DEGRADATION OF POTATO PEELS USING AMYLASE AND PECTINASE PRODUCING FUNGAL STRAIN IN ELECTROCHEMICAL CELL AND BYPRODUCT ANALYSIS

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

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Certificate of Evaluation

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GLOSSARY ACRONYMS

APS	Ammonium persulfate
µl	Microliter
COD	Chemical oxygen demand
CTAB	Cetyltrimethylammonium bromide
CV	Cyclic voltammetry
DNS	Dinitrosalicylic acid
g	Gram
h	hour
HPLC	High pressure liquid chromatography
L	Liter
M	Molar
m^3	Cubic meter
MFC	Microbial fuel cell
mg	Milligram
mg/L	Milligram per liter
min	Minute
mL	Milliliter
mm	Millimeter
mM	Millimolar
mV	Millivolt
mW	Miliwatt
MWCNT	Multi-walled carbon nanotube
NCBI	National Centre for Biotechnology Information
nm	Nanometer
^0C	Degree centigrade
OCV	Open circuit voltage

PANI	Polyaniline
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PEM	Proton exchange membrane
PPW	Potato peel waste
rpm	Rotation per minute
TSS	Total suspended solid
UV	Ultraviolet
V	Volt
v/v	Volume per volume
VSS	Volatile suspended solid
W	Watt

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ABSTRACT

Potato peels are the mostly abundant waste in Asian household. Degradation of the waste using amylase and pectinase producing fungal strain in Microbial fuel cell (MFC) can be a sustainable and economic strategy for solid waste management and hence alternative electricity generation. A fungal strain Isolated from soil showed both amylase and pectinase activity and molecular characterization of isolated strain was done which was found similar with *Aspergillus niger*. Potato peel waste had pH 6.51± 0.08, Total suspended solid (TSS) 21.4±1.27%, moisture content 78.6±1.26%, ash content 11.68±7.05%, volatile suspended solid (VSS) 88.31±7.056 %, chemical oxygen demand (COD) 10.24±0.12 mg/g, reducing sugar 1.061±0.64 mg/g, ammoniacal-nitrogen 0.01±0.01 mg/g and phosphorus 0.015±0.017 mg/g. Similarly potato peels contained iron 0.167 mg/g, copper 0.007 mg/g, zinc 0.007 mg/g and manganese 0.005 mg/g but lead and nickel was not found. Microbial fuel cell was constructed using different concentration of finely pasted potato peel waste sample in anode and from which 1:10 dilution showed better result used for further operation. After that effect of different electron acceptor in catholytes, inoculum added to sample, electrode modification were observed, each experiment was done in triplicate. Open circuit voltage (OCV) generation was highest while using $KMnO_4$ in catholyte (505±18 mV). The electrode was modified by coating of the graphite electrode with MWCNT, which was used as an anode in the OCV generation. Microbial fuel cell in fed batch was also performed by adding 10% of sample in every 24 h, and clearly improved result was obtained in this operation. Power density was determined using 100 ohm and 1000 ohm external resistors which was found 119±7 W/m^3 and 42±9 W/m^3 respectively. From Microbial fuel cell operation at optimized condition removal rate of COD, ammoniacal-nitogen, reducing sugar and total suspended solid were found to be 37.69%, 67.72%, 72.64% and 65.95% respectively. The Microbial fuel cell electrical performance was examined and analyzed by oxidation and reduction peaks in cyclic voltammetry (CV) technique. Sugar analysis in sample and byproduct analysis after MFC operation was done by HPLC using suitable mobile phase and concentration of each compound was determined. Finally, this research conveys that the use of enzyme produced by fungal strain can be a novel alternative approach in bioelectricity production and is beneficial in management of solid waste also, as it does not require separate pretreatment.

Keywords: Potato peel waste, Microbial fuel cell (MFC), Chemical oxygen demand (COD), Multi-walled carbon nanotube (MWCNT), Total suspended solid (TSS), Cyclic voltammetry (CV), High pressure liquid chromatography (HPLC)

CHAPTER 1: INTRODUCTION

1.1 Background

Scientists and researchers began looking for solutions to extract energy from renewable natural resources in this age of increasing energy demand and continual depletion of fossil fuels (Armaroli and Balzani, 2007). Solid waste is generally composed of three groups of materials: organic waste (kitchen waste, garden waste, etc), non-recyclable inorganic waste (coal ash, cinder, dust, etc), and recyclable waste (paper, plastics, glass, metal, etc) (Zhang et *a*l., 2010). In this solid regard organic waste can be used as renewable natural source to convert into products such as bioelectricity, bioethanol, etc.

Potato peel waste (PPW) is a major byproduct of the potato processing industry and a potential source of functional and bioactive substances such as antioxidants, pigments, dietary fiber, vitamins, and minerals, among others (Singh et al., 2007). Despite the fact that fresh potato consumption is declining in many countries, more potatoes are being processed into value-added products to meet demand, particularly from the fast food and convenience food industries. Depending on the type of product, potatoes are usually peeled during processing using steam, lye, or abrasive peeling. As a result, significant quantities of peels are produced, posing a serious disposal dilemma for the business, especially given the growing awareness and goals of reducing environmental impact and ensuring long-term sustainability (Schieber et al., 2009).

Microorganisms or enzymes can be utilized to generate electricity in microbial fuel cells (MFCs), which has been known for several years (Kim et al., 2005). Solid phase organic waste is a major source of contamination in the environment; enormous amounts of agricultural waste, livestock excreta, and municipal garbage are all environmental challenges that must be addressed but are difficult to manage (Roger et al., 2005). The use of a microbial fuel cell to treat organic waste (such as household waste water, waste water treatment plant waste water, contaminated soil and livestock waste water, food waste water, and lingocellulosic biomass, etc.) and obtain additional electrical capability is possible thanks to the actions of microorganism and enzymes. The microbial fuel cell, on the other hand, does produce electricity (Wang et al., 2013).

1.1.1 Vegetable waste management

Vegetable wastes include the rotten, peels, shells, and scraped portions of vegetables or slurries. These wastes can be treated for biofuel production through fermentation under controlled conditions or else used for composting (Singh et al., 2012). There are various conventional methods of organic waste management, mainly used medhod is composting, which can be the reason of air and soil pollution by producing CH4 and CO_2 (Ayilara et al., 2020).

1.1.1.1 Conventional techniques of waste management

1. Briquetting

For decades, academics have been interested in the compaction and binding of bulky food waste products as a fuel supply. Due to their excellent thermal efficiency, agro-waste and coal-agro-waste mixes have acquired popularity among the typical varieties of briquettes. The main mechanism involves coal and biomass combustion; because vegetable waste ignites at lower temperatures than coal, less smoke is produced while more heat is released (Raju et al., 2014)

2. Landfills and animal feeding

Landfills are one of the most common and straightforward methods of disposing of solid waste. These vegetable/fruit wastes are disposed of in landfills because they are more biodegradable than other trash. Landfill gases (LFG) are one of the main anthropogenic sources of methane, accounting for 8% of global emissions, according to the United States Environmental Protection Agency (USEPA). Fruit and vegetable valuing, as well as their plant components, has long been considered a customary practice for their use in animal feed.

3. Composting

Composting is a biological process in which organic matter is treated to produce manure or fertilizers. Microorganisms are used to carry out the process. Catabolism of hydrolysis and oxidation of carbonaceous substrate provide CO2 and heat, which are required for microbial growth and metabolic demands. Composting produces stable chemicals primarily made up of carbon and nitrogen (Chang et al., 2006).

4. Anaerobic Digestion

Different types of fruit and vegetable waste can be handled to produce value-added goods in families, canteens, industries, and commercial places. Single-stage and two-stage

anaerobic digestion processes are the most often used anaerobic digestion processes. As a result, methane and other by-products are produced (Chang et al., 2006).

1.1.1.2 Emerging techniques of waste management

In recent years, opportunities in 379 utilizing fruit and vegetable waste have grown in nutraceuticals, flavoring agents, bioactive compounds in packaging, and waste nanoparticles. Compared to traditional methods, which require large amounts of fruit and vegetable waste to make value-added goods, low-volume, high-value options with increased sustainability are predicted to be a viable option.

1. Nutraceuticals

Nutraceuticals are substances produced from food or a portion of food that have been shown to improve human health by treating or preventing diseases. Wadhwa et al. (2015) classify it as a substance comprising antioxidants, dietary fibre, fatty acids, and polyphenol.

2. Films and packaging

In comparison to glass, these petroleum-derived materials are lightweight, low-cost, and long-lasting. Its non-biodegradability, on the other hand, has had an impact on the entire ecosystem. Various researchers have been interested to generate sustainable materials as an alternative to plastics by combining diverse fruit/vegetable-derived compounds as viable films for bio-packaging.

3. Flavouring agents

Flavors are crucial in determining whether or not a dish will be accepted. Natural scents found in plants and flowers have a wide range of applications as additives in fragrance, cosmetics, perfumery, surfactants, and the food industry.

4. Waste induced nanoparticles

Cost, environmental feasibility, and safety, on the other hand, must be prioritized. The green synthesis of nanoparticles using various elements of vegetable or fruit waste has interested researchers in recent years due to its sustainability and lower cost than the physical/chemical process.

1.1.2 Microbial Fuel Cell

Microbial fuel cells (MFCs) are bioelectrochemical fuel cells that generate electricity by redirecting electrons from microbial oxidation of reduced compounds on the anode to oxidized compounds on the cathode via an external electrical circuit (Logan & Rabaey, 2012). MFCs, which have recently been expanded into a variety of Bio-Electrochemical Systems (BESs), are an exciting and rapidly developing field of science and technology that combines biological catalytic redox activity with traditional abiotic electrochemical reactions and physics (Santoro C. et al., 2017). MFC represents the noval approach for generating electricity from biomass using microorganism.

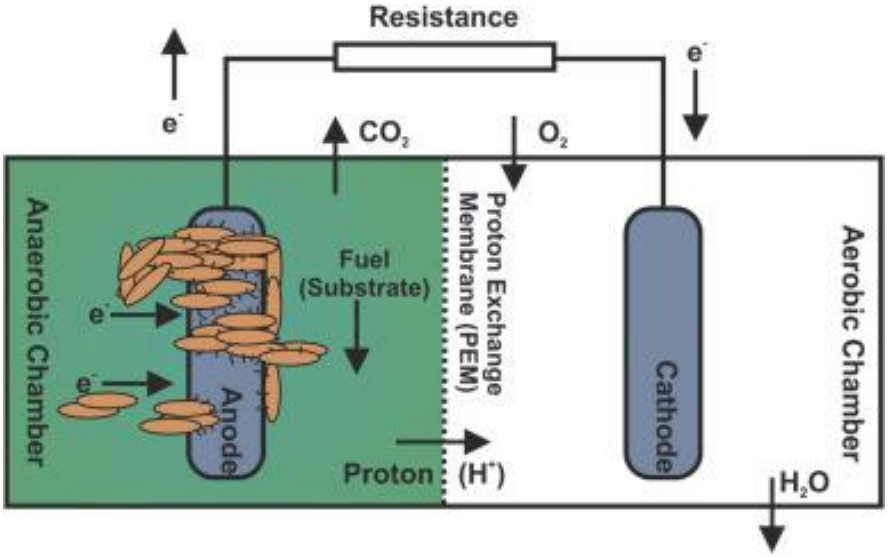

Figure 1.1: The scheme of a two-chamber microbial fuel cell (Slate et al., 2019)

The electrons generated during oxidation are directly transported to an electrode or a redox mediator species. The electron flow is transferred from the anode to the cathode. Ionic migration within the cell, usually across an ionic membrane, maintains the system's charge equilibrium. Most MFCs oxidize an organic electron donor to create CO2, protons, and electrons. Other electron donors, such as sulfur compounds or hydrogen, have been reported (Pant D. et al*.,* 2010). The cathode reaction employs a number of electron acceptors, the most common of which being oxygen (O2). Metal recovery via reduction, water to hydrogen, nitrate reduction, and sulfate reduction are among the other electron acceptors investigated.

