabricating the MFC which was low cost, chemical free and disposable. The design was simple which consists of anode and cathode where graphite particles were deposition on the paper using four different strokes of pencil act as an electrode. As proton exchange membrane, parchment paper was used which allows the H^+ to pass through them. The crayon was added to the corners to make it hydrophobic. The microbe was added to the anode chamber along with few microliters of growth media. The air cathode was used, where electrons were accepted by $O_{2.}$ The maximum voltage and current generated was found to be 300mV and 11µA, respectively (Lee *et al*., 2016).

2.7 Performance parameter of MFC

Power Density: Power Density is the power output of the cell per anode surface area. High internal resistances of MFCs limit the maximum power density that can be achieved using MFCs. Therefore, reactor configuration and electrolyte has to be in such a way to reduce the internal resistance in order to operate the full microbial potential (Kim *et al*., 2007). According to the studies done on a circulating column MFC with air cathode density generated per unit of COD is around 0.01 Wm^{-2}/mgL^{-1} (Kargi and Eker, 2009).

Coulombic Efficiency (CE): CE is the percentage of electrons received from the organic matter versus the theoretical maximum whereby all electrons go to current generation. Following are the factors that reduce Coulombic Efficiency (Logan and Regan, 2006).

- Part of the substrate is used for cell synthesis of electricity generating bacteria.
- If oxygen is used at the cathode and oxygen diffusion through membrane to anode chamber can be used for aerobic respiration by same electricity

generating bacteria if they are facultative or by other bacteria if a mixed culture is used.

- Substrates lost to alternative electron acceptors if they are present in the medium (eg: Nitrate of Sulphate through fermentation and methanogenesis)

2.8 Application of MFC

2.8.1 Hydrogen production

Apart from electricity generation using microbial fuel cell, it can also be used for hydrogen production. Conventionally, the two chambers (anode and cathode) are separated by proton exchange membrane and proton passed through this membrane whereas electron passes through the external circuit. The proton combines with oxygen and electron in cathode chamber and forms water. If the cathode chamber is maintained in an anaerobic condition and a small amount of external potential (to break thermodynamic barrier) is provided, a thermodynamically favorable reaction takes place in the cathode chamber. The protons (H^+) combine with electrons (e^-) to form Hydrogen molecules (H_2). Theoretically, only 110 mV is required to break the thermodynamic barrier whereas about 1210 mV is required to split the water molecules (electrolysis). Approximately, about 8 to 9 mole H_2 generated for 1 mole of glucose which is 2 times greater than conventional fermentation about 4 mole of H_2 generated for each mole of glucose (Liu *et al*., 2005).

2.8.2 Methane production

MFC technology can also generate methane where methanogen bacteria are used. The design contains two compartments anode and a cathode separated by proton exchange membrane similar to those design used for hydrogen and electricity production but the operation mode varies. A small power source is supplied for splitting water molecules in an anode compartment under anaerobic conditions without any microbes. CO_2 is supplied in cathode compartment and proton that is produced near anode passes through proton exchange membrane, reacts with CO_2 to form CH_4 and H_2O. The methane that is produced is pure and can be utilized directly (Wagner *et al*., 2009).

2.8.3 Wastewater Treatment

Today, treating wastewater is a major problem which has to be solved in much cost-effective manner. In 1991, the MFC was used to treat the wastewater (Habermann *et al*., 1991). The sources of wastewater could be from municipal wastewater, domestic

wastewater and industrial wastewater. Out of this entire source, it is found that the municipal wastewater is very difficult to treat because of its most diversified composition. Apart from treating wastewater, they can also be used for generating electricity that can be utilized as an energy source. It is found that less solid waste (50 – 90% less) is generated for disposal and it can also convert molecules such as acetate, butyrate and propionate etc. into CO_2 and H_2O before releasing into the environment (Li *et al*., 2007).

2.8.4 Biosensors

MFCs can also be used as biosensors to detect the pollutants level in the environment. There is a correlation between wastewater strength and coulombic yield. Hence, it can be used as BOD (biological oxygen demand) sensors. There is a linear relationship between BOD and Coulombic yield. The current increases linearly with increase in BOD. MFCs BOD sensors are more reliable when compared to other BOD sensors because of stability and accuracy. Also, MFC BOD biosensors have more lifespan (over 5 years) without maintenance when compared to other sensors (Li *et al*., 2007).

2.9 Types of Anodes and their modifications

The anode plays an important determinant of MFC performance and is often the limiting factor for high power output. The anode material and its structure can directly affect the bacteria attachment, electron transfer and substrate oxidation. The anode material should be highly conductive, non-corrosive, highly porous, non-fouling, and inexpensive, easily made and scale to larger sizes and should have high specific area (area per volume). The most important parameters of them is the high electric conductivity since this few ohms of added internal energy can greatly reduce the power and non-corrosiveness which rules out many metals.

Carbon cloth and carbon paper are the most applied MFC anodes due to their stability in a microbial inoculum mixture, high conductivity and high surface-area. The association between anode electrode and microbial inoculums has a great impact on MFC power density. There are some improvements done in anode electrodes by impregnating it with different chemical catalysts. Research has shown improvement with the electron transfer between microbial cells to anode by immobilizing microorganisms or mediators (Logan, 2008).

Optimization of porous structure of the anode with higher specific surface area increases the power density but by observation, the pores are clogged by the entering bacteria resulting in cell death and reduction of the electrochemical reaction surface. Conventional carbon based anodes, such as carbon felts and porous carbon papers

suffer from this problem. Development of a new nanostructure for anode material with high specific area favorable for both bio and electro catalytic process is critical for improving the power density (Qiao *et al*., 2007).

2.9.1 CNT based anode material

Carbon nanotubes (CNTs) are allotrope of carbon in which the structure consists of enrolled tubular graphene in the configuration labelled armchair (Slate *et al*., 2019). CNTs consist of one or more layers of graphene either single-wall (SWCNT) or multi-wall (MWCNT) with open or closed ends (Komarov *et al*., 2004). MWCNTs with hydroxyl functional groups are a possible alternative anode material to traditionally used carbon cloth due to greatly improved performance in electron transfer capabilities, microbial attachment and substrate diffusion (Thepsuparungsikul *et al*., 2014).

2.9.2 Polymer-carbon composites

The combination of CNT with polymers could also introduce the new electronic properties based on the new morphological disposition or based on the electronic interaction between the two components which leading to new functionalities and applications (Cabezas, 2013)**.** Mostly used conductive polymers are conjugated polymers with heteroatoms in the main chain as polyaniline (PANI), polypyrrole (PPy), polythiophene (PTh) and their derivatives. Conducting polymers such as PPy and PANI have a good biocompatibility and are commonly used to immobilize enzymes and fabricating enzyme electrodes (Scott, 2010). Therefore, conductive polymers can be used as effective supports for microorganism in MFCs. The anode carbon supports have been coated with PANI (*Lai et al*., 2011) and PPy (Yuan and Kim, 2008). It is reported that CNTs incorporated with a conductive polymer can lead to a synergistic effect (Qiao *et al* 2007) showed that a CNT-polyaniline composite enhanced the electro catalytic property and adhesion with the bacterial cell. They assumed that it was because of the protective effect of polyaniline and large increase in the surface area.

2.10 Scanning electron microscopy (SEM)

SEM was performed to study the morphological change of the coated and uncoated electrodes during MFC experiment and to evaluate the morphology of biofilm formed on the surface of the electrodes (Nandy *et al*., 2019). Scanning Electron Microscopy helps to visualize the surface of the anode in order to determine microbial attachment and formation of a biofilm on the anode electrode surface (Khater *et al*., 2017). In one of the studies, SEM was applied on anodophilic electrode in order to analyse the presence of microbial attachment and biofilm formation on anode electrode. They conducted SEM in bare electrode surface (before inoculation on MFC with aerobic

activated sludge) and in inoculated cells, which revealed the presence of abundant microbial attachment on the carbon paper (Khater *et al*., 2015).

2.11 Molecular characterization

Molecular characterization determines the genetic characteristics of the organism. These are usually measured by 16S rRNA in the case of prokaryotes, or 18S rRNA in the case of eukaryotes. 16S rRNA is the mostly used technique to analyze microbial communities involved in electricity production since bacteria are the major microbes. The molecular method includes Denaturing Gradient Gel Electrophoresis (DGGE), commonly used for analyzing microbial community composition, diversity and dynamics (Khater *et al*., 2017).16 srRNA gene sequencing is the most relevant method for the identification of the Bacteria.

CHAPTER THREE
MATERIALS AND METHOD

3.1 Materials

All the regents were provided by Central Department of Biotechnology, Tribhuvan University Laboratory. All the reagents were analytical grades unless stated. For MFC operation, Electrodes (Graphite from Nippon co. Japan and Platinum wire from Sigma Co.) and carbon nano tubules (CNT) were used. Nafion Membrane 117 (DuPont, USA) was treated and was used as proton exchange membrane (PEM).

3.1.1 Chemicals

1. Nutrient Broth (Himedia, India Pvt. Ltd)
2. Lurea Bertani Broth (Himedia, India Pvt. Ltd)
3. Potassium Dichromate (Merck Pvt. Ltd)
4. DNS (3,5- Dinitrosalicyclic acid)
5. Nesslers' Reagent (Himedia, India Pvt. Ltd)
6. Silver Sulphate (Siga Co., provided by Central Department of Environment)
7. Mercuric Sulphate (Siga Co., provided by Central Department of Environment)
8. Glucose (Merck Pvt. Ltd)
9. Boric Acid solution (Merck Pvt. Ltd)
10. Antimony potassium tartarate solution (Merck Pvt. Ltd)
11. Ammonium molybdate solution (Merck Pvt. Ltd)
12. Ascorbic Acid (Merck Pvt. Ltd)
13. Agarose (Himedia, India Pvt. Ltd)

3.1.2 Glass wares

1. Glass petri plates
2. Reagent bottles
3. Conical Flask
4. Resistor (provided by physics department)

3.1.3 Equipments

1. Microscope
2. Magnetic Stirrer and magnetic bar
3. Autoclave
4. Vortex

5. Laminar flow
6. Centrifuge (Hettich)
7. Incubator (Mammert)
8. Hot air oven (Mammert)
9. pH meter (Hanna)
10. UV spectrophotometer (Thermo scientific)
11. Gel electrophoresis tank (Cleaver scientific)
12. PCR machine (Biorad)
13. Multimeter (Fluka)

3.2 Methodology

3.2.1 Collection of Sample

The cauliflower waste sample was collected from Balkhu vegetable market and Lagankhel vegetable market. Then, the sample was ground and was stored for further analysis.