Substrate, microorganisms and their metabolism, electron transfer mechanism in an anodic chamber, electrode material shape and size, type of membrane, operating conditions such as temperature, pH, and salinity, electron acceptor in a cathodic chamber,

internal resistance and geometric design of the MFC are considered to be the most important factors among the numerous factors affecting MFC performance (Mehrdad et al., 2015)

1.2 Current Studies

The handling of solid waste has always been more advanced in the bigger cities. As the population density rises, so does the difficulty of disposing of waste. Simultaneously, garbage production per unit area is increasing, but the amount of land accessible for waste disposal is decreasing. Also Reduction and recycling of waste are very serious problems all over the world due to the limitation of final disposal site and decreasing environmental loads. According to a study of the characteristics of solid waste in many developing countries, the majority (more than 80%) of total solid waste is organic waste, which does not normally receive much attention for recycling or resource recovery (Moqsud et al., 2008). Most of the waste is directly incinerated with other combustible waste, and the residual ash is disposed in land fill, however water containing waste is energy consuming.

Microbial fuel cells (MFCs) are a promising electrochemical technique for treating wastewater and generating sustainable energy. Microorganisms can convert organic components in wastewater to electricity through bioconversion. The key advantages of MFC for wastewater treatment include the generation of safe, clean, efficient, and direct power as well as the elimination of organic components found in wastewater (R. Sindhu, et al., 2019). Similarly composite vegetable waste was used as renewable source in the MFC for bioelectricity generation (Venkata Mohan et al., 2010). Microbial fuel cell (MFC) technologies are the most recent attempt to utilize microorganisms to generate power from biomass (M.A. Moqsud et al., 2013). The MFCs were fed potato peels until they reached a stable state. Chemical oxygen demand (COD) removal in the current study grew consistently with time after voltage stabilization and as the only electron donor. It dropped from 3800 mg/L to 3000 mg/L in 5 days, and the clearance rate increased to 18% (Flimban, S. G et al., 2018).

1.3 Research Hypothesis

Potato Peels mainly consists of starch and pectin, which are degraded by the activity of enzymes amylase and pectinase respectively. Degradation of these compounds in electrochemical cell generates the energy. Degradation can be done by using the isolated fungal strain which produce both enzymes. Thus generated energy can be enhanced by performing various optimization of fuel cell.

Null Hypothesis (H_0)

The Potato Peels waste cannot be used by isolated fungal strain for electricity generation in MFC.

Alternative Hypothesis (H_1)

The Potato peels waste can be used by isolated fungal strain for electricity generation in MFC.

1.4 Research Objectives

1.4.1 General Objectives

Use of Microbial Fuel cell to observe the degradation efficiency of potato peel waste and to determine electricity generation efficiency.

1.4.2 Specific Objectives

1. Analysis of potato peel waste for its Chemical Oxygen Demand (COD), Reducing sugar, Nitrogen, Phosphorus contents and metal ions and trace elements.

2. Isolation of amylase and pectinase producing strain from potato peel waste

3. Operation of microbial fuel cell for enhancement of electricity generation using thinly pasted potato peel waste sample.

4. Observation of organic waste degradation status and electricity generation in all successive waste degradation process.

5. Analysis of byproducts generated in MFC by HPLC.

1.5 Rationale

Producing, harvesting, sorting, and packing fruits and vegetables generates a large amount of waste, which has the potential to pollute the environment (EI-Ramady et al., 2014). Similarly, global energy demand continues to rise, putting the globe on the edge of a global energy crisis and pollution. The main focus of this research is on the management of organic waste, which is generated on a daily basis and from which alternative energy is generated using MFC. The use of MFC technology for waste treatment has recently become the most promising use of this innovative technology for energy production due to their mild working conditions and ability to use a variety of biodegradable substrates (Nastro R.A et al., 2017). The potential of sustainable energy generation from various

substrates, such as organic wastes, has prompted increased research in this subject in recent years. MFCs can be used for a variety of purposes such as bioelectricity generation, biohydrogen production, biosensors etc. The generated power in MFC is still too low for commercial use, and researchers are attempting to enhance it by using various class of nanomaterials which makes the MFC more efficient and cost effective (Maski et al., 2018)

1.6 Research Scope

Microbial fuel cells (MFCs) are bio-based reactors that convert chemical energy from a substrate into electrical energy (Chandrasekhar K. et al., 2018). Sustainable clean energy and climate change issues are the center of attraction in today's world. MFC technology is a novel way of utilize microorganisms to generate bioelectricity by oxidizing organic substrates ranging from synthetic substrates like acetate and glucose to a complex mixture of organic substrates. By modifying electrode surfaces and putting active catalyst coatings to the electrode surface, ongoing attempts are being undertaken to build improved e transfer mechanisms between the electrode and the biocatalyst (Chandrasekhar K. et al., 2018). Extensive research has been conducted on microorganism selection, proper MFC designs, ideal electrode materials, and the optimal level of process parameters, all of which will help to speed up the commercialization of this technology in the near future.

CHAPTER 2: LITERATURE REVIEW

2.1 History

Microbial fuel cells (MFC) rely on the power of microbes to generate electricity and transform the energy generated in the environment. Electrical energy is produced by metabolic reactions. This procedure is safe for the environment generates power without requiring combustion the use of fossil fuels. The use of microbes to create electricity implies that the activities in an MFC are self-sustaining; the bacteria proliferate and continue to produce power indefinitely as long as there is a food source to feed them.

M. C. Potter, a botany professor at the University of Durham, first proposed using microorganisms to generate energy in 1911. His research resulted in the development of a primary microbial fuel cell. Dr. M. C. Potter may also be able to demonstrate that variables such as temperature, concentration, and nutritional media can affect the amount of "electricity created" (Potter et al., 1911). Potter came up with the notion of harvesting this newly discovered source of energy for human use. He was able to build a crude microbial fuel cell, but not enough was understood about bacteria metabolism at the time to enhance the design. Professor Barnet Cohen was able to create around 35V and two milliamps of current exactly two decades later, in 1931. To get such a high potential and relatively excellent current, Mr. Cohen aligned and connected a number of microbial fuel cells in series (Sami et al., 2018). Later, in 1962, Rohrback et al., developed a glucose-generating MFC. Since then, researchers have been studying bacteria's ability to create an electric current while designing and testing various designs that eventually evolved into what we now term a microbial fuel cell in the 1970s. Researchers are now striving to improve electrode materials, microbe species and combinations, and electron transmission in microbial fuel cells. Despite the fact that the concept of harnessing the energy produced by bacteria has been around for than a century, researchers are only now beginning to fully comprehend the MFC and how to maximize its full potential (Santa et al., 2014).

MFC is primarily a biochemical device that extracts the energy of various respiring microorganisms and converts organic matter in waste to electrical power. The succession of oxidation-reduction events that occur at its center is the basic principle (Goutam Mukherjee, A. et al*.,* 2021). Many MFC designs have been used to increase the efficiency and product increment of this process.

2.2 Waste biomass as renewable energy source

Since ancient times, biomass has been used as a key energy source all across the world. In contrast to fossil fuels, the use of biomass to produce energy is just one type of renewable energy that may be used to lessen the impact of energy production and usage on the global environment. Wood and wood wastes, agricultural crops and waste byproducts, municipal solid waste, animal wastes, food processing waste, aquatic plants, and algae are all examples of biomass resources (Gavrilescu and Chisti, 2005). Biomass can be converted into three main types of product: electrical/heat energy, transport fuel and chemical feedstock. In the UK, the Government's target is to generate 10% of the national electricity need of 60 GW/year from renewable sources, of which biomass will form a significant part. To date, the 10 or so biomass projects working/under construction will generate about 100 MW (McKendry et al., 2001). The type of biomass chosen determines the most likely form of energy conversion process based on the moisture content of the biomass. Biomass with a high moisture content, such as sugarcane, lends itself to a 'wet/aqueous' conversion process involving biologically mediated reactions like fermentation, while a 'dry' biomass such as wood chips, is more economically suited to gasification, pyrolysis or combustion (McKendry, 2002). Apart from moisture content, there are other aspects to consider when selecting a conversion procedure, particularly in respect to biomass types that reside below the water table. Between the two extremes of 'wet' and 'dry,' there is a middle ground.

As a result, substituting biomass for fossil fuels will result in a net reduction in greenhouse gas emissions as well as the replacement of a non-renewable energy source. Many significant power providers in developed nations are turning to biomass to satisfy their greenhouse emission reduction goals (Demirbas, 2001). Biomass energy, unlike other nonrenewable sources of energy, can be recycled. There is no net contribution of carbon dioxide to global warming because it is produced and utilized in a sustainable manner. Biomass energy, if managed properly, can have a substantial environmental impact. Compared to the utilization of fossil fuels, there are several advantages. An optimum degree of biomass energy utilization can have less negative environmental consequences than our existing methods of energy production (Ciubota-Rosie et al., 2008).

Biomass fuels have negligible sulfur content and, therefore, do not contribute to sulfur dioxide emissions, which cause acid rain. The combustion of biomass produces less ash than coal combustion, and the ash produced can be used as a soil additive on farm targets. Many significant power producers in developed nations are looking to biomass to help them reach their greenhouse emission reduction goals (Demirbas, 2001).

2.2.1 Organic waste status in Nepal

The organic waste composition in municipal waste of Nepal was highest (54.0%) in 2075/76 compared to the inorganic waste (33.3%) and other wastes (12.7%) according to waste management baseline survey of Nepal, 2020. Kalimati Fruits and Vegetables Market is a wholesale market of Kathmandu city, where 650 metric tons of vegetables arrive every day, out of which 15-25 metric tons of vegetables are discarded daily because they are found to be damaged (Global Press Journal, 2014). In Nepal biofuel production from waste materials is not industrialized yet, researcher doing work on it in recent days. Bioethanol is produced from the red potatoes grown in hilly region of Nepal, maximum yield of ethanol was 5.2% obtained at a temperature of 30℃ (Joshi J., 2014).

2.3 Role of MFC in solid waste treatment

MFCs are a new technology that can extract energy from biomass while also treating trash. In recent years, this approach has advanced quickly in combination with environmental procedures such as wastewater treatment, toxic pollutant degradation, and desalination. With the rise in solid waste, using MFC in composting holds promise because to its waste disposal and simultaneous energy generating properties. Composting, anaerobic digestion, and carbonization are all environmentally friendly waste disposal processes, but they have drawbacks such as generating global warming and requiring a lot of energy. Both increased renewable energy recovery and lower environmental contamination necessitate more efficient technologies. MFC is a technique that uses anaerobic biodegradation to turn organic materials into power (Logan et al., 2006). MFC has the advantage of immediately recycling the cleanest energy (electricity) from organic waste while producing less secondary pollutants in the water, soil, and atmosphere. MFC has been used to treat organic waste (including food waste, activated sludge, and animal waste) in a number of studies thus far.

Bioelectrochemical systems have the ability to play a vital role in producing sustainable waste recycling systems, with lower consumption of energy and, at the same time, creating usable chemicals. In 2011, Mohan and Chandrasekhar released their first work on the effects of operational parameters on the performance of MFCs fed with canteen food waste, with an emphasis on electrode distance and feed stock pH. Solid phase MFCs (SMFCs) have recently been studied in conjunction with a composting process using soybean, rice husk, leaf mold, and coffee wastes to create mixtures with various C/N ratios (Wang et al. 2015).The products of hydrolysis/acidification are converted to gases (including methane and carbon dioxide) primarily through the involvement of methanogens in conventional anaerobic digestion; however, in MFC, a portion of the

products is preferentially used by electrogenic bacteria for electricity generation, with methanogen growth within the MFC reactors being limited (Krishna and Mohan, 2016; Quan et al.)

2.4 Working Principle of MFCs

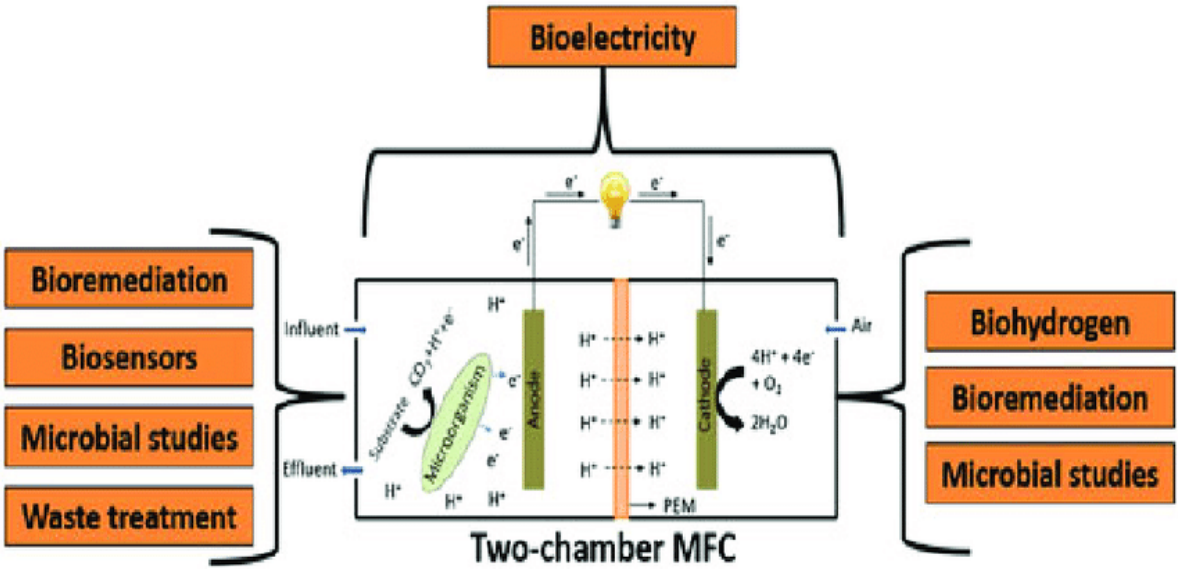

Figure 2.1: Electricity generation in dual chambered MFC (Kumar R. et al., 2017)

A fuel cell is an electrochemical device that turns the chemical energy of a fuel directly into electrical energy rather than generating heat. The first step in MFCs towards current generation is the acclimatization of the exoelectrogens and enzymes produced by microorganism in the anode chamber and subsequent biofilm formation on the electrode surface (anode). The principle of redox reactions underpins the operation of microbial fuel cell (MFC) technology. Carbon dioxide (CO2), electrons, and protons are produced when organic matter is oxidized. The inherent metabolism microorganism is used to create electricity. Electrons are generated from the substrates. Electrochemical reactions occur at the electrodes to generate an electric current that flows through the electrolyte while driving a complementary electric current that works on the load (K SCOTT *et al.*, 2014). This molecule is oxidized (Equation [1]) at the anode of, say, an acid electrolyte fuel cell using methanol, releasing electrons and generating H + ion (protons), releasing energy:

$$C_6H_{12}O_6 + 6H_2O \longrightarrow 6CO_2 + 24H^+ + 24e^- \qquad [1]$$

At the cathode, oxygen combines with protons that have migrated from the anode to the cathode internally, as well as electrons given from the anode via the external electrical circuit, to generate water:

$6O_2 + 24H^+ + 24e^- \longrightarrow 12\ H2O$ [2]

2.5 Components of MFCs

A typical two chambered MFC consists of an anodic chamber and a cathodic chamber separated by a Proton exchange membrane (PEM). The two chambers are connected externally by electrode connection. Substrates are the basic components of MFC which are utilized by microbes and generates electricity.

2.5.1 Anode

The anodic chamber is a critical aspect of the MFC setup and anodic materials must be conductive, biocompatible and chemically stable in reactor solution. To reach the cathode, electrons are routed through the anode electrode and transferred through an external circuit. The proton released from the substrate diffuses into the cathode compartment via an ion exchange membrane, where they mix with the final electron acceptor, such as molecular oxygen, to produce water, completing the circuit and generating electricity via the redox reaction process (Y. Hindatu et al., 2017). The commonly utilized anode electrodes in MFC include carbon paper (CP) , carbon cloth (CC), activated carbon cloth (ACC) , carbon felt (CF), activated carbon fiber felt , graphite felt (GF), tungsten carbide , graphite foil amongst other.

2.5.2 Cathode

The cathode serves as an electron sink, accepting electrons and protons created by anodic oxidation of the substrate. Because of its infinite supply, high redox potential, and lack of chemical waste, oxygen is commonly used as an electron acceptor (Zhang, Y et al., 2012). A wide variety of materials have been examined as bio-cathodes in MFCs, including carbon paper , graphite fiber brush , graphite felt , and stainless steel mesh. De Schamphelaire et al., (2010) reported that carbon felt was more suitable than stainless mesh for biocathode. The interaction between microbial biofilms and electrode surfaces is critical for overall cathode performance, including microbial adhesion, electron transport, electrode resistance, and electrode surface reaction rate.

2.5.3 Membrane

The PEM is an important component of an MFC because it separates the anaerobic anodic and aerobic cathodic chambers while also assisting proton transfer between them. Charge separation between the anode and cathode happens as a result of proton transport via PEM, which creates electricity. Nafion is the most widely used PEM, with good proton conductivity and is regarded as one of the best-performing PEMs. Several modifications of Nafion, such as low-cost polyvinyl alcohol, were tested to improve proton conductivity, proton selectivity, and cost (Das I et al., 2020)

2.5.4 Substrate

One of the most important biological elements affecting energy generation in MFCs is substrate. MFCs can use a wide range of substrates for energy generation, from pure chemicals to complex combinations of organic materials found in wastewater. Main substrate used in MFCs are acetate, glucose, lignocellulosic biomass, synthetic wastewater, Brewery wastewater, Starch processing wastewater, Dye wastewater, Landfill leachates, cellulose and chitin, Inorganic and other substrates etc (Pant D et al*.,* 2010).

2.5.5 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. When the cathode has an acceptable electron acceptor (for example, oxygen) and the cell's overall thermodynamics are favorable, electrons flow spontaneously through the external circuit to the cathode for reduction processes. The cathode catalysts are categorized into carbon-based catalysts, metal-based catalysts, carbon–metal hybrids, metal–nitrogen–carbon complexes and biocatalysts. Similarly activated carbon, carbon nanotube, graphite/grapheme, metal oxides, etc.

Chemicals such as ferricyanide, potassium dichromate and potassium permagnate 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 et al., 2006). According to Cai et al., 2015 MFCs with potassium permanganate and potassium ferricyanide generate relatively higher voltage in comparison to those containing oxygen (Equations (1) and (2))

$MnO_4^- + 4H^+ + 3e^- = MnO_2(s) + H_2O \qquad E^0 = 1{:}68\ V$ (1)

$Fe(CN)_6^{3-} + e = Fe(CN)_6^{4-}$ E^0= 0:36 V (2)

Table 1.1: Basic components of MFC with most commonly used materials (Z. Du et al., 2007)

Items	Materials
Anode and Cathode	Graphite, graphite felt, carbon paper, carbon cloth, pt, pt black
Anodic and Cathodic Chambers	Glass, polycarbonate, Plexiglas
Proton exchange system	Proton exchange membrane: Nafion,
Electrode catalyst	Pt, Pt black, MnO2, Fe3+, polyaniline, electron mediator immobilized on anode

2.6 Electron Transfer in MFC

2.6.1 Direct Electron Transfer (DET)

The electrons are transferred directly from the microorganisms to the electrode in this process. A physical contact between the bacterial cell membrane and the electrode is required for this to happen (Lovley et al., 2006). There is no dissolved redox species involved in this pathway for electron transport. DET is carried out instead by outer membrane cytochromes (OMCs), conductive pili, or self-assembled nanowires. It was discovered that the protein has self-assembling characteristics, forming nanowires that might facilitate electron transmission. LSCV cyclic voltammetry, electron impedance spectroscopy, and other techniques are used to investigate direct electron transfer in exoelectrogenic bioflms (EIS) (Winaikij et al., 2018).

2.6.2 Mediated Electron Transfer (MET)

Many microorganisms are unable to perform DET either because they lack the necessary mechanisms or because they are not in contact with the electrode surface. In such instances, they transport electrons in a roundabout way. A redox carrier works as a shuttle to transport electrons to the TEA in mediated electron transfer (MET). This redox mediator in MFCs takes electrons from bacterial cells, leaving them reduced, and transfers them to the anode, oxidizing them in the process. After that, the oxidized form is free to begin another round of electron transfer. The properties of a good mediator, according to Ieropoulos et al. (2005), include: (1) non-toxicity toward bacteria; (2) ability to easily cross

the bacterial cell membrane; (3) a sufficiently positive redox potential to facilitate electron transfer; (4) good solubility in the anolyte; and (5) low cost and easy commercial availability.

2.6.3 Cathodic Electron Transfer (CET)

In comparison to anodic electron transfer methods, there is a scarcity of information on cathode to microbe electron transmission pathways. Direct and indirect electron transfer processes are commonly described for electron transport from the cathode to the bacterial cell (Kumar et al., 2016). A fundamental characteristic of this mechanism is the generation of a proton gradient during cathode oxidation (Rowe et al., 2018). An important application of the cathodic electron transfer to the microbes is in bioremediation of environmental pollutants.

2.7 Biofilm Anode

Understanding the numerous biological and electrochemical mechanisms that drive the oxidation of the fuel in the anode is a key scientific challenge in MFC research. Biofilm mathematical models are helpful in comprehending and developing biofilm reactors with complicated interconnected phenomena (Wanner et al., 2006). The ED substrate, the bacteria active in oxidizing the ED, and the electrons (e) extracted in the oxidation and delivered to the anode are all critical components of the MFC from bacterial cells to a soluble EA (i.e., the shuttle, also known as a mediator). The decreased shuttle diffuses to the anode, discharges its electrons, and then returns to the bacterial cells to begin the process all over again. Bacteria transmit electrons from their membrane-bound electron carriers to the electrode via conductive elements in the biofilm matrix via the conduction mechanism.