3.2.2 Environmental analysis of water

3.2.2.1 Determination of Ammonical nitrogen (Standard method for examination of water) (Pisal, 2010)

Standard curve of Ammonia-Nitrogen

A series of concentration ranging from 0.1mg/L, 0.2mg/L, 0.4mg/L, 0.5mg/L, 0.8mg/L, 1mg/L, 1.6mg/L, 2.0mg/L of Ammoniacal nitrogen working solution was prepared by pipetting suitable volumes of ammonia-nitrogen stock solution. Then 2ml of Nesslers' reagent was added to each of the flasks and mix thoroughly. The solutions were allowed to sit for 20 minutes for color development. Background correction was performed with a blank solution and the absorbance of the solutions was taken at 425 nm using spectrophotometer.

Determination of Ammonia-Nitrogen

First of all 400 ml of distilled water was added to a 1000-ml Kjeldahl flask with previously treated boiling chips to avoid bumping. The distillate was steamed until it showed positive reaction with Nessler reagent and collected. Then again 500 ml of sample (waste) was added in Kjeldahl flask and 25 ml of borate buffer was added. 150 ml of the sample was added into 25 ml of boric acid solution in 250 ml Erlenmeyer flask. The distillate was made up to 250 ml of distilled water. To 50 ml of the diluted distillate 2 ml of Nessler reagent was added and was mixed well. The solutions were allowed to incubate for 20 minutes for color development. Background correction was performed with blank solution followed by sample analyses.

3.2.2.2 Determination of Chemical Oxygen Demand (Standard method for examination of water) (Pisal, 2010)

Standard Curve of Chemical Oxygen Demand

A series of concentration ranging from 20mg/L, 50mg/L, 100mg/L, 200mg/L, 400mg/L, 600mg/L and 900mg/L of phthalate working solution was prepared, by pipetting suitable volumes of the phthalate stock solution (1 g/L) in 50-ml volumetric flasks. Then 10 ml of the reference solutions were added to the culture tubes. After then 6ml of digestion solution was added to each of the references and mix thoroughly. To the tubes, 14 ml of the catalyst solution was added to each of the references, down the side of the culture tube. It was capped tightly and was shaken to mix the layers. The culture tubes were placed in an oven at 150^0C for 2 hours. The tubes were cooled and any precipitate if was allowed to settle. Background correction was performed with the blank solution (without sample) and the absorbance of the solutions was taken at 600 nm using the cuvette.

Determination of Chemical Oxygen Demand

Finally, 10 ml of the sample was taken into a culture tube and the same procedure was followed as in the standard curve preparation and COD was determined. The color change obtained during COD concentration determination is shown in figure (12) in APPENDIX (III).

3.2.2.3 Determination of reducing sugar using DNS method (Miller *et al.*, 1959)

Standard Curve of reducing sugar

Firstly stock solution of glucose (10 mg/L) was prepared. Then prepared solutions of concentrations 0.05mg/L, 0.10mg/L, 0.2mg/L, 0.4mg/L, 0.6mg/L, 0.8mg/L, 1.0mg/L by using distilled water. A 500µl of sample was taken into which 500µl of citrate buffer and 3ml of DNS reagent was added. After the addition of DNS it was kept in boiling water for 10mins. It was allowed to cool and then absorbance was taken at 540nm.

Determination of reducing sugar

500µl of sample was taken and the same procedure was followed along with the preparation of standard curve.

3.2.2.4 Determination of Phosphorous (Temminghoff and Houba, 2004)

Standard Curve of phosphorous

A series of reference solution ranging from 0mg/L, 1mg/L, 2mg/L, 3mg/L, 4mg/L, 5mg/L was prepared by pipetting suitable volumes of the stock solution. The reference

solutions were added to 100 ml volumetric flasks, which already contained 40 ml water. Then, 4.5 ml of concentrated sulphuric acid (96%) was added. Then the mixture was cooled down and the solution was made up to the mark with water. Then the standard series were diluted in 1:9 (v/v) with water. The diluted standard series were pipetted out into the test tubes. Then, 3.8 ml of diluted mixed reagent was added and mixed. Then, the solution was allowed to stand for 10 minutes. Background correction was performed with blank solution and the absorbance was measured at 880 nm using glass cuvette.

Preparation of Digest

0.6g dried plant was taken in 50 ml volumetric flask. Then, 6.6 ml of digestion mixture was added with 4 carborundum beads and was swirled carefully until all plant material was moistened. Then, it was allowed to stand overnight. Two blank solutions (sulphuric acid and salicylic acid) were prepared.

The flask was allowed to heat on a hot plate at 180°C for about 1 hour, while also adding 5 drops of hydrogen peroxide. The treatment was repeated until the digest turned colorless. Then, it was cooled to room temperature with 10 ml of water was added and mixed. Then it was swirled until most of the precipitate had dissolved.

Determination of Phosphorous

Digest was diluted in 1:9 (v/v) with water. Then, the diluted digested sample was pipetted out in the test tubes. 3.8 ml of diluted mixed reagent was added and mixed. Then, the solution was allowed to stand for 10 minutes. Background correction was performed with blank solution and was followed sample absorbance measurements.

3.2.3 Construction of MFC

The MFC set up was done by joining the two plastic bottles each capacity of 1000 ml via a glass tube with the help of a rubber gasket. Nafion 117 was treated and was used as a PEM. The graphite felt was used as anodic material and platinum wire was used as cathodic material. In the cathode compartment 800 ml of phosphate buffer, pH 7.6 was kept whereas in the anode compartment 1% of bacterial culture was kept along with 800 ml of solid waste. The electrode was dipped. The wires arising from the anode and the cathode compartment were connected to the multimeter (FLUKA) and open circuit voltage was observed. The closed circuit voltage was obtained by connecting the external resistance of 1000Ω and 100Ω. In each case power, voltage and current was measured. The MFC was constructed and operated for about 5-7 days. Each day the anodic sample was taken for the determination of removal efficiency of reducing sugar. The initial and final COD, Phosphorous and Nitrogen was determined and compared for

the percentage reduction. The open circuit reading was taken for the optimization of MFC. Afterwards, power, voltage and current were obtained from the closed circuit possessing 1000Ω and 100Ω resistance. Also the normal graphite electrode, acidified CNT treated graphite electrode and acidified CNT treated graphite electrode after MFC operation were subjected to Jawaharlal Nehru University (JNU), New Delhi, India to study the adherence of microbial load at different condition.

Further improvements were done using in the cathode compartment 800ml of phosphate buffer whereas in the anode compartment 800 ml of solid waste was taken inoculating 1% bacterial mix culture. The graphite felt coated with PANI/MWCNT was taken as an electrode. The Nafion117 was used as PEM and external resistance of 1000Ω and 100Ω was used separately to form a closed circuit. Thus, voltage, current and power was obtained with the help of multimeter. In another condition, phosphate buffer was replaced by potassium ferricyanide buffer to see the efficiency of MFC..

3.2.3.1 Synthesis of PANI/MWCNT nanocomposites (Abdulla, 2015)

In-situ oxidative polymerization method was adopted for the synthesis of Polyaniline multiwalled carbon nanotubules (PANI/MWCNT) nanocomposites. A 1mg of C-MWCNTs was mixed in aniline/HCl (1:1) solution at 0°C which leads to the adsorption of monomer on the MWCNT surface. The reaction time was optimized to be 12 hr. A solution of (0.1M) ammonium per sulphate (APS) dissolved in 1M HCl was added drop wise into the mixture. Polymerization process was carried out at 0°C for 6 hr. The PANI/MWCNT nanocomposite was obtained by washing the material several times by repeated centrifugation with deionized water and methanol to obtain a greenish black powder. The material was further dried under vacuum at 60°C for 24 hr.

3.2.3.2 Treatment of Graphite electrode

The surface of graphite felt was modified by sonication using ultrasonication bath containing H_2SO_4 and HNO_3 (3:1, v/v) for 6 hrs. Then graphite felt was washed with distilled water for four times until pH of washing solution reached 7. Then it was air dried before use.

3.2.3.3 Coating of Anode Electrode with carbon nanotube

The treated PANI/MWCNT was mixed with solvent such as dichloromethane, N-methyl - 2 Pyrrolidone (NMP). Clean graphite electrode was dipped in the mixture and sonicated for about 15 minutes. Then, it was dried in an oven for about 12 hours at 50-60°C.

3.2.3.4 Treatment of Proton Exchange Membrane (PEM) (Najafpour et al., 2010)

Proton exchange membrane (PEM; NAFION 117) was used to separate the two compartments. Nafion proton exchange membrane was subjected to a course of pretreatment to take off any impurities that was boiling the film for 1 hour in 3% H_2O_2, washed with deionized water, 0.5 M H_2SO_4 and then washed with deionized water. The anode and cathode compartments were filled by deionized water when the biological fuel cell was not in use to maintain membrane for good conductivity.

3.2.4 Isolation and storage of Bacteria

3.2.4.1 Isolation of microorganism

About 1g of grounded sample was diluted upto 10^{-5} dilution using serial dilution. 0.1 ml from each dilution was spread plated on the agar plate. The plates were sealed with parafilm, labeled and kept in incubator for 24 hrs. The process was repeated until isolated colony was obtained. The isolated colony is shown in figure (13) in APPENDIX (III).

The different colonies of bacteria on the plate were chosen. By using aseptic technique, bacteria were streaked on the agar plates. The streaked plate was sealed and kept in 37^oC incubator for growing. Besides that, from the petriplate, gram staining was done. Samples were observed under microscope and characterized.