2.8 MFC Designs

2.8.1 Two compartment Microbial fuel cells

This is the most widely used design of MFC. The double-chamber MFC was built utilizing a previously published design (Ye et al., 2019), which included a two different chambers; anode chamber and cathode chamber. The two rooms were formed of plexiglass and were separated by proton exchange membrane. Anode chamber contains microbes, media, sample and cathode chamber contain buffer, fresh water, and oxygen supply etc. The standard MFC consists of an anode compartment and a cathode compartment separated by a cation exchange membrane (CEM) (Yan et al., 2018). The anode compartment is in charge of microbial substrate breakdown and electron and proton generation, while the

cathode chamber, which is equipped with electron acceptors, completes the electrical loop.

2.8.2 Single compartment Microbial Fuel cell

More recently, single chambered, membrane-less MFCs with an open air–cathode have been proved to be capable of higher power production than two-chambered MFCs (Liu and Logan, 2004; Oh and Logan, 2005). The anode electrode's large active surface area has been demonstrated to provide plenty of reaction sites for microorganism growth and oxidant reduction, resulting in a faster reaction rate. The single compartment membrane combined electrode (SCME) was designed based on Min and Logan's structure (2004).

2.8.3 Up-flow Microbial Fuel cell

The upflow microbial fuel cell (UMFC) was created to generate power and treat wastewater at the same time. The UMFC constantly generated electricity with a maximum power density of 170 mW/m^3 during a five-month period of feeding a sucrose solution as the electron donor. Internal resistance, estimated as 84Ω at maximum power density in this investigation, was the overall limiting factor for the UMFC, restricting power output by generating a considerable fall in operating potential (Zhen he et al., 2005). Hence, this design can be used to treat wastewater where electricity generation is not a first priority (Jang et al., 2004).

2.8.4 Stacked Microbial Fuel Cells

Combining multiple small MFC modules to form a larger stack may be a more feasible and efficient strategy to scale up MFC systems than merely increasing the size of an individual reactor. So far, there have been a variety of researches focusing on the performance of scaled-up MFC stack, but the reported power densities were still too low to make MFC system comparable with conventional anaerobic treatment in terms of energy recovery (Rabaey and Verstraete, 2005). To enhance the power output of scaled-up MFC systems, more efficient MFC stack and electrode configuration are required, as well as more data to expose the impact of external circuit connection on MFC stack performance. The electrode chambers were fabricated as narrow and flat (90 cm height, 40 cm length with only 5 cm width) to reduce electron transfer distances and be easily stacked together to a larger dimension, and the stack configuration should have larger ion exchange membrane areas per volume to facilitate ion transport efficiency. GAC packed bed electrodes were used because their increased accessible surface area might provide more active sites for the attachment of anode and cathode biofilms, resulting in significantly higher biomass contents and improved kinetic performance of MFC electrodes. GAC's

adsorptive action was pre-verified to considerably reduce MFC anode mass transfer resistance at low substrate concentrations and boost anode biofilm bioactivity (S. Wu et al., 2016).

2.8.5 Paper Microbial fuel cell

In low-resource areas around the world, extremely low-cost, yet highly sensitive and selective diagnostic equipment are now required (Yager et al., 2008). These requirements may be met by paper-based diagnostic instruments. To improve sensitivity, paper-based diagnostic systems have recently begun to use a battery-operated luminescence detector (Wang et al., 2012). Traditional batteries, on the other hand, are expensive and even wasteful for this single-use, low-power device, as it only takes a few minutes of power to get outputs from the devices. This limitation prompted us to develop paper-based MFCs that may be used as an integrated power source in paper-based diagnostic devices to power on-chip functionality. We created a paper-based MFC that is both flexible and lightweight. We created a paper-based MFC that is exceedingly flexible and has a low moisture content. The anode/cathode electrodes were made of flexible carbon clothing, and the paper chambers were delineated by photoresist hydrophobic barriers. In areas with limited resources, this low-cost, highly portable MFC is intended to be employed as a power source for single-use diagnostic instruments (A. Fraiwan et al., 2013).

2.9 Types of Anode and their modification

The anodic compartment is an important aspect of the MFC setup because it serves as a microbial metabolic electron sink. To reach the cathode, electrons are routed through the anode electrode and transferred through an external circuit. The proton released from the substrate diffuses into the cathode compartment via an ion exchange membrane, where they mix with the final electron acceptor, such as molecular oxygen, to produce water, completing the circuit and generating electricity via the redox reaction process (Juang D et al., 2011). Poor microbial adhesion on the electrode reduces the efficiency of electron transmission between the microbe and the anode, resulting in low power output and preventing the widespread adoption of MFC technology. The creation of a low-cost anode that also aids in microbial development/attachment, substrate metabolism, and extracellular electron transfer (EET) will provide a significant component enhancement to increase MFC performance and viability.

The commonly utilized anode electrodes in MFC include carbon paper (CP), carbon cloth (CC), activated carbon cloth (ACC), carbon felt (CF), activated carbon fiber felt, graphite felt (GF), tungsten carbide, graphite foil amongst others. Despite the fact that they were

stable, reasonably inexpensive, and had decent conductivity, their intrinsically hydrophobic nature made them unsuitable for robust microbial adherence, resulting in low electron transfer capabilities. Surface fouling from microbial secretion added to the problem. A good MFC anode material should be biocompatible, promote strong microbial adhesion, and allow for effective electron transmission. It should also have strong electrical conductivity, as well as low resistance and a big surface area (Huggins T et al., 2014).

However, synthetic components with desired qualities could be used to improve the electrodes' physicochemical properties, allowing for easier electron transfer and microbial adherence. In the recent years, conventional anode electrode (CAE) materials have been functionalized with nanostructural implements such as carbon nanotube (CNT), graphene (GR) and conducting polymers such as polyaniline (PANI) etc.

2.9.1 Anode modification with carbon nanotube

Carbon nanotube (CNT) has been used as anode electrode modification in MFCs and is a promising electrode material. The CNT is a cylinder-shaped carbon substance with a huge surface area. It's also chemically inert, mechanically stable, and has excellent electrical conductivity. Modification procedures including CNT functionalization are typically difficult synthetic methods involving corrosive chemicals (HNO_3/H_2SO_4), as well as time-consuming, resulting in application infeasibility. Thepsuparungsikul et al., (2012) evaluated the impact of diverse CNT morphologies on MFC performance, including single-walled CNTCOOH, MWCNT-COOH, and MWCNT-OH.

2.9.2 Anode modification with nanocomposite of metal oxides

Because of the reduction in ohmic loss as a result of improved bacterial cell adherence, electrode modification with metal nanocomposite or its oxides considerably improved MFC performance. Metal oxides such as goethite and rutile, for example, not only improve long-distance EET in MFCs, but they also promote the development of chemotrophic and heterotrophic bacteria using solar energy (Lu A et al., 2014). Titanium (Ti), tin (Sn), manganese (Mn), iron (Fe), and other metal oxides have been combined with carbonaceous materials to make nanocomposite anode electrode modifiers.

2.9.3 Anode modification with conducting polymer

Conductive polymers have been used as anode doping material in MFC because of their conductivity and environmental endurance. They're mostly used to improve bacterial cell adherence by modifying anode electrodes. Furthermore, when the polymer is doped with nanomaterial in the form of composites, improved anodic performance is reported.

Previously, electrophoretic deposition of MWCNT onto GF that had been electropolymerized with PANI improved MFC performance. The PANI improved the electrostatic contact of the GF electrode with the negatively charged bacterial cell wall by increasing its hydrophilicity (Cui H-F et al., 2015).

Although it is electrically conductive and environmentally safe, the chemical structure of polypyrrole is less well-defined. This is because the polymer side chain has a lot of ' coupling, which can cause structural instability and limit its electrochemical response (Xiao Y et al., 2004). In the building of the MFC anode, Kang et al. took use of the wide pH working circumstances and the outstanding property of the continuous redox activity of Poly(3,4-ethylenedioxythiophene) (PEDOT). A hydrogel is a water-based network of swelling polymers. We recently reported on the ability of biocompatible hydrogels based on medium-chain-length poly-3-hydroxyalkanoates to expand and hold a large amount of water without being dissolved (Gumel AM et al., 2014).

2.10 Performance parameter of MFC

Power Density: MFCs are derived from fuel cells, and their electrochemical characterization is comparable to that of fuel cells. In MFCs, power density is frequently employed as a fundamental measure to describe "electricity generation." Power output over the surface area of an anode electrode is commonly measured in "watts per square meter" (W m–2). Later research reported power densities dependent on the surface area of the cathode electrode or even the surface area of the ion exchange membrane. The use of surface area-based power density to present MFC performance has significant drawbacks. We must understand that power density does not reflect energy generation, regardless of the type of power density. Of course, power is defined as the amount of energy expended per unit of time. In MFC research, power density is still an essential measure that may be used to compare electrodes, catalysts, and microorganisms (Zhen He et al., 2017).

2.11 Application of MFC

2.11.1 Electricity generation

It is obvious that the majority of MFC research is focused on power generation, as this is the technology's primary use (Orellana et al., 2013). The microorganisms in the MFC's anode chamber oxidize the substrate into protons and electrons, which are then delivered to the cathode via PEM and electrical connection, respectively. To measure the voltage,

the two chambers of the MFC can be electrically connected to a multi meter and an external resistor box, and the power can then be computed using Ohm's law.

2.11.2 Wastewater treatment

MFCs have demonstrated their ability to treat a variety of industrial, urban, and home wastewaters. Though very toxic wastewaters cannot be entirely treated in MFCs, the COD of wastewaters can be reduced sufficiently to meet discharge requirements before being released into the environment. The MFCs have proved up to 98 % COD removal from the wastewater (Oh et al., 2010). Before and after the MFC operation, basic wastewater treatment assays (COD, BOD, total solids, nitrogen removal) can be used to assess the MFCs' treatment efficiency (2013, Zhou et al). COD removal in MFCs can be improved further by running the MFCs under optimal circumstances, such as mesophilic temperatures, has been proven to improve COD removal.

2.11.3 Biohydrogen

The typical double-chamber MFC can be amended to microbial electrolysis cell (MEC) for hydrogen production. The basic principle of a MEC is identical to that of a cathodic chamber, except that electric current is given at the cathodic chamber. The anode and cathode chambers make up the anode and cathode of a MEC. MEC, like MFC, has an ion exchange membrane that divides the two chambers (Zhou et al., 2013). Exoelectrogens digest the substrate and create electrons and protons in the anode chamber. MFCs transfer protons to the cathode in a similar way. The reaction of protons and electrons to form hydrogen at the cathode is thermodynamically impossible so that, electric current is provided at cathode to carry out this reaction.

2.11.4 Biosensor

Aside from electricity generation and wastewater treatment, MFC technology can be used as a biosensor for pollution detection in water (Shantaram et al., 2005; Zhou et al., 2013). The linear relationship between the coulombic yield of MFC and wastewater strength appoints MFC as a BOD sensor. Compared to traditional biosensors, MFC-based biosensors have several advantages. These biosensors are less expensive than traditional biosensors since they do not require the usage of a transducer. Furthermore, they can function for an extended period of time, such as 5 years, without requiring any maintenance.

2.12 Atomic Absorbance Spectroscopy

Bunsen and Kirchhoff, as well as a few others, constructed the first atomic absorption spectroscopes in the second half of the nineteenth century, which used a continuous source because it was the only trustworthy light source available at the time (Welz B. et al*.,* 2004). Atomic Absorbance Spectroscopy (AAS) is a technique for determining the amount of chemical elements contained in an environmental sample by determining the amount of radiation absorbed by the chemical element of interest (García, R *et al*, 2012). This is accomplished by analyzing the spectra generated when a sample is excited by radiation. Single-beam, double-beam, dual-channel double-beam, and double-beam sequential multielement are the four basic types of AAS instruments. Matrix interferences can be thought of as indirect effects caused by differing equilibria in reactions between molecules, neutral atoms, and ions in unknown samples versus prepared standards.

2.13 High Performance Liquid Chromatography (HPLC)

2.13.1 History of HPLC

Liquid chromatography was first used to separate colored substances in the early twentieth century, when it was developed as an analytical tool. Mikhail S. Tswett, a Russian botanist, employed a crude kind of chromatographic separation to separate combinations of plant colours into their pure constituents. He separated the pigments using the interaction of the pigments with a stationary phase, which is required for any chromatographic separation. Powdered chalk and aluminia served as the stationary phase, while the solvent served as the mobile phase in his separation. He put the mixture of plant pigments and solvent in the top of the column after packing the solid stationary phase into a glass column (basically a long, hollow glass tube). He then poured more solvent into the column until the samples were completely eluted at the bottom. Kuhn and Ledere P separated pigments following Tswett's procedures in 1931 (Touchstone et al., 1993). The most important outcome of this method for his research was that as the plant pigments moved through the stationary phase, they split into bands of pure components. This separation, the first kind of liquid chromatography, is where modern high performance liquid chromatography, or HPLC, gets its start. Over the last century, the chromatographic process has advanced dramatically, resulting in increased separation efficiency, variety, and speed.

2.13.1 Principle of HPLC

HPLC (high performance liquid chromatography) is an analytical technique for separating, identifying, and quantifying each component in a mixture (Swartz, 2005). Purification

occurs in a separation column that separates a stationary and mobile phase. In a separation column, the stationary phase is a granular substance with very small porous particles. The mobile phase, on the other hand, is a solvent or solvent mixture that is pushed through the separation column under high pressure. The sample is injected into the mobile phase flow from the pump to the separation column using a syringe and a valve with a connected sample loop, i.e. a tiny tube or a stainless steel capillary. Individual components of the sample migrate through the column at different rates as a result of interactions with the stationary phase, which retain them to varying degrees. Individual compounds are detected by an appropriate detector after exiting the column and sent as a signal to the computer's HPLC software. A chromatogram is generated in the HPLC software on the computer at the conclusion of this operation/run. The chromatogram allows the different compounds to be identified and quantified (Aryal, 2018).

2.13.2 Components of HPLC

This technique is currently gaining favor among many analytical techniques as the preferred method for fingerprinting studies for herbal plant quality control. HPLC's resolving capability is appropriate for the quick analysis and preparation of such multi-component mixtures on both an analytical and preparative scale (Martin et al., 2005).

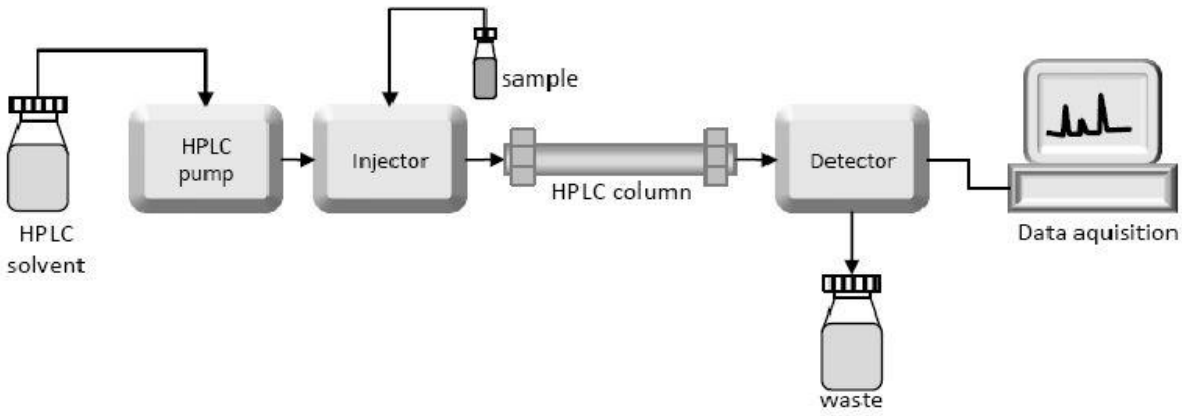

Figure 2.2: Components of HPLC system (Aryal, 2018)

1. Pump

The pump system was developed as a result of the development of HPLC. The pump is located in the liquid chromatography system's upper stream and creates a flow of eluent from the solvent reservoir1 into the system. Pumps must be able to generate high

pressure and offer a consistent pressure under all conditions, as well as a regulated and repeatable flow rate (Aryal S., 2018)

2. Injector

Next to the pump, there is an injector. The simplest way is to use a syringe to insert the sample into the eluent flow. Sampling loops are the most extensively utilized injection mechanism. The autosampler (auto-injector) device, which allows for repeated injections at predetermined times, is also commonly utilized.

3. Column

The separation takes place within the column. Instead of glass columns, contemporary columns are frequently manufactured in stainless steel housing. In comparison to calcium carbonate, silica or polymer gels are commonly utilized as packing materials. The majority of column housing is built of stainless steel, which is resistant to a wide range of chemicals. The column is made using a novel sol-gel method that involves the hydrolysis and polycondensation of alkoxysilanes in the presence of water-soluble polymers (Karin et al, 1999). To the extent that the functional groups circled, silica–silanol groups (Si–OH) can contribute hydrogen-bonding interactions that distinguish prednisone and prednisolone. Van der Waals interactions with hydrophobic molecules are responsible for retention. The length and inner diameter of the packing bed are the column dimensions. The diameter range of the particles is 1.5–20 m, and they are either spherical or irregular. In square meters per gram, the surface area is the sum of the particles outside surface and internal pore surface. On the basis of bonding type and carbon load, monomeric and polymeric materials exist (Craig S. Young, 2002).

4. Detector

To use HPLC to identify any component, you must first choose a detector. A separation assay must be established after the detector has been chosen and configured to optimal detection parameters. UV detectors υ Refractive index detector υ Flourimetric detector υ Conductivity detector υ Amperometric detector. UV detectors are the most popular of all detectors due to their great sensitivity and the fact that the majority of naturally occurring chemicals exhibit some UV absorption (Sashidharan et al., 2011). At wavelengths 190-380 nm, UV-VIS and Photodiode Array (PDA) detectors are often used to identify phenolics. The most promising, quick, and reliable analytical approach for sugar measurement of lignocellulosic hydrolysates is HPLC coupled with a refractive index detector (RID) (Tamara et al., 2017). The difference in optical refractive index between the mobile phase and the sample is measured using RI detectors; no chromophore on the solute molecule is required.

5. Recorder

The change in eluent detected by a detector is in the form of an electronic signal, and thus it is still not visible to our eyes. The assay parameters should be set so that a clean peak of the known sample may be seen on the chromatograph.

2.13.3 Applications of HPLC

According to Sagar Aryal, 2018 HPLC can be used in following areas,

1. Drug evaluation
2. Synthetic polymer analysis
3. In environmental analytics, contaminant analysis is performed.
4. Drug determination in biological matrices
5. Isolation of high-value goods
6. Controlling the purity and quality of industrial and fine chemicals
7. Biopolymers such as enzymes and nucleic acids are separated and purified.
8. Purification of water
9. Trace component pre-concentration
10. Ligand-exchange chromatography (LEC) is a type of chromatography that uses
11. Protein chromatography via ion exchange
12. High-pH anion-exchange chromatography of carbohydrates and oligosaccharides

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

All the reagents were provided by Central Department of Biotechnology, Tribhuvan University Laboratory. All the reagents were of analytical grades.

3.1.1 Chemicals

1. Potato Dextrose Agar (PDA) (Himedia, Indian Pvt. Ltd)
2. Potato Dextrose Broth (Himedia, Indian Pvt. Ltd)
3. Potassium Dichromate (Fisher Scientific)
4. Potassium Permagnate (Himedia, Indian Pvt. Ltd)
5. Potassium Ferricyanide (Merck Pvt. Ltd)
6. Nessler's Reagent (Himedia, Indian Pvt. Ltd)
7. Silver Sulfate (Qualigens- Thermofisher Scientific)
8. Mercury Sulfate (Qualigens- Thermofisher Scientific)
9. Glucose (Merck Pvt. Ltd)
10. Boric Acid Solution (Merck Pvt. Ltd)
11. Antimony potassium tartarate solution (Merck Pvt. Ltd)
12. Ascorbic Solution (Merck Pvt. Ltd)
13. Agarose (Himedia, Indian Pvt. Ltd)
14. Acetonitrile (Qualigens- Thermofisher Scientific)
15. Sulfuric acid (Fluka Analytical)
16. Acetone (Qualigens- Thermofisher Scientific)
17. Methanol (Qualigens- Thermofisher Scientific)
18. Hydrogen Peroxide (Qualigens- Thermofisher Scientific)
19. Sodium Acetate (RFCL Limited RANKEM)

3.1.2 Glass Wares

1. Glass petri plates
2. Reagent Bottles
3. Conical Flask
4. Beakers
5. Microbial Fuel Cell (Adams and Chittenden Scientific glass)
6. Culture tubes

3.1.3 Equipments

1. Magnetic Stirrer and Magnetic Bar
2. Autoclave (Indfos Piezostat)
3. Vortex
4. Hot air oven (Memmart)
5. Ultrasonicator (Indo Sati Instruments)
6. HPLC (Agilent 1260 infinity II)
7. Centrifuge (Eppendorf AG)
8. Grinder

3.2 Methodology

3.2.1 Sample collection

During the month of February 2021, potato peels were collected from the Tribhuwan University Girls Hostel, where potatoes were brought from the Kalimati vegetable market. The sample was then ground and kept in plastic bag then stored at -20^{o}C for future use.

3.2.2 Physical Characterization of Sample

3.2.2.1 pH measurement (soil, plant and water analysis, 2013)

Sample was mixed with distilled water up to 1:10 dilution and was allowed to stand for 30 minute. The sample was stirred and after 1 h pH was measured with calibrated pH meter.

3.2.2.2 Moisture Content and TSS (AOAC, 2000)

Moisture content was determined according to American society of Analytical chemist 2020, method by heating sample at 105℃ overnight and calculated using following formula:

%Total suspended solid (TSS) = (dried sample weight/ fresh sample weight) ×100

% Total Moisture = 1- TSS %

3.2.2.3 Ash Content (AOAC, 2000)

Dried sample from moisture content determination was transferred to the crucible and weighted. These sample were placed in muffle furnace at 550℃ for 2 h. Final weight was measured after cooling of residue and then ash content and volatile suspended solid (VSS) were calculated.