Similarly, for the growth of the mix culture of microorganisms, about 10g of waste sample was taken and cultured in 100 ml LB broth. The culture was incubated at 37^oC for 2 weeks for acclimatization. The culture is primarily used in microbial fuel cell for waste treatment.

3.2.4.2 Maintenance of microorganisms

All the organisms were maintained in Nutrient Agar Plate. The organisms were sub-cultured at 15 days interval and were preserved using glycerol stock.

3.2.4.3 Screening for cellulase production

For the screening of organism, in the middle of CMC ager plate hole was made and 100µl inoculum of each isolates were inoculated in each plate and incubated at 28º C for 24 hrs. After incubation the plate were flooded with 0.1% congo red and shaken at 100 rev/min for 15 min. After that the plate was destained with 1N Nacl and shaken at 100rev/min for 15 min. Observation was done for clear zone of hydrolysis and the

promising isolates were further characterized at molecular level. The composition of CMC agar is given in APPENDIX (I).

3.2.5 Molecular Characterization

3.2.5.1 Extraction of Genomic DNA from Bacteria (Nishiguchi et al., 2010)

For extraction of DNA from liquid culture, 1-3 ml of culture was kept in an eppendorf tube and centrifuged at 13000 rpm for about 2 minutes and the supernatant was removed. A 567µl of TE buffer was added to the pelleted cells. The pellet was resuspended by repeated pipetting or by gently vortexing so that the cells become resuspended. A 30µl of 10% SDS and 3µl of 20mg/ml solution of proteinase k was added, mixed and incubated for 1 hour at 37°C. After incubation, 100µl of 5M NaCl was added and mixed. Afterwards, 80µl of CTAB/NaCl solution (0.7M NaCl, 10% CTAB) was added. This solution was incubated at 65°C for 10 minutes. After incubation, equal volume of chloroform:isoamyl alcohol (24:1) was added and mixed. Centrifugation was done for 5 minutes and the aqueous solution was transferred to the new tube. Again, centrifugation was done at 14000 rpm for 5 minutes and transferred the supernatant to the new tube. Again, 0.6µl of isopropanol was added and mixed gently until the DNA precipitates. Then centrifugation was done to remove the isopropanol and 1 ml of 70% ethanol was added to wash the salt away from DNA. Centrifugation was done to discard ethanol, drying and the pellet was resuspended in 50µl of TE buffer and kept at 4°C. The obtained g-DNA was undergone through gel electrophoresis and visualized under UV-transilluminator.

3.2.5.2 Polymerase chain reaction

After the genomic DNA was extracted, PCR was performed. The PCR reaction mixture and thermocyclic condition of PCR is given in table (3) and (4) respectively.

Table 3: PCR Reaction Mixture

Reagents	Amount(µl)
Master Mix	5
Forward primer (10pm)	1
Reverse primer (10pm)	1
Template (100ng)	1
Nuclease Free Water	2
Total	10

Table 4: Thermocyclic conditions of PCR

Stage	Cycle	Step		Temperature (^oc)	Time
1	1	1	Enzyme activation	95	2 min
2	35	1	Denaturation	95	30 sec
		2	Annealing	52	30 sec
		3	Extension	72	2-5 min
3	1	1	Final Extension	72	5 min
		2	Hold	4	Hold

The PCR product after amplification was subjected to Xceleris Laboratory, Ahmedabad, India for sequencing which was done by Sanger Sequencing method ie Illumina sequencing. The sequences obtained were identified through BLAST search at NCBI and sequences were aligned with help of Bioedit software and finally the sequences were deposited in Genbank database and accession number was received.
Similarly, for evolutionary relationship Phylogenetic tree were constructed by Neighbor-joining method using MEGA software.

CHAPTER FOUR
RESULT AND DISCUSSION

The standard curve of absorbance against ammoniacal-nitrogen concentration drawn to determine the concentration of ammoniacal-nitrogen present in the waste. Similarly, the standard curves for COD, soluble reducing sugar and phosphorus were plotted to determine the concentration [APPENDIX (II)].

4.1 Determination of Environmental parameters of Cauliflower waste

Cauliflower waste contained variable amounts of ammoniacal-nitrogen, Chemical Oxygen Demand (COD), reducing sugar, phosphorus which is shown in table (5). It had COD reading (143.48 ± 5.60 mg/g). For phosphorous, our data showed 67.82 mg/g. The result was different with (Baloch et al., 2015), according to whom, phosphorous content was 61.35 mg/100g. In literature (Baloch et al., 2015), sample was different (edible portion of cauliflower) and atomic absorption spectrophotometer process was used. The soluble reducing sugar was less i.e. (0.53 mg/g). The waste contained less soluble reducing sugar because it is rich in insoluble lignocellulosic biomass (Joshi *et al*, 2011; Rasmussen *et al*., 2017). For Ammoniacal Nitrogen, our study showed (0.90 ± 0.4 mg/g), which was near value to research done by (Chang *et al*., 2009). According to the literature (Chang *et al*., 2009), Nitrogen content was (0.7 ± 0.03), where the sample was *Brassica* waste compost.

Table 5: Different parameters in cauliflower waste sample

Analytical parameters of cauliflower waste	Concentration(mg/g)
Reducing sugar (soluble)	0.530
Chemical Oxygen Demand (COD)	143.48 ± 5.60
Phosphorous	67.82
Ammoniacal Nitrogen	0.90 ± 0.4

The data in Table 5 was the initial concentration of fresh cauliflower leaves before microbial fuel cell (MFC) treatment. This process was carried out in order to compare the data with final concentration of cauliflower waste after MFC treatment under various conditions. Then, removal efficiency (COD, Nitrogen, Phosphorous and soluble

reducing sugar) was calculated in percentage by taking difference in final and initial concentration.

4.2 Optimization of substrate concentration in MFC operation

Figure (3) showed the time versus open circuit voltage curve obtained when different concentrations of substrate were used in MFC operation. The data showed that 10% of substrate was best for operation with highest open circuit voltage (OCV) of 420 mV after 48hr of operation. Ghoreyshi et al., 2011 studied on the effect of type and concentration of substrate on the MFC performance. They took glucose as substrate over concentration range of 2-20 g/L. The data in the literature (Ghoreyshi *et al*., 2011) could be compared with our data. As the substrate concentration increased, the OCV value was minimal. This might be because of most of substrate must have remained unconsumed at high concentrations. Also, in lower substrate concentration i.e. 5%, the OCV value was minimum due to limitation of substrate. Higher concentration of substrate adversely effects MFC operation (Gurung *et al*, 2012). The 10% of waste biomass as substrate was taken for all MFC operation.

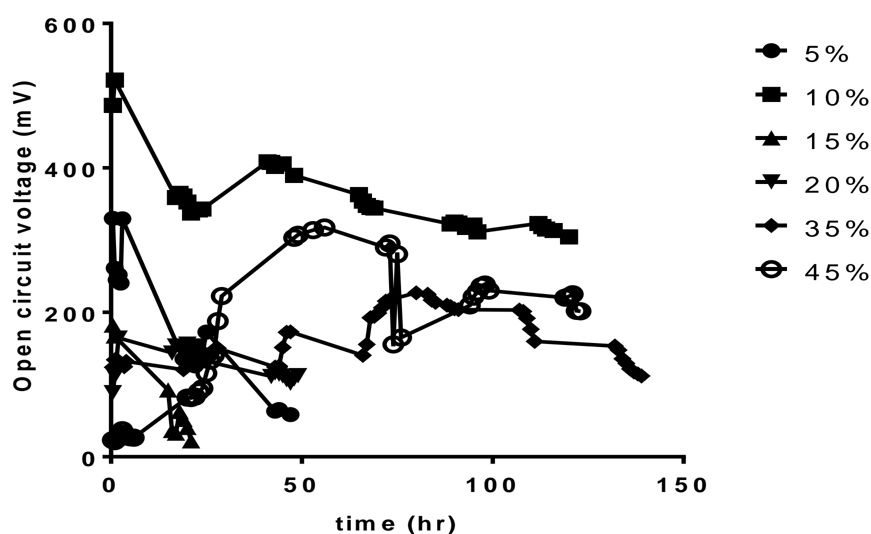


Figure 3: Time versus open circuit voltage curve with different concentration of substrates

4.3 Optimization of electrode in MFC operation

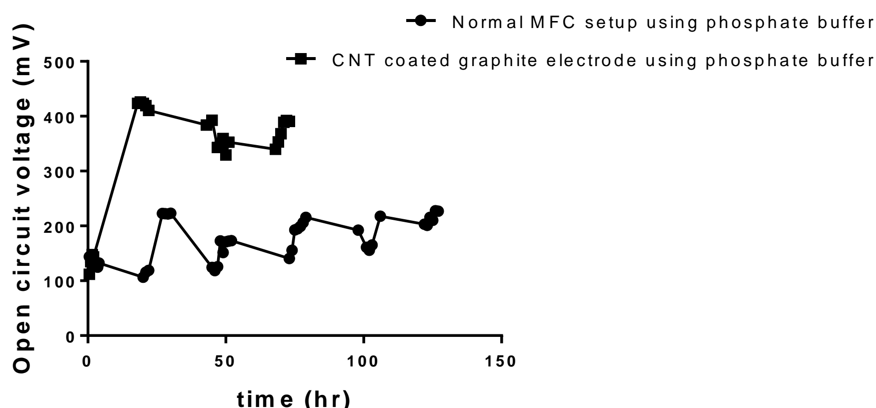


Figure 4: Time versus open circuit voltage of normal and coated electrode using phosphate buffer

Figure 4 showed time versus open circuit voltage reading of normal and coated electrode using phosphate buffer. This process in figure 4 was an attempt made on electrode modification i.e. anode treatment in order to produce improved output depending upon the type of modification. For this process, the comparative study was done using carbon nanotubes (CNT) coated electrode (modified graphite felt) and normal graphite electrode (not coated) using phosphate buffer in both cases. From the experiment, we could conclude that the use of carbon nanotubes showed improvement in open circuit voltage (OCV) reading i.e. 426 mV than normal buffer i.e. 200 mV. The result thus obtained might be due to changes in physical and chemical properties (after modification) in an electrode might have enhanced the surface area for the microbial attachments and electron transfer. Therefore, anode modification was done for dual objectives of increasing active surface area for bacterial attachment and improving electrical conductivity of surface (Scott *et al.*, 2007). Thus, modification of the anode materials to increase affinity of the bio-films proved to be an efficient way to enhance the performance of the MFCs (Yang et al., 2016). A recent study showed that polymer implied as the molecular wire connecting the bio-film to the anode increased the power density from 1,479 mW/m^2 to 2,355 mW/m^2 (Yuan *et al.*, 2016).