% Ash content = (weight of residue after heating / weight of initial sample residue) ×100

%VSS = 1- % Ash content

3.2.3 Environmental Analysis of Sample

Digestion of sample with H_2SO_4-Salicylic acid-H_2O_2 (Temminghoff and Houba 2004)

In a 50 mL volumetric flask, 0.6 g of each sample was taken. In each flask, 3.3 mL of digestion mixture (Appendix I) was added, followed by four carborundum beads. The mixture was carefully swirled until the entire sample was soaked. It was then allowed to stand at room temperature overnight. For the preparation of blank, water was utilized instead of sample. The following day, sample and blank were taken on a hot plate at 180°C for 1 h. The flask was removed from hot plate, cooled down and 5 drops of pure hydrogen peroxide was added. The flask was again heated on hot plate at 280℃ for 10 min until the water was evaporated. The flask was removed from the hot plate, allowed to cool down, again 5 drops of hydrogen peroxide was added and heated again for 10 min. The treatment was repeated until the digest had turned colourless. The flask was removed from the plate and cooled to room temperature. About 10 ml of water was added and mixed, swirled until most of precipitate had dissolved. The mark was made up with water, mix well and filtered over course filter paper. The calibration blank solution was prepared in the same way as the sample prepared for further analysis. The final medium of thus prepared solution was 0.8M H_2SO_4.

3.2.3.1 Determination of Total Phosphorus (Pisal A., 2003)

Standard curve for Phosphorus

Stock solution of 1000mg/L PO_4 was prepared using potassium dihydrogen phosphate, and then 0, 1, 2, 3, 4, 5 mL of the stock solution was pipetted into a 100mL volumetric flask that already contained 40 mL ultrapure water. Then 4.5 mL of conc. H_2SO_4 was added to each volumetric flask and final volume 100 mL was made with addition of ultrapure water. A series of reference solution ranging from 0mg/L, 10mg/L, 20mg/L, 30mg/L, 40mg/L and 50mg/L were prepared.

Determination of phosphorus

All of the digested samples, standard series and blanks were diluted in water at a ratio of 1:9 (v/v). After that, 1 mL of each diluted sample was pipetted into a test tube followed by 3.8 mL of diluted mixed reagent [Appendix I]. After allowing the solution to stand for 10 minutes, the absorbance was measured in a spectrometer at 880 nm.

3.2.3.2 Determination of trace element and heavy metals

The prepared digested solution along with blank was subjected to trace elements and heavy metals estimation at National Academy of Science and Technology (NAST).

3.2.3.3 Determination of Chemical Oxygen Demand (COD) (Pisal A., 2003)

Standard curve for COD

Stock solution (1000 mg/L) of hydrogen phthalate was prepared. Then a series of reference solution ranging from 50 mg/L to 1000 mg/L were prepared from the stock phthalate solution. 2 mL of solution from each reference solution with water as blank was taken in culture tube and 1.2 mL of digestion solution was added with mixing thoroughly. Then 2.8 mL of catalyst solution was added to each tube, cap tightly and shaken properly to mix the layers. Then culture tubes were taken to digester within oven at 150℃ for 2 h. After that solution were allowed to cool down and any precipitate to settle then absorbance was measured at 600 nm using blank for background correction.

Determination of COD of sample

Similarly 2 ml of each sample was taken in culture tube and the same procedure was followed as in the standard curve preparation. Then COD of sample was calculated using standard curve.

3.2.3.4 Determination of Ammoniacal-nitrogen (Pisal A., 2003)

Standard curve of Ammoniacal-nitrogen

Ammonia nitrogen stock solution (100mg/L) was prepared using ammonium chloride in distilled water. A series of reference solution ranging from 0.1mg/L, 0.2 mg/L, 0.4mg/L, 0.6mg/L, 0.8mg/L, 1mg/L, 1.6mg/L and 2mg/L prepared by using appropriate volume of stock solution, final volume should be 5 mL. Then 2 mL of nessler's reagent was added to each tubes and mixed thoroughly. The solution was allowed to stand for 20 minutes for colour development. Then absorbance was measured at 425 nm by using blank as background correction.

Determination of ammoniacal-nitrogen

100 mL of each sample were taken in Kjeldahl flask and 5 mL of borate buffer was added. From mixed sample 30 mL of sample was taken and placed in heating gauge, distillate was collected in flask which already contained 5 mL boric acid. There the tip of condenser should be dipped into boric acid. After collecting all the distillate final volume was made 50 mL with distilled water. After that 2.5 mL of distillate was taken in test tube and 0.1 mL of nessler's reagent was added. Solution was allowed to stand for 20 minutes for colour development and absorbance was measured at 425nm using blank for background correction. Similarly water was used instead of sample for determination of ammonia nitrogen in water.

3.2.3.5 Determination of Reducing Sugars (Miller et al., 1961)

Stock solution of glucose (10mg/L) was prepared. Then a series of working solutions 0.05mg/L, 0.1mg/L, 0.2mg/L, 0.4mg/L, 0.6mg/L, 0.8mg/L, 1mg/L were prepared from stock solution using distilled water. Then, 0.2 mL of each standard, digested sample, and blank was placed in a test tube with 0.2 mL of DNS reagent. It was maintained in boiling water for 10 minutes after adding the DNS reagent. It was allowed to cool for a while and later 2mL of distilled water was added. Then absorbance was measured at 540nm for both sample and standard, background correction was done using blank.

3.2.4 Microbial strain analysis for amylase and pectinase production

3.2.4.1 Isolation of Microbial strains

Isolation of Fungal Strains from soil was done using Potato Dextrose Agar (PDA). Soil Sample was collected from the moist places where kitchen waste was predominant. Thus taken sample was serially diluted then inoculated in PDA plate and incubated at 28℃ for 48 h. After incubation, isolated colonies were selected and inoculated in PDA plate separately for subculture.

3.2.4.2 Characterization for amylase producing strain

A 48 hour incubated culture of each fungal isolate in potato dextrose broth was inoculated in well on modified starch agar media (appendix I). After inoculation plate was incubated at 28℃ for 48 h. Thus incubated plate was flooded with iodine solution then observed for clear zone around the colonies.

3.2.4.3 Characterization for pectinase producing strain

Similarly a well was prepared in Pectin agar (appendix I) plate. Then isolated strain cultured in PD broth with pectin (appendix I) was inoculated inside well of agar plate and plates were incubated at 30℃ for 24 h. After incubation plate was flooded with 1:1 v/v conc. HCl and water then observed for clear halo zone around the well.

3.2.4.4 Optimization of pH for amylase production (Alexander V. Gusakov et al., 2011)

Amylase production broth (appendix I) was prepared and then different pH were maintained in different conical flask ranging from 3.5 to 7 at 0.5 pH interval. Thus produced media were autoclaved at 121℃ for 15 minutes. After sterilizing the media 0.5 mL of 48 h fungal culture was suspended in each conical flask containing media broth. Then incubated in shaking incubator at 28℃ and 200 rpm for 48 h. After incubation the fermented broth was centrifuged at 7000 rpm for 30 minutes and then the fungal cell free supernatant was used for the estimation of amylase.

A 0.5 mL of culture extract from each flask was pipetted into test tube, then 0.5ml of 1% soluble starch in citrate phosphate buffer (pH 6.5) was added. All test tubes were incubated in water bath at 40 ℃ for 30 minutes. After that 0.5 mL of DNS reagent was added to each tube. Then reaction was stopped by boiling these tubes in water for 5 minutes. Then tubes were allowed to cool at room temperature and 10 mL of distilled water was added to each tube. Finally absorbance was measured at 540 nm using spectrophotometer. The blank was prepared in the same way using distilled water instead of culture extract.

3.2.4.5 Optimization of pH for pectinase production (Alexander V. Gusakov et al., 2011)

Pectinase production media (appendix I) was prepared similarly having different pH ranging from 3.5 to 7 in interval of 0.5. After sterilizing the media 1 mL of 48 hour incubated fungal culture was suspended in each flask with media broth. Then every flask was incubated at 28℃ and 200 rpm for 48 h. After incubation fungal cells and mycelia were removed from culture broth by centrifugation followed by filtration. Thus filtered

broth was cooled by placing in ice water bath and 20% by volume of cold acetone was added from which negligible precipitated indicated production of pectinase. Precipitate was dissolved in minimum amount of sodium acetate buffer (0.1 M, pH 4.2).

A 0.1 mL of crude enzyme was taken in a test tube and 0.4 mL of acetate buffer was added in each tube. Then 0.5 mL of 0.5% pectin solution dissolved in sodium acetate buffer was added to each tube and incubated at 30^0C for 10 minutes. After incubation 4 mL of DNS reagent was added to every tube and then tubes were taken in boiling water for 15 minutes to stop the hydrolysis. Then allowed to cool at room temperature and absorbance was measured at 575 nm. Background correction was done using blank.

3.2.5 Molecular Characterization of isolate

3.2.5.1 Extraction of gDNA

Whole DNA was isolated from amylase and pectinase producing isolate from broth culture using Murray and Thompson's modified CTAB approach (1980). The CTAB extraction buffer (2% CTAB [sigma-Aldrich, USA], 1.4 M NaCl, 0.02 mM EDTA, 100mM Tris-HCl [pH 8.0], 1% PVP) was prepared in a water bath at 60-65 ^{0}C in a 50mL falcon tube. 50-100 mg fungal colonies were mashed with 500 µL preheated CTAB extraction buffer in a sterile mortar and pestle. It was then transferred to a 2 mL Eppendorf's tube and incubated for 30-60 minutes at 60-65℃. The tubes were allowed to cool to room temperature before being filled with 500 µL chloroform: isoamylalcohol (24:1) and well mixed after incubation. The tubes were then centrifuged at 9000 rpm for 8 minutes. The upper aqueous layer was transferred to a new Eppendorf's tube, and an equal volume of cold isopropanol was added, carefully mixed, and incubated at -20^0C for 1 h. The tubes were centrifuged at 9000 rpm for 8 minutes at room temperature. The pellet was washed twice with 200-300 µL of 70% ethanol and centrifuged for 1-2 minutes at 9000 rpm, with the supernatant discarded. The ethanol was completely discarded. Before being resuspended in 100 µL tris buffer, the DNA pellets were dried in the air. Electrophoresis on a 1% agarose gel was used to confirm the genomic DNA. The remaining DNA was stored at -20^0C for future use.

3.2.5.2 PCR Amplification of gDNA

Newengland Biolab's 18S rRNA universal primers were used to amplify the gDNA. The forward and reverse primer sequences were 5'GGTCTTGTAATTGGAATGAG 3' and 5'CTTCCGTCAATTCCTTTAAG3', respectively.

Table 3.1: PCR components used for amplification of gDNA

Component	Volume
Master mix	10 µL
$MgCl_2$	0.6 µL
Forward primer	1 µL
Reverse primer	1 µL
Nuclease free water	6.4 µL
Template	1 µL
Total	20 µL

Table 3.2: PCR Condition for amplification of gDNA by 18S rRNA

Steps	Temperature /time	
Initial denaturation	95 ^{0}C for 2 minutes	
Denaturation	90 ^{0}C for 30 seconds	
Annealing	51 ^{0}C for 30 seconds	30 cycles
Extension	72 ^{0}C for 70 seconds	
Final extension	72℃ for 5 minutes	

The PCR was carried out in a Biorad thermal cycler using different components and condition as given in table 3.1 and 3.2 respectively. Aliquots of 5.0 µL of PCR amplified products and a 100-bp ladder (Invitrogen Life Technologies, USA) were loaded onto a 1% agarose electrophoresis gel (Sigma Chemical, USA) and run at 50 V for 1 h with 1x Tris-acetate-EDTA buffer (40 mM Tris-HCl, pH 8.3, 20 mM acetic acid [Merck, Germany], and 1 mM EDTA [Ethidium bromide was used to stain the gel. After that, it was examined using a UV transilluminator.