4.4 Power generation in MFC using different resistors

Figure (4) is the curve showing power generation in MFC during operation with 1000Ω and 100Ω external resistances. This experiment was done to observe effect of external resistance on the overall performance of microbial fuel cell. External resistance acts as an integral part of an electrical grid that controls the output of fuel cells (Risamani *et al.,*

2011). The data of two external resistance viz 100 Ω and 1000 Ω were compared to study optimal conditions for power production. A steady state power of 7.2 W/m^3 was observed when MFC was operated after 48hrs with 1000Ω external resistance. At 1000 Ω, the steady reading was observed. This might be due to increase in external resistance might have lowered internal resistance that overall improved the rate of reaction. The optimal external resistance usually correlated with the internal resistance of MFCs. Internal resistance is not a system constant, and depends on the external load applied to the MFC (Manohar *et al.*, 2008). A slight increase in internal resistance can dramatically decrease microbial fuel cell performance (He *et al.*, 2005). External resistance could also relate to anode potential. Du *et al.*, 2015, studied variations of the anode potential with external resistances. Changing in external resistance showed the highest anode potential. When the anode potential is higher, more free energy can be obtained, which could enhance the startup of electricity generation (Goud *et al.*, 2011).

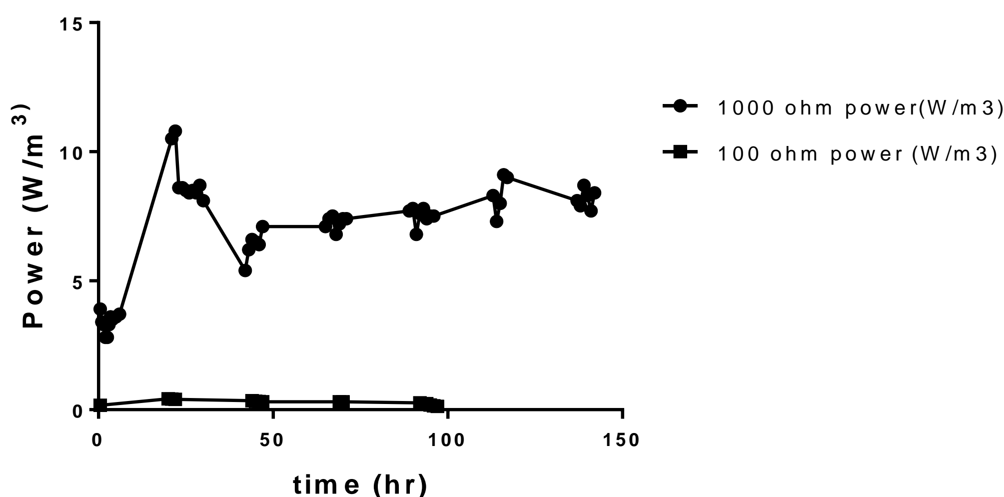


Figure 5: Power generation in MFC using CNT treated acidic electrode, with external resistance of 1000Ω and 100Ω

Power Density was calculated based on the anodic volume liquid in order to better reflect three-dimensional properties of both electrodes and MFC reactors. Power Density was introduced in the unit watts per cubic meter (W/m^3) (Rabaey *et al.*, 2005).

4.5 Analysis of different components reduction during MFC operation

Table (6) showed the degradation of waste biomass and reduction in COD, ammoniacal-nitrogen, phosphorous, reducing sugar values after MFC operation at eight day time interval. Acidified MWCNT coating in electrode was found to be effective for waste removal as there was COD reduction by 13.52% where as in normal system COD

reduction was only 10.75%. The data shown by (Chaulagain, 2016) also revealed that CNT enhanced electrical performances. CNT actually have shown to be promising alternative material for MFC electrode because of their electrical conductivity, chemical stability, biocompatibility and high specific area (Mustakeem, 2015). The 1000Ω resistor found to reduce more COD than 100Ω. There was huge reduction in ammoniacal-nitrogen by 76.9% as given in table(7), phosphorus by 22.52% given in table(8) and soluble reducing sugar by 53.40% given in table(9) when acidified CNT was used as electrode with 1000Ω resistor.

Table 6: Removal of COD with different modes of operation of MFC

Samples	Electrode coating	Buffer	Initial concentration (mg/g)	Final concentration (mg/g)	% Reduction
Mix culture strains (Graphite electrode)	Normal graphite electrode	Phosphate buffer	143.48	128.06	10.75
	Acidic/CNT with coating	Phosphate buffer	143.48	124.07	13.52
	Acidic/CNT with resistor(1000 Ω)	Phosphate buffer	143.48	108	24.73
	Acidic/CNT (with resistor-100Ω)	Phosphate buffer	143.48	121.91	15.03

Table 7: Removal of Ammonia-Nitrogen with MFC operation

Samples	Initial concentration (mg/g)	Final concentration (mg/g)	% Reduction
Mix culture strains (acidic CNT treated, phosphate as buffer with 1000 Ω resistor)	0.90 ± 0.4	0.208	76.9

Table 8: Removal of Phosphorous with MFC operation

Samples	Initial concentration (mg/g)	Final concentration (mg/g)	% Reduction
Mix culture strains (acidic CNT treated, phosphate as buffer with 1000Ω resistor	67.82	52.55	22.52

Table 9: Removal of reducing sugar with MFC operation

Sample	Electrode coating	Buffer	Initial concentration (mg/g)	Final concentration (mg/g)	% Reduction
Mix culture strains	no coating	Phosphate buffer	0.530	0.225	57.55
	CNT treatment with resistor (1000Ω)	Phosphate buffer	0.530	0.247	53.40

4.6 Effect of ferricyanide in MFC operation

The MFC was operated by adding 1.0% potassium ferricyanide ($K_3[Fe(CN)_6]$) in phosphate buffer and used as cathodic solution to see the effect of ferricyanide in MFC. The comparison between the OCV obtained in case of normal MFC set-up (with phosphate buffer only) and the set-up with addition of ferricyanide in phosphate buffer is shown in Figure (5) below.

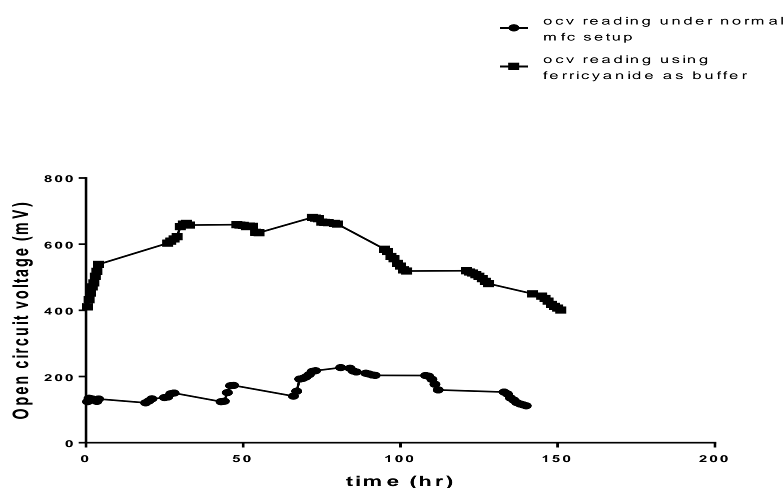


Figure 6: Time versus open circuit voltage curve with variation in buffer contents

Addition of ferricyanide found to enhance MFC operation. The performance of potassium ferricyanide was the best with maximum OCV of 681mV where as normal MFC set-up with phosphate buffer only showed OCV of 227mV. The performance of the mediator (ferricyanide) was probably due to the fact that ferri/ferrocyanide $[Fe(CN)_6^{3-}]$ is highly diffusible and can be easily reduced to its ferrous counterpart by the well-defined reversible reaction simultaneous to an increase in the redox potential of the solution (Parkash *et al.*, 2015). (Adebule *et al.*, 2018) studied on evaluating the potentials of different exogenous compounds to enhance bioelectricity generation by MFC using kitchen waste as substrate.

4.7 Scanning electron microscopy of graphite electrodes during MFC operation

Scanning electron microscopy (SEM) was done to observe the surface and morphological information and to see the behavior of mix culture of microbes to adhere in graphite electrode surface. The pictures were observed as shown in figure (7- A, B and C). There was adherence of huge amount of microbes in electrode which could enhance MFC efficiency. Mohamoud, M (2014) in his study on redox enhancement and electrochemical pseudo-capacitance performance of polyaniline/poly (vinyl alcohol) (PAn/PVA) composite films concluded that formation of film in electrode enhances electron transport. SEM image of graphite showed uniform surface initially, where after CNT treatment and MFC operation thick layer bacterial adhesion was observed in approximately 261 μm^2 (measured from ImageJ software). SEM image of CNT treated graphite electrode after MFC operation showed that efficient bacterial adhesion, so that it showed good MFC operation with lower resistance.

Figure 7: SEM images of graphite electrode. A: Normal graphite electrode, B: Acidified CNT treated graphite electrode and C: Acidified CNT treated graphite electrode after MFC operation.

4.8 Screening for cellulose degrading microorganisms

From the mix culture of microbes in media plate, seven different colonies were selected. Among seven different isolated colonies screened for cellulase production, the colonies labeled R2, R3 and R4 showed clear halozone on CMC agar plate. The photograph of halozone formation for screening of cellulase production for three different isolates R2, R3 and R4 is given in figure (8) below.

Figure 8: Halozone formation in CMC agar plate by isolates R2, R3 and R4.

Presence of halozone indicates that potent isolates could produce cellulase enzyme with optimal cellulolytic activities, which might have some active protein molecules that increase the potential level for cellulose degradation. Our study obtained bacterial isolates R2, R3 and R4 from the cauliflower waste, which have a great efficacy to convert organic waste into simple molecules due to the presence of cellulase enzyme. Lu *et al*. (2006) reported that the mesophilic cellulose degrading bacteria obtained from vegetable waste showed hydrolytic capacity. Cellulose degrading organisms help in bioelectricity production by using cellulose as substrate. In cauliflower, it contains 16.6% cellulose (Khedkar et al., 2017).

4.9 Characterization of microbes

4.9.1 Colony morphology

The isolated bacterial colony were subjected to observation of their morphological characteristics in which its shape, size, color, consistency, margin and elevation was noted. The observed bacteria was circular shaped, small sized, creamy white, sticky, uniform margined with convexed elevation.

4.9.2 Microscopic structure of isolated colony

Under microscopic observation (100x) by Gram staining technique the rod shaped, purple colored bacteria was observed. This indicated that the isolated bacteria were Gram positive. The bacterium as observed under microscope is shown in figure (9) below.

Figure 9: Gram stained bacteria under microscope (100x)

4.9.3 Molecular characterization of microbes in MFC

Among all of the different putative bacterial strains, 3 bacterial strains were selected based on the basis of morphology, and its characteristic (cellulose degradation). The genomic DNA was extracted from CTAB (Cetyl Trimethyl Ammonium Bromide) method. Distinct band of DNA was observed in 3 different putative strains of bacterial species. The concentration of genomic DNA was found to be approximately ~100ng by nanometer reader and was used as template for PCR (polymerase chain reaction). Then PCR products showed visible distinct band of size 1500bp in uv transilluminator and then products were subjected to Sanger sequencing. The different strain of bacteria was characterized by 16S rRNA gene sequence analysis. After sequencing, chromatogram files were obtained through Chromas software which was shown in Appendix III. The chromatogram file showed different color peaks. The evenly spaced peaks, each with

only one color determines the quality of sequences. The sequence was submitted to NCBI andis available for public under following accession number.
R4 Bacteria : SUB6956271 R4 MT040749

The identity of the bacterial species were determined by comparing the sequences obtained with the gene sequences available in the Genbank database using Basic Local Alignment Search Tool (BLAST) software at NCBI site. While doing the sequence similarity search with BLAST it showed the maximum similarity with *Bacillus* species of different strain. Phylogenetic tree for the sequenced strains R2, R3, R4 and closely related species extracted from NCBI was carried out to elucidate the evolutionary relationship of bacteria. The tree was constructed using Neighbor Joining method using MEGA software. From the tree diagram of R4, R3, R2 bacteria with different bacterial species it showed that it is closely related with *Bacillus* bacteria. *Bacillus subtilis* is one of the microorganisms which are highly enriched in various environment having degradation capabilities of different wastes producing enzymes like protease, lipase, amylase, catalase etc (Fergus, 1977).

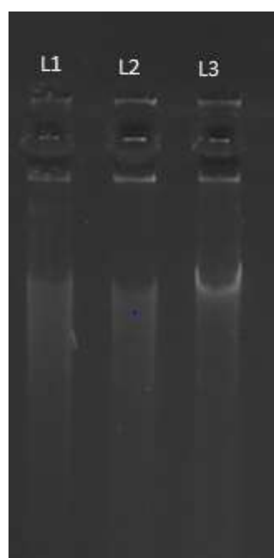


Figure 10: Gel electrophoresis in 0.8% agar of Bacterial genomic DNA extraction using CTAB method. L1: R2 L2:R3 and L3: R4

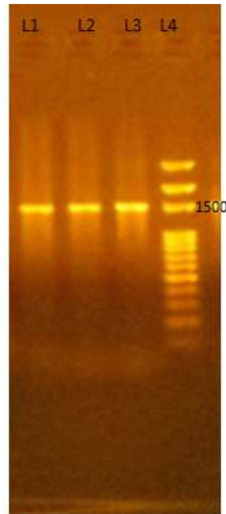


Figure 11: Gel electrophoresis of Molecular amplification of 16srRNA gene of bacterial DNA using universal primer (1500bp) in 1% agarose gel. L1: R2 bacteria L2: R3 bacteria L3: R4 bacteria L4: ladder 100bp

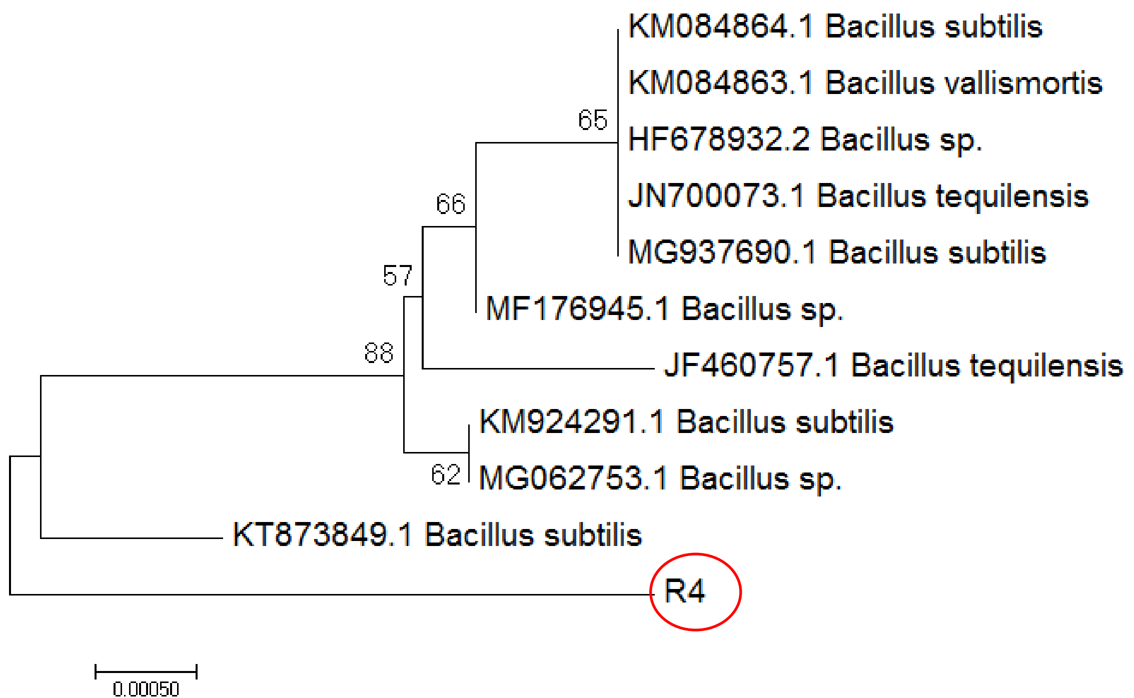


Figure 12: Phylogenetic tree of R4 microbe obtained by Neighbor joining method using MEGA software

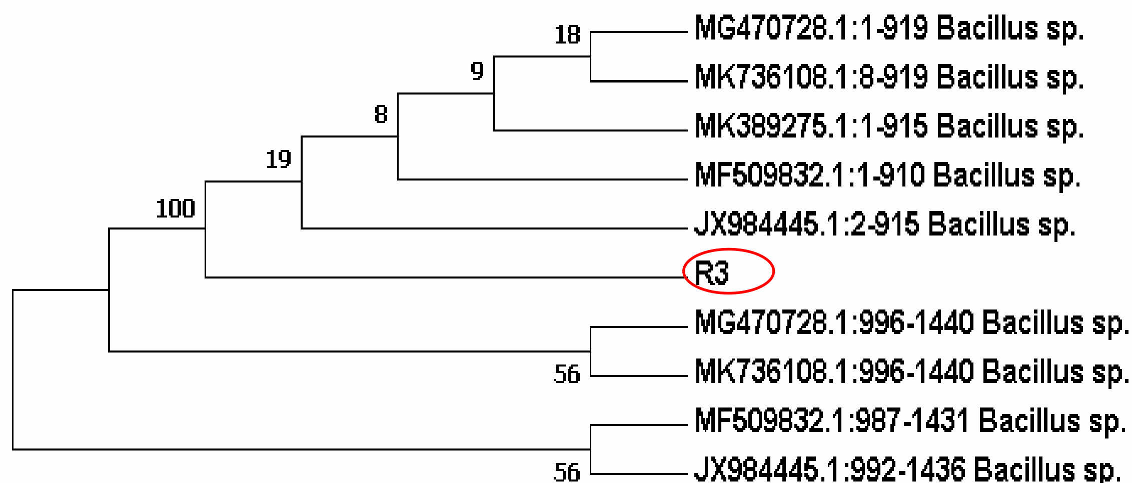


Figure 13: Phylogenetic tree of R3 microbe obtained by Neighbor joining method busing MEGA software

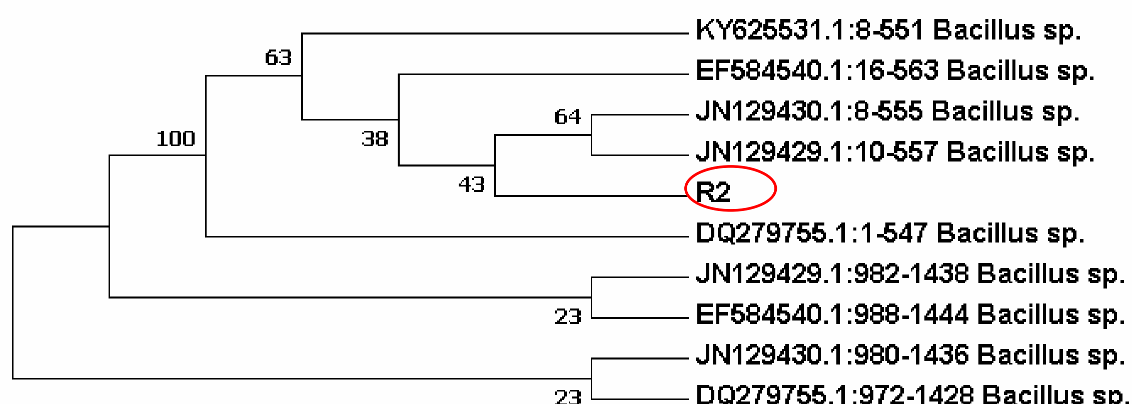


Figure 14: Phylogenetic tree of R2 microbe obtained by Neighbor joining method using MEGA software

For a MFC to operate efficiently, the microbial strain should be electro active and able to convert a chemical energy into an electrical energy and to transfer electrons direct, indirect or mediated electron transfer pathways (Cao, *et al*., 2019). In this MFC set up, community of bacteria were sampled from a real environment of cauliflower waste. From the pure culture isolation to molecular characterization, the microbial strains were found to be different strains of *Bacillus* spp. The phylogenetic tree for bacterial strain R4, R3 and R2 were shown in figure 12, 13 and 14 respectively. All the strains R4, R3 and R2 were found to be closely related with *Bacillus* spp. Logan *et al*., 2019 proposed that *Bacillus spp.* are classified as weak exoelectrogens and typically produce quite low

current densities in pure cultures. Production of low current densities is associated with unique roles in biofilm microbial ecology (Doyle *et al*., 2018).