3.2.5.3 Phylogenetic Analysis

The isolate's PCR product was forwarded to the Nepal Academy of Science and Technology (NAST) to be sequenced. Sequencher 4.1.4 was used to modify the sequences obtained. NCBI Blast was used to compare sequences. Finally, the MEGA11 software was used to align the sequences and create a phylogenetic tree using the neighbor-joining approach.

3.2.6 Construction of MFC

MFCs are made up of two chambers, anodic and cathodic chambers, two electrodes (anode and cathode), and a salt bridge. At anaerobic conditions, the anodic chamber contains a substrate and biocatalyst. At the cathodic chamber, an aerobic environment is maintained. Dual Chambered fuel cell of 350 mL capacity was taken and marked as an anode and cathode. In the anodic chamber, 300 mL of sample was used as an anodic inoculation, whereas in the cathodic chamber, 300 mL of acetate buffer solution was utilized as a cathodic solution. Proton exchange membrane (PEM) situated in between anodic and cathodic chambers act as salt bridge. Graphite sheets were used as electrodes and Nafion 177 was used as proton exchange membrane.

3.2.6.1 Membrane Treatment (Najafpour et al., 2010).

Nafion membrane having diameter of 5 cm treatment was done in four steps. Firstly membrane was boiled in 3 % H_2O_2 at 100℃ for 2 h. Then membrane was taken in distilled water and boiled for 2 h at 100℃. Again membrane was boiled at 100℃ in 0.5 M sulfuric acid for 2 h and finally in distilled water for 2 h at 100℃.

3.2.6.2 Electrodes Treatment (Swain G.M. 2007)

Graphite sheets (10cm×2.8cm) were used as anode and cathode. Copper wires were used to connect the electrodes on each side of the chamber. These electrodes were chosen because they are affordable and readily available. Before use, electrodes were ultra sonicated with 70% methanol, 70% acetone, followed by distilled water and finally 15 minute under UV light.

3.2.6.3 Electrode Modification with PANI/MWCNT nanocomposite (Abdulla, 2015)

3.2.6.3.1 Purification of MWCNT

MWCNT was first acid treated under reflux conditions using an H_2SO_4/HNO_3 mixture to increase dispersion and surface reactivity, as well as to eliminate other carbonaceous compounds created during synthesis. In a round bottom flask equipped with a condenser,

0.1 g of MWCNTs were treated with a mixture of sulfuric acid (3 M) and nitric acid (3 M) (3:1 ratio). The mixture was ultra sonicated for 30 minutes, stirred for 30 minutes, and then refluxed for 8 h at 120°C. The resultant solution was diluted in water and cleaned until it reached a pH of neutral. The sample was dried overnight in oven at 60℃.

3.2.6.3.2 Synthesis of PANI/MWCNT nanocomposite

PANI/MWCNT nanocomposites were synthesized using an in-situ oxidative polymerization technique. At 0℃, 1 mg of MWCNTs was combined in an aniline/HCl (1:1) solution, resulting in monomer adsorption on the CNT surface. The reaction time was set at 12 h. A solution of (0.1 M) APS dissolved in 1 M HCl was used to drop the aforementioned mixture drop by drop. The polymerization process took 6 h at 0 °C. The PANI/MWCNT nanocomposite was created by centrifuging the material several times with deionized water and methanol until it turned into a greenish black powder. After that, the material was vacuum dried for another 24 h at 60°C.

3.2.6.3.3 Treatment of Graphite Electrode

For 6 h, the surface of the graphite electrode was modified using an ultrasonication bath containing H_2SO_4 and HNO_3 (3:1 v/v). The Graphite Electrode was then repeatedly washed with distilled water until the pH of the washing solution reached 7. After that, it was air dried before being used.

3.2.6.3.4 Coating of Electrode with Nanocomposite

Finally, synthesized PANI/MWCNT composite was combined with a solvent like N-methyl-2 pyrrolidone. After dipping a clean graphite electrode in the mixture and ultrasonicated for about 15 minutes. Then electrode was dried in an oven for about 12 h at 50-60 ℃.

3.2.6.4 MFC with fed-Batch mode

Dual chambered MFC containing both electrode made of graphite sheet in two chambers called anode and cathode. The working volumes of anode and cathode were 300 mL of each substrate and acetate buffer respectively. Then, anode chamber was inoculated (4% v/v) with the enrichment fungal culture and operated at room temperature in fed-batch mode. OCV generation was observed up to 72 hours and the recycling of sample was done in every 24 h. During each feeding, 30 mL (10% of initial sample) sample was taken from the anolyte and the same volume was replaced with fresh medium (C.-H. Lay et al., 2015).

3.2.7 Cyclic Voltammetry

Hokuto-Denko HA151 potentiostat was used in combination with national instrument Lab View work station. During the stabilized period of operation, CV investigated the bio-electrochemical behavior of MFC during power generation, measurement were performed in a three electrode arrangement. CV was performed by applying a potential ramp to the working electrode (anode of MFC; graphite) against the Ag/AgCl reference electrode (RE) at a scan rate of 10 mV/s over the potential range of -0.8 to +0.8 V. All electrochemical experiments were carried out in situ in MFCs, with the anode (graphite) working as the working electrode and the cathode (graphite) working as the counter electrode against RE. The cycle voltammetry was done for 1 cycle only, in Central Department of Chemistry, Tribhuvan University (CDC, TU). (Venkata Mohan et al*.,* 2010). The current generated from the electrochemical cell was observed in different interval of potential applied in graphite electrode (anode).

3.2.8 Analysis of substrate and byproducts by High Performance Liquid Chromatography (HPLC)

3.2.8.1 Sugar Analysis in Sample (Van Wychen S. et al., 2015)

Digestion of sample and generation of calibration standard

Five different concentration i.e 0.05, 0.25, 1.25, 2.5 and 4 mg/mL of various sugars were prepared in different reagent bottle. Glucose, Fructose, Maltose, Galactose, Xylose and Arabinose were used for calibration curve. A 3 mL of 72% H_2SO_4 was added to each reagent bottle and incubated at 30±3℃ for 60 minutes. All bottles were stirred in every 10 min without removing from water bath. Similarly 0.3 g of sample was taken in heat resistant reagent bottle and 3 mL of 72% H_2SO_4 was added. The bottle was placed in water bath at 30±3℃ and incubated for 60 minutes. Sample was stirred in every 10 minutes without removing from water bath. After that concentrated acid was diluted up to 4% by adding 54 mL deionized water and mixed sample by inverting several times to eliminate phase separation between high and low concentration acid layers. Then mixed samples were autoclaved at 121℃ for 1 h and allowed to cool to room temperature.

Sample Preparation for HPLC Analysis

From hydrolysis liquor 20 mL was transferred to another flask and each sample was neutralized up to pH 6 using calcium carbonate in stepwise swrilling the samples and pH was checked periodically during the addition of calcium carbonate. After reaching pH, sample was allowed to settle decant off the supernatant into tubes. The neutralized

sample was centrifuged and supernatant was filtered through 0.2 mm syringe filter. Thus filtered hydrolysate was placed in clean and labelled HPLC vial and cap tightly.

HPLC Analysis for Sugar

The Calibration Standard and sample were analyzed by using carbohydrates specific column; Zorbax carbohydrate analysis column (4.6×250 mm, 5 micron). Mixture of HPLC water and acetonitrile (25:75) was used as mobile phase with flow rate 1 mL/min. Column temperature was 25℃ and detected on the basis of Refractive Index (RID). Injection volume of sample was 20 µL.

3.2.8.2 HPLC Analysis for MFC end products

For standard calibration, organic acids such as acetic acid, citric acid, propionic acid, succinic acid, and lactic acid, Glucose, fructose, and ethanol are used. For quantitative study of compounds produced in MFC, all standard were taken in five different concentrations: 0.1, 1, 5, 10, and 15 mg/mL. Thus prepared series of calibration standard compound were filtered through 0.2 mm syringe filter and taken in HPLC vial. Sample taken from MFC in eppendorf tube and centrifuged. Supernatant was filtered using 0.2mm syringe filter and the kept in HPLC vial. Aminex HPX 87H (250×4 mm) column was used for end product analysis. 5mM sulfuric acid was used as eluent (mobile phase) with flow rate 0.6mL/minute. Column temperature was 50℃ and detected on the basis of Refractive Index (RID) with sample injection volume 10 µL (Bio-Rad, 2012).

CHAPTER 4: RESULTS AND DISCUSSION

Despite the fact that there are limited research available on solid waste management, several studies were done on the degradation of potato peels for energy generation. The degradation efficiency of waste depends on the content present in potato peels waste (Achinas S. et al., 2019). Potato peels mainly consist of starch, non-starchy polysaccharides such as pectin, xylose etc. Similarly it contain magnesium, potassium, phosphorus, iron etc as microelements. So that in this research various compounds present in potato peels waste sample were determined. The standard curve of absorbance against concentration of various chemicals drawn to determine the concentration of parameters present in the waste sample. The standard curves for COD, ammoniacal-nitrogen, phosphorus, reducing sugar were plotted to determine the concentration [Appendix II].

4.1 Determination of Environmental parameters of Sample

Table 4.1: Different physical parameters in potato peels waste sample

Characteristics of Sample	
pH	6.51±0.081
Total Suspended Solid (TSS) %	21.4±1.275
Volatile Suspended Solid (VSS) %	88.31±7.056
Ash Content %	11.68±7.056
Moisture Content %	78.6±1.267

Table 4.2: Different analytical parameters of potato peels waste sample

Analytical Parameters of Samples	**Concentration mg/g**
Reducing Sugars	1.061±0.64
Ammonical Nitrogen	0.011±0.01
Chemical Oxygen Demand (COD)	10.24±0.12
Phosphorus	0.015±0.017

Table 4.3: Analysis of different metal ions in potato peels waste sample by AAS

Analytical Parameters	**Concentration mg/g**
Iron	0.167
Copper	0.007
Lead	0
Nickel	0
Zinc	0.007
Manganese	0.002

The analytical test of the collected samples was performed for various parameters such as pH, moisture content, ash content, chemical oxygen demand (COD), ammoniacal nitrogen , reducing sugar, phosphorus, total suspended solid (TSS), volatile suspended solid (VSS) which is shown in table above. Potato peels waste contained moisture content 78.6±1.267 %, TSS 21.4±1.275%, VSS 88.31±7.056 % and ash content 11.68±7.056 %. Similarly the pH of sample was 6.51±0.081 which is shown in table 4.1. The moisture level of potato peels was determined to be 70-75%, with just 25-30 % dry matter left, according to Norell R. J., 2016.

The waste sample contained variable amounts of Ammoniacal-nitrogen, reducing sugars, Chemical Oxygen Demand (COD), phosphorus which is shown in table 4.2. According to Liang S. et al., 2015 total carbohydrates in potato peels is 8.7–12.4 mg per 100 gram. Starch, organic acids, and micro and macro-elements such as sodium, potassium, phosphorus, and calcium are abundant in the peel. It's also high in vitamin C, fat, and glucose.