The weak electroactivity of *Bacillus* has been repeatedly reported. *Bacillus thuringiensis* DRR-1 from cow rumen, produced a small potential and current, when cultivated in an MFC (Jothinathan and Wilson, 2017). *Bacillus cereus* was also cultivated on an MFC anode and produced a high current output (Islam *et al*., 2017). *Bacilus subtilis* in an MFC with glucose as carbon and energy source and 2, 4-dichlorophenol as pollutant produced a significant current output (Hassan *et al*., 2016). Such studies demonstrate that *Bacillus spp.* can perform extracellular electron transfer in an MFC. Also, *Bacillus spp.* can produce cell free bioactive compounds that make them capable of complete cellulose hydrolysis invitro (Li *et al*., 2009).

CHAPTER FIVE
SUMMARY

The MFC is a renewable and sustainable technology that produces electrical energy using microorganisms as a biocatalyst to oxidize the biodegradable substrates. The fact that bacteria can oxidize the substrates to produce electricity makes MFCs an ideal solution for waste treatment and energy production.

Initially, treatment of fresh cauliflower leaf waste before MFC operation contained COD (143.48±5.6 mg/g), ammoniacal-nitrogen (0.90±0.4 mg/gm), phosphorous (67.82mg/g) and soluble reducing sugar (0.530 mg/g).

The results revealed the feasibility of bioelectricity generation and waste treatment using two chambered MFC with graphite felt as an electrode and cauliflower leaf waste as substrate. After eight days treatment in MFC, the concentration of COD reduced from 143.48±5.6 mg/g by 24.73%. Similarly phosphorous, soluble reducing sugar and ammoniacal-nitrogen with initial concentration 67.82mg/g, 0.53 mg/g and 0.90±0.4 mg/gm has been reduced by 22.52%, 53.40% and 76.9% respectively.

During comparative study of MFC operation, the use of ferricyanide in phosphate buffer showed increase in voltage reading i.e. 681mV than phosphate buffer only 227mV. Further, a power of 7.2 W/m^3 was observed when MFC was operated with 1000Ω external resistance. This indicates a positive approach in treatment of cauliflower leaf waste as well as in potent power production which aid in solving the daunting problems of household waste management.

From the screening of seven different isolated colonies for potential cellulase producers, three isolates labeled as R2, R3 and R4 were able to degrade cellulose.

Scanning Electron Microscopy shows the adherence of microbes in graphite electrode thus helping the transfer of electron efficiently. The microbes when characterized by 16S rRNA sequence analysis revealed all the three microbes resembling as *Bacillus spp.*

CHAPTER SIX
CONCLUSION

Degradation of the cauliflower waste in microbial fuel cell can generate electricity with the observable reduction of Chemical Oxygen Demand (COD). In this study, COD, ammoniacal-nitrogen, phosphorus and soluble reducing sugar were decreased after treatment of cauliflower leaf waste in microbial fuel cell (MFC) for eight days. Open circuit voltage (OCV) data showed that the addition of ferricyanide in phosphate buffer solution at cathodic chamber enhanced electricity generation in the course of MFC operation. MWCNT coating on graphite electrode found to enhance the power output in MFC. Scanning Electron Microscopy showed microbial adherence in graphite electrode. Molecular characterization confirmed that the isolated colonies were *Bacillus sps*. Hence, the chemical energy in cauliflower leaf waste can be converted into electrical energy using MFC as an alternative technology which can eventually aid in managing waste to worth in context to landlocked country like Nepal.

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APPENDICES

Appendix I: Composition and Preparation of different Microbiological Culture Media and Reagents.

A. Culture Media

Nutrient Agar (NA)

Peptic digest of animal tissue	5.0g
Beef extract	1.5g
Yeast Extract	1.5g
Sodium chloride	5.0g
Agar	15.0g
Distilled water	1L

Carboxy methy cellulase agar

Ammonium hydrogen phosphate	1g
Potassium chloride	0.2g
Magnesium sulphate	1g
Yeast Extract	1g
Carboxymethyl cellulose	26g
Agar	15g
Distilled water	1L

Luria Burtani broth (LB- broth)

Yeast Extract	5.0 g
Sodium chloride	10.0 g
Enzyme casein hydrolase	10.0g
Distilled water	1L

Nutrient Broth (NB)

Peptic digest of animal tissue	5.0g
Sodium chloride	5.0g
Beef extract	1.5g
Yeast Extract	1.5g
Final pH at 25˚C	7.4±0.2

B .Reagents

1. DNS reagent: 1g of 3, 5-Dinitrosalicylic acid was dissolved in 20 ml of 2N NaOH at room temperature and then 50ml of D/W was added. To this, 30g of Rochelle salt (sodium potassium tartarate) was added and volume of 100 ml was made with D/W.

2. Phosphate buffer: 8.02g of KH_2PO_4 and 7.0368g of K_2HPO_4 was dissolved in 1000 ml of distilled water to make 0.1M solution of pH 7.6.

3. Sodium tetraborate solution 0.025mol/l: 5g of anhydrous sodium tetraborate in was dissolved in 1000ml distilled water.

4. Digestion solution: 10.2g of potassium dichromate, 167ml of conc. sulfuric acid and

33.3g of mercury sulfate was dissolved in 500ml distilled water and diluted to 1000ml with distilled water.

5. Catalyst solution: 22g of silver sulfate was added to 4.09 kg bottle of concentrated sulfuric acid and let it stand for 2 days until dissolved.

6. Borate buffer: 88ml of 0.1mol/ltr of sodium hydroxide solution was added to 500ml of 0.025mol/ltr of sodium tetra borate solution and diluted the mark to 1000ml with distilled water.

7. Boric acid solution: 20g of boric acid was dissolved in distilled water and made the mark upto 1000ml with distilled water.

8. Potassium hydrogen pthalate stock solution: 0.850g of potassium hydrogen phthalate was dissolved in 800ml of distilled water and diluted the mark upto 1000ml in volumetric flask.

9. Nessler reagent: Dissolve 100g mercuric iodide and 70g of potassium iodide in small amount of water. Add this mixture to a cooled solution of 160g NaoH in 500 ml of distilled water and dilute up to mark with distilled water in a 1000 ml volumetric flask.

10. Ascorbic Acid solution: Dissolve 1.76g ascorbic acid, $C_6H_8O_6$, in 100ml of ultrapure water and mix .Prepare freshly daily.

11. Ammonium molybdate solution: Dissolve 40g ammonium molybdate tetrahydrate, $(NH_4)_6Mo_7O_{24}.4H_2O$, in ultrapure water and make up into 1000ml. This solution should be stored in a bottle made of hard glass.

12. Potassium Antimoyl Tartrate Solution: Dissolve 0.274g potassium antimoyl tartrate, $KSbOC_4H_4O_6.5H_2O$, in ultrapure water and make up to 100 ml with ultrapure water.

13. Sulphuric Acid solution 2.5 ml/L : Dilute carefully , in portion, 140 ml concentrated Sulphuric acid (96 %) in about 500ml ultrapure water in a 1000ml volumetric flask. Allow the mixture to cool off and makes up to volume with ultrapure water.

14. Mixed reagent for phosphorus: Add successively with a graduated cylinder and mix after each solution: 50ml sulphuric acid(6.5), 15ml ammonium molybdate solution (6.3), 30ml ascorbic acid solution(6.2) and 5ml potassium antimoyl tartrate solution (6.4). Prepare fresh daily.

15. Digestion solution for Phosphorus: Mix 18ml of ultra-pure water and 100ml 0f 96% sulphuric acid. Then dissolve 6g of salicylic acid powder in the mixture.

APPENDIX II Standard Curve

1. Standard Curves of Ammonical Nitrogen

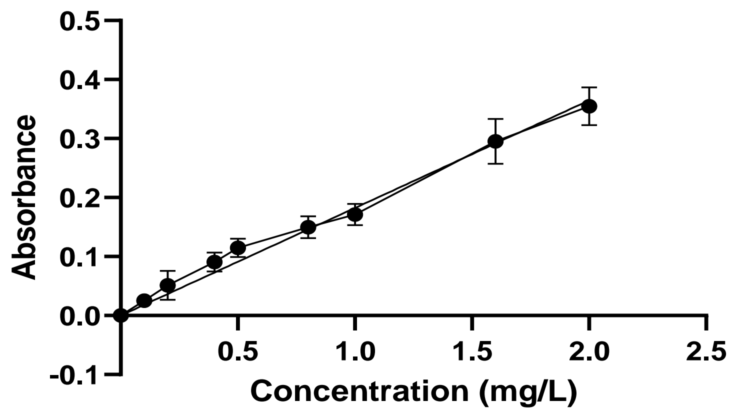

Figure: Standard curve of Ammonical Nitrogen (y= 0.1823*x + 0.000)

2. Standard curve of Chemical Oxidation Demand

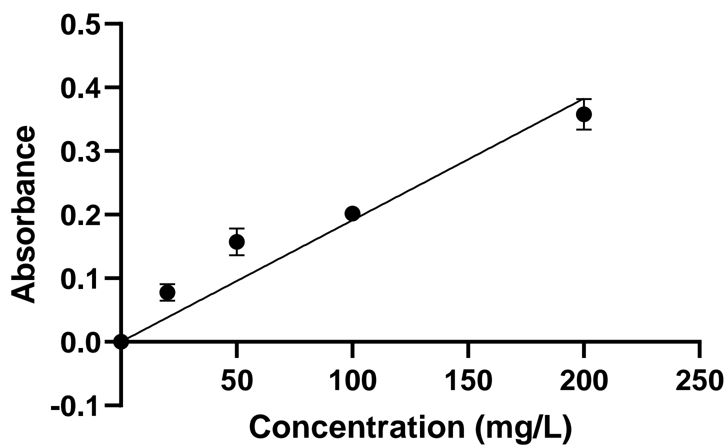

Figure: Standard curve of chemical oxygen demand (y = 0.001912*x + 0.000)

3. **Standard curve of reducing sugar**

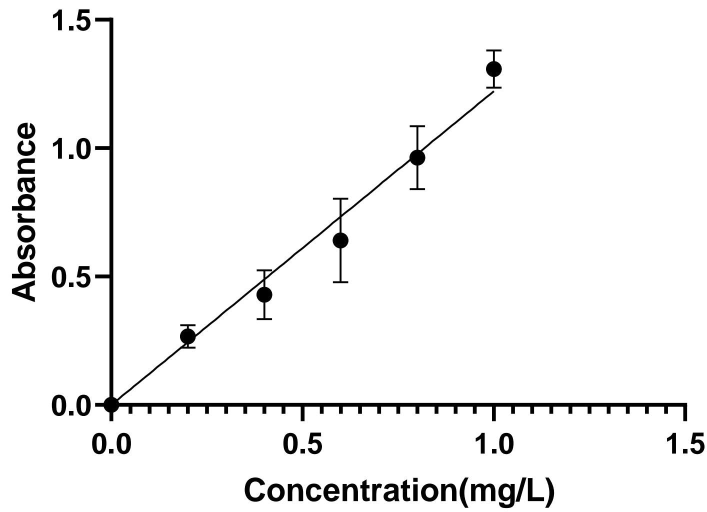

Figure: Standard curve of reducing sugar (y = 1.285*x + 0.00)

4. **Standard curve of Phosphorous**

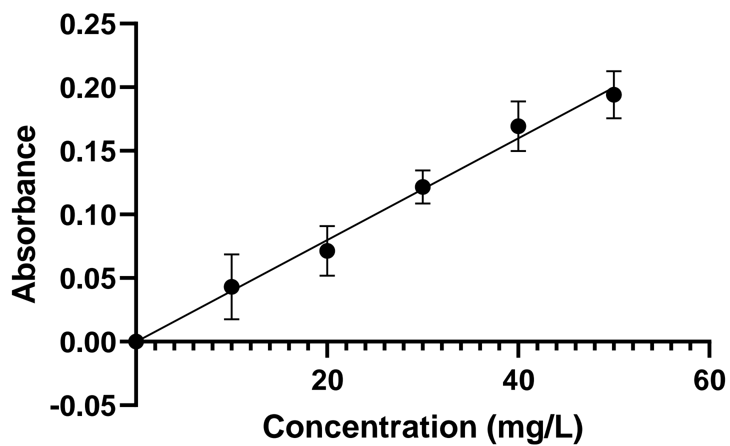

Figure: Standard curve of Phosphorous (y = 0.003996*x + 0.000)

APPENDIX III: Photographs of Laboratory work

Digested waste during COD determination

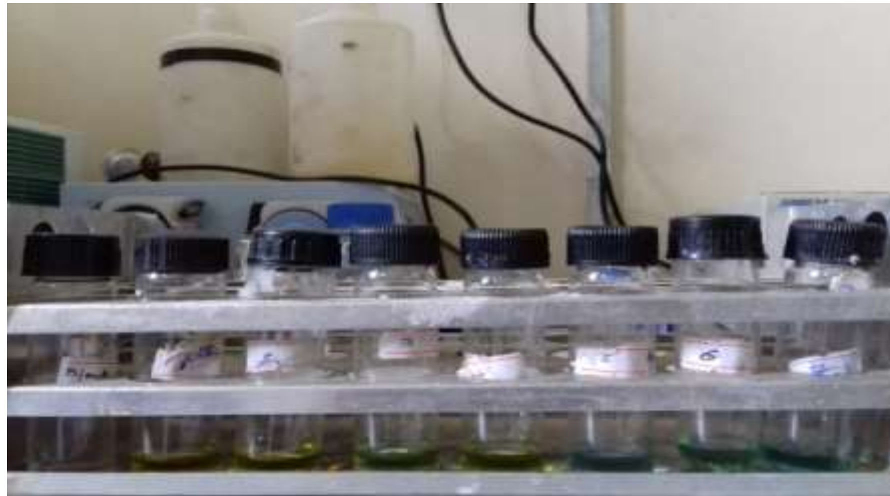

Figure: color observed of digested waste during COD determination

MFC setup

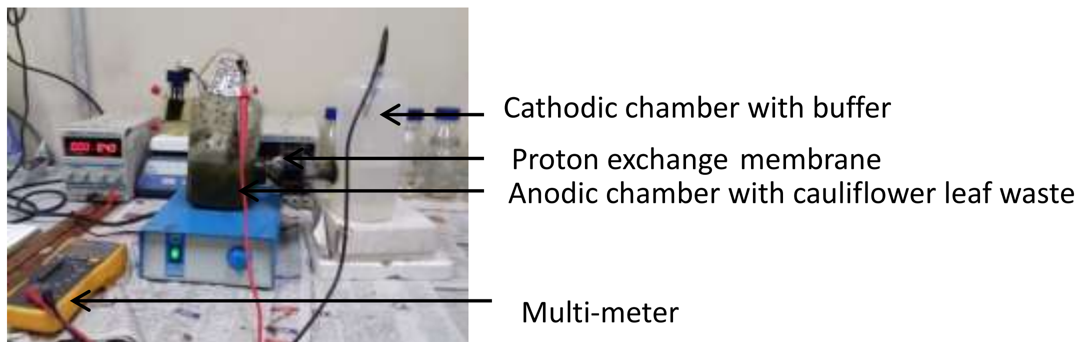

Figure: Dual-chambered MFC setup at CDBT (Biofuel lab)

Isolated bacterial colonies

Figure: An isolated bacterial colonies from cauliflower waste in nutrient agar (R4)

APPENDIX IV: Chromatogram file of 16SrRNA sequencing

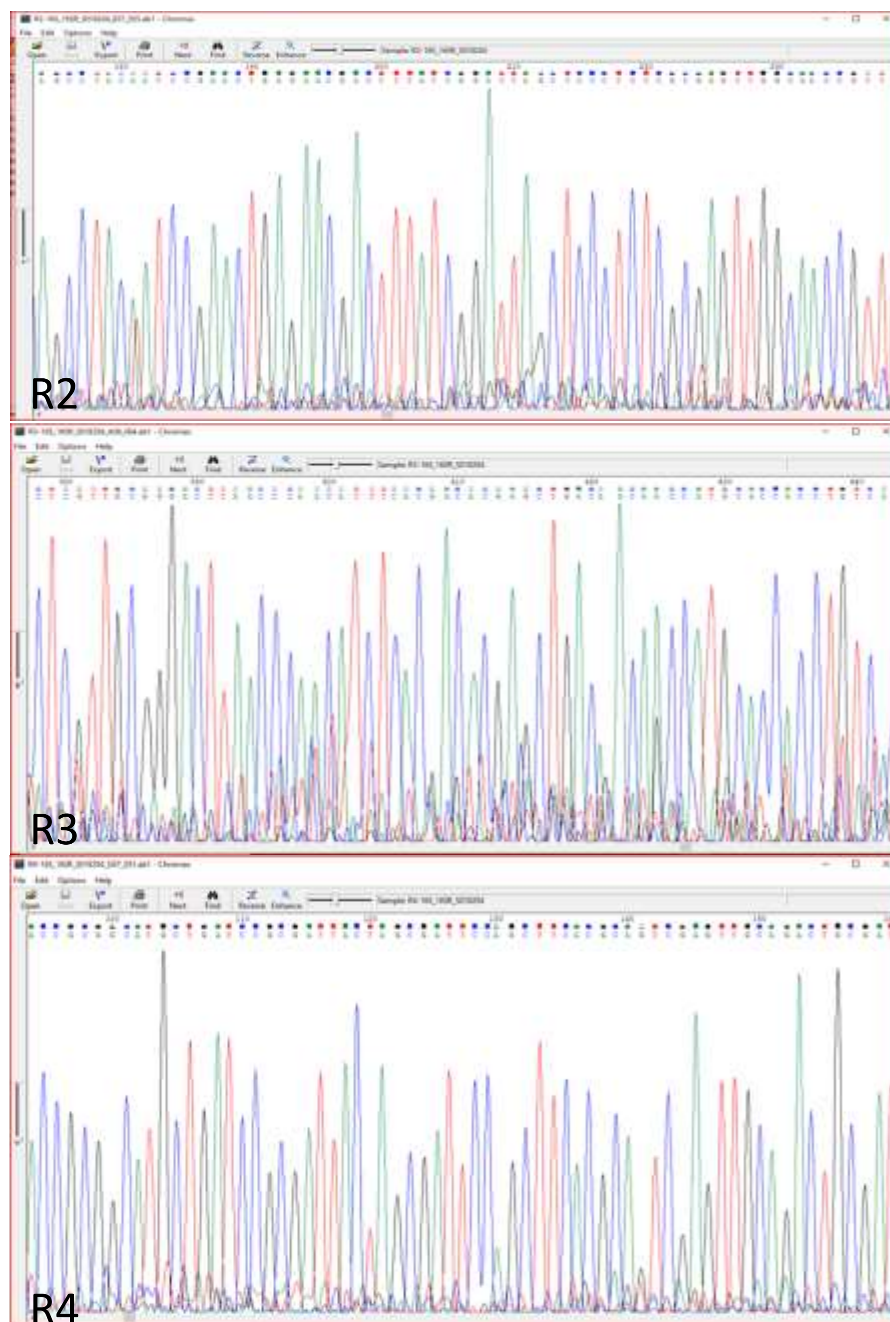

Figure: Chromatogram file of Bacteria R2, R3 and R4 16srRNA gene using Chromas software

APPENDIX V: BLAST result

Sequences producing significant alignments:

Select: All None Selected 0

Alignments

Description	Max Score	Total Score	Query Cover	E value	Per Ident	Accession
Bacillus subtilis strain IJ_19 16S ribosomal RNA gene, partial sequence	2206	2286	97%	0.0	92.31%	[illegible]
Bacillus subtilis strain IJ_15 16S ribosomal RNA gene, partial sequence	2205	2286	97%	0.0	92.31%	[illegible]
Bacillus subtilis strain YNA13 16S ribosomal RNA gene, complete sequence	2204	2284	97%	0.0	92.30%	[illegible]
Bacillus subtilis strain 1776 16S ribosomal RNA gene, partial sequence	2204	2284	97%	0.0	92.30%	[illegible]
Bacillus sp. (in: Bacteria) strain K35mCTM4 16S ribosomal RNA gene, partial sequence	2203	2283	97%	0.0	92.23%	[illegible]
Bacillus subtilis 16S ribosomal RNA gene, partial sequence	2203	2283	97%	0.0	92.34%	[illegible]
Bacillus subtilis strain 1245 16S ribosomal RNA gene, partial sequence	2201	2282	97%	0.0	92.23%	[illegible]
Bacterium L3 16S ribosomal RNA gene, partial sequence	2199	2280	97%	0.0	92.22%	[illegible]
Bacillus subtilis strain DYU1 16S ribosomal RNA gene, partial sequence	2199	2280	97%	0.0	92.23%	[illegible]
Bacillus subtilis strain CICC10153 16S ribosomal RNA gene, partial sequence	2199	2280	97%	0.0	92.23%	[illegible]
Bacillus subtilis strain HD-1 16S ribosomal RNA gene, partial sequence	2198	2279	97%	0.0	92.17%	[illegible]

Figure: BLAST result of R4 strain which showed 97% similarity with *Bacillus subtilis*

Sequences producing significant alignments:

Select: All None Selected 0

Alignments

Description	Max Score	Total Score	Query Cover	E value	Per Ident	Accession
Lysinibacillus sphaericus strain DSM 28 chromosome, complete genome	962	21084	96%	0.0	98.91%	[illegible]
Lysinibacillus varians strain GY32, complete genome	962	17550	96%	0.0	98.91%	[illegible]
Bacillus sphaericus 16S rRNA gene, isolate JG-7B	962	1762	96%	0.0	98.91%	[illegible]
Bacillus sp. JDM-2-2 16S ribosomal RNA gene, partial sequence	962	1762	96%	0.0	98.91%	[illegible]
Bacillus sphaericus partial 16S rRNA gene, strain JG-A12	962	1762	96%	0.0	98.91%	[illegible]
Lysinibacillus manniferthami strain clv011 16S ribosomal RNA gene, partial sequence	958	1759	94%	0.0	99.63%	[illegible]
Uncultured bacterium clone L141 16S ribosomal RNA gene, partial sequence	958	1759	94%	0.0	99.63%	[illegible]
Lysinibacillus sp. Z5Bx-33 16S ribosomal RNA gene, partial sequence	958	958	51%	0.0	99.63%	[illegible]

Figure: BLAST result of R2 strain which showed 96% similarity with *Bacillus spp*

Sequences producing significant alignments:

Select: All None Selected 0

Alignments

Description	Max Score	Total Score	Query Cover	E value	Per Ident	Accession
Bacillus subtilis strain IADCASCX 16S ribosomal RNA gene, partial sequence	760	760	95%	0.0	92.16%	[illegible]
Bacillus subtilis strain Azhar 16S ribosomal RNA gene, partial sequence	748	748	95%	0.0	91.59%	[illegible]
Bacillus subtilis strain CNM1L 16S ribosomal RNA gene, partial sequence	746	746	95%	0.0	91.42%	[illegible]
Bacillus amyloliquefaciens strain U79BA 16S ribosomal RNA gene, partial sequence	742	742	95%	0.0	91.42%	[illegible]
Bacillus subtilis subsp. inaquosorum strain F147-46251 16S ribosomal RNA gene, partial sequence	739	739	95%	0.0	91.21%	[illegible]
Bacillus velezensis strain JS25R 16S ribosomal RNA gene, partial sequence	739	739	95%	0.0	91.21%	[illegible]
Bacillus sp. strain O34 16S ribosomal RNA gene, partial sequence	739	739	95%	0.0	91.21%	[illegible]
Bacillus amyloliquefaciens strain RC9 16S ribosomal RNA gene, partial sequence	739	739	95%	0.0	91.21%	[illegible]
Bacillus amyloliquefaciens strain S13 16S ribosomal RNA gene, partial sequence	739	739	95%	0.0	91.21%	[illegible]
Bacillus sp. (in: Bacteria) strain v2 16S ribosomal RNA gene, partial sequence	737	737	95%	0.0	91.20%	[illegible]
Bacillus siamensis strain HBUM07082 16S ribosomal RNA gene, partial sequence	737	737	95%	0.0	91.23%	[illegible]
Bacillus amyloliquefaciens strain EB-HR2 16S ribosomal RNA gene, partial sequence	737	737	95%	0.0	91.23%	[illegible]

Figure: BLAST result of R3 strain which showed 95% similarity with *Bacillus spp*

APPENDIX VI: Participation in Extra Activities

i) **Poster presentation** in 2nd INTERNATIONAL CONFERENCE ON BIOSCIENCE & BIOTECHNOLOGY (ICBB-2018) Held in Dhulikhel, Nepal February 17-20,2018 organized by RIBB and FIMM ,Finland

ii) Completion of 8 weeks online course „Research Writing in the Sciences" of the AuthorAid Programme at INASP from 4 September to 2 October

APPENDIX VII: PUBLICATIONS

frontiers in Energy Research

ORIGINAL RESEARCH
published: 31 July 2019
doi: 10.3389/fenrg.2019.00070

Enhancement of Ethanol Production in Electrochemical Cell by *Saccharomyces cerevisiae* (CDBT2) and *Wickerhamomyces anomalus* (CDBT7)

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Bioethanol (a renewable resource), blended with gasoline, is used as liquid transportation fuel worldwide and produced from either starch or lignocellulose. Local production and use of bioethanol supports local economies, decreases country's carbon footprint and promotes self-sufficiency. The latter is especially important for bio-resource-rich

applied sciences

MDPI

Article

Secretory Laccase from *Pestalotiopsis* Species CDBT-F-G1 Fungal Strain Isolated from High Altitude: Optimization of Its Production and Characterization

Mukesh Yadav [1], Garima Bista [1], Rocky Maharjan [1], Pranita Poudyal [1], Milan Mainali [1], Lakshmaiah Sreerama [2,*] and Jarina Joshi [1,*]

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Abstract: Microorganisms producing laccases may be used for the pretreatment of lignocellulosic biomass to recover fermentable sugar. Very few fungi and other microbes growing in high altitudes have been tested for this purpose. As part of this study, we have collected soil samples from different parts of the Kathmandu Valley and the Rautahat at district of Nepal (1600 to 2303 m above sea level) and successfully cultured 53 different isolates of microorganisms. Among the 53 isolates obtained 30 were *Actinomycetes*, 20 were *Streptomycetes*, and three were fungi). These isolates were tested for laccase expression using guaiacol, tannic acid, and 1-naphthol as substrates. Twelve of the 53 isolates tested positive for the expression of laccase. Among the laccase- positive isolates, a fungal species designated as CDBT-F-G1 was found to produce high levels of laccase. This isolate was identified as *Pestalotiopsis* species based on 18S rRNA sequencing. *Pestalotiopsis* spp. CDBT-F-G1 isolate grows efficiently in PDB media containing 1% Kraft lignin at pH 5 and 30 °C and secretes 20 ± 2 U/mL laccase in culture medium. Further optimization of growth conditions revealed that addition of (i) metal salts, e.g., 1 mM magnesium sulfate (51 ± 25 U/mL); (ii) agitation of cultures at 200 rpm (51 ± 9 U/mL); (iii) surfactants, e.g., 0.75 mM Tween 80 (54 ± 14 U/mL); (iv) 40% dissolved O_2 (57 ± 2 U/mL) and inducers, e.g., 1 mM gallic acid (69 ± 11 U/mL), further promote laccase production by *Pestalotiopsis* spp. CDBT-F-G1 isolate. On the other hand, 0.1 mM cysteine inhibited laccase production. The secretory laccase obtained from fermentation broth of CDBT-F-G1 was partially purified by ammonium sulfate (13-fold purification with specific activity 26,200 U/mg) and acetone (14-fold purification with specific activity 31,700 U/mg) precipitation methods. The enzyme has an approximate molecular mass of 43 kDa, pH and temperature optima were pH 6 and 60 °C, respectively. V_{max} and K_M were 100 µmol/min and 0.10 mM, respectively, with ABTS as the substrate. Given the above characteristics, we believe *Pestalotiopsis* spp. CDBT-F-G1 strain native to high altitudes of Nepal could be used to pretreat lignocellulosic biomass to efficiently recover fermentable sugars.

Keywords: laccase; lignin; *Pestalotiopsis* spp. CDBT-F-G1; guaiacol; tannic acid and 1 naphthol