The data in table 4.3 was the concentration of various metals and trace elements in waste sample. According to this result potato peels contained iron 0.167 mg/g, trace amount of copper and zinc 0.007 mg/g and manganese 0.002 mg/g. According to Lopez A. et al., (1980) amount of iron was 0.59±0.12 mg, copper 0.13±0.03mg, zinc 0.27±0.05mg and manganese 0.24±0.12mg in per 100 g of sample. Amount of iron in our sample was higher than the literature mentioned. Similarly, amount of Lead and Nickel was below the detection limit of the AAS instrument.

4.2 Screening of Fungal isolate for enzyme production

4.2.1 Test of Fungal isolate for amylase production

A clear zone of starch hydrolysis was seen around the well on media plate showed that the organism produces amylase which hydrolyzed the starch present in media.

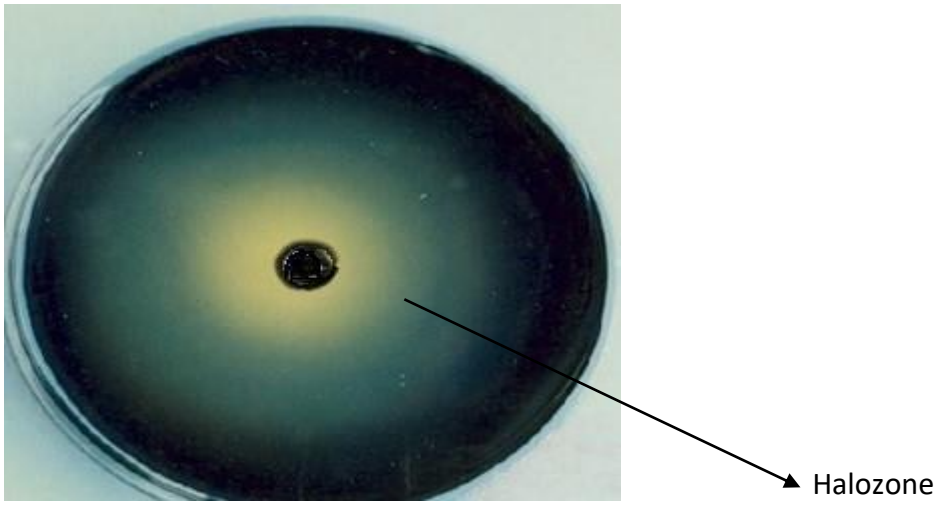

Figure 4.1: Test for amylase activity by starch hydrolysis test; halozone formed by isolate on starch agar media flooded with iodine

4.2.2 Test of fungal isolate for pectinase production

A halo zone around the well was seen in the pectin media plate flooded with 1:1v/v HCl indicated the production of pectinase by the isolate.

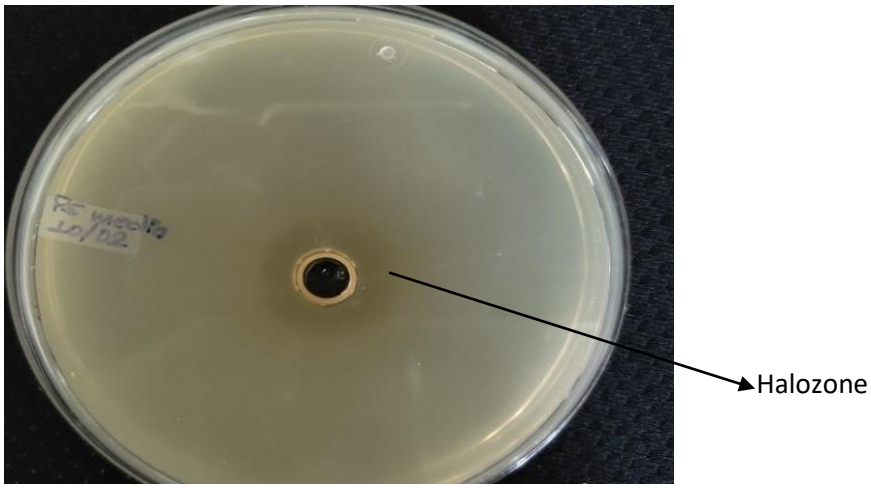

Figure 4.2: Test for pectinase activity halozone formed by isolate on pectin media flooded with 1:1 v/v HCl

4.2.3 Optimization of pH for amylase and pectinase

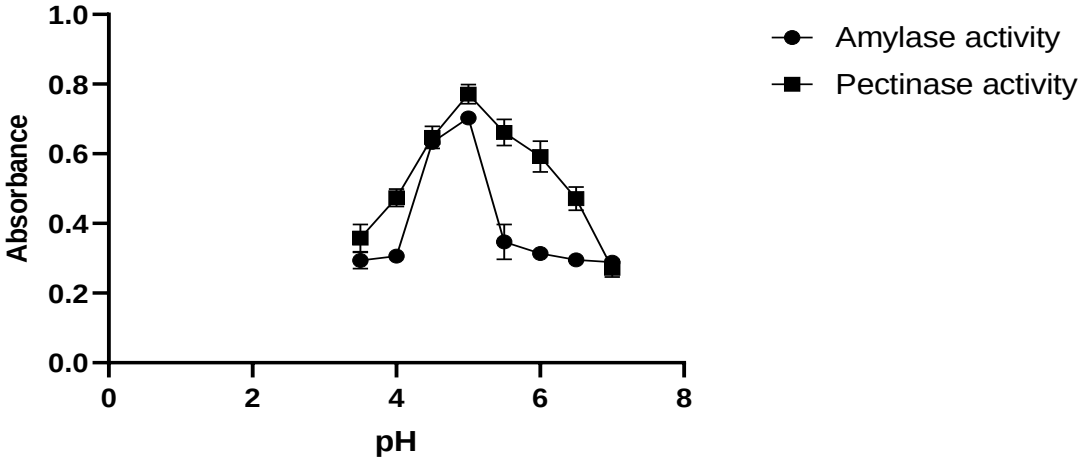

Figure 4.3: Activity of enzymes produced by fungal isolate in media having different pH

Figure 4.3 shows the effect of pH on activity of amylase and pectinase enzymes. The highest activity of both enzyme was found at pH 5, which was used further in MFC operation

Screening test of fungal isolate for enzyme production was done and results shows that the isolate produced both amylase and pectinase enzymes. Amylase production of isolate was confirmed by starch hydrolysis test using iodine which was shown in figure 4.1. Similarly pectinase production was confirmed by observing halozone around isolate in pectin media when flodded with 1:1 v/v HCl shown in figure 4.2. According to Wang et al., 2017 *Aspergillus niger* have the ability to produce amylase enzyme which can be used in starch degradation process. Similarly Qian li et al., 2020 showed that pectinase producing ability of same organism which can be used in degradation of pectin component.

Figure 4.3 depicted the synthesis of enzymes based on the pH of the media. The pH of media was adjusted from 3.5 to 7 in the interval of 0.5, and the enzyme activity was determined using the DNS technique. In the case of amylase, the greatest enzyme activity was observed at pH 5, followed by a quick reduction at pH 5.5. In the case of pectinase, the highest activity was found at pH 5 and slightly lower at pH 5.5. As a result of this finding, pH 5 is a favorable condition for both enzyme production and the optimal condition for organisms to produce enzyme. According to Ogbonna et al., 2014 highest activity of amylase produced by *Aspergillus niger* was found in pH 5 followed by pH 6 then pH 7.

4.3 Molecular Characterization of Fungal strain

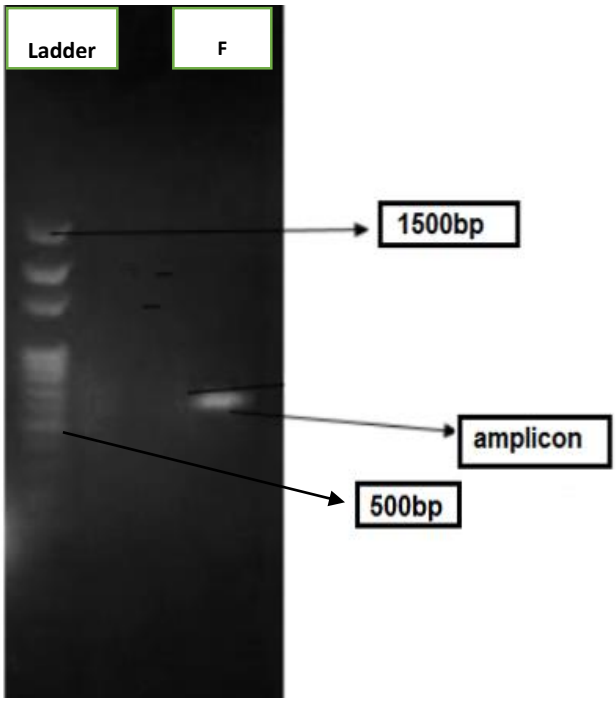

Figure 4.4: PCR product of fungal isolate amplified by 18S rRNA primer

Table 4.4: Molecular Characterization of fungal Isolate

Isolate label	Activity Showed	Isolate Identified as	Genebank accession number
F	Amylolytic and pectinolytic	*Aspergillus niger*	OK353813

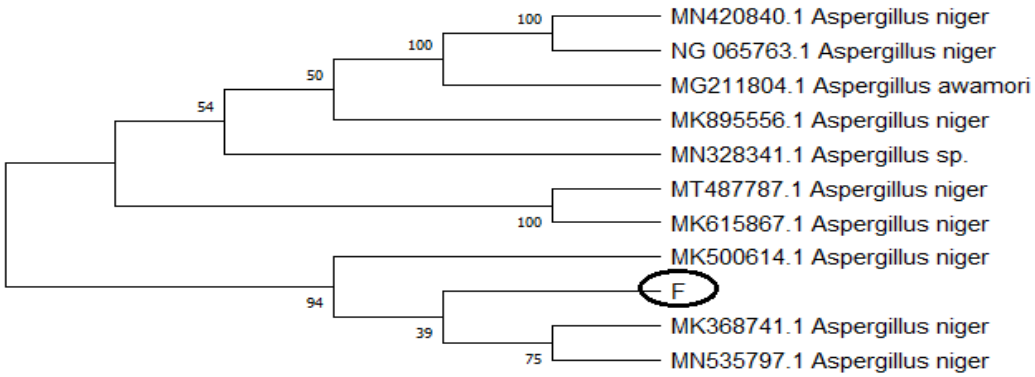

Figure 4.5: Phylogenetic tree construct by neighbour-joining method of strain F

DNA of fungal isolate was extracted using modified CTAB method. The 18S rDNA region of fungal isolate was amplified by universal 18S RNA primer. The amplified DNA regions were confirmed by electrophoresis in 1% agarose gel using 100 bp ladder. The size of isolated fungal amplicon was around 700 bp shown in figure 4.4. Amplified products was sent for sequencing in National Academy of Science and Technology. The obtained sequences were edited by sequencher software, aligned by Bioedit software and analyzed by NCBI blast. Isolate was found to resemble as *Aspergillus nige*r. Genebank accession number of isolate is shown table 4.4. A phylogenetic tree was developed neighbour-joining method using MEGA11 software shown in figure 4.5.

4.4 Construction of MFC for enhancement of electricity

4.4.1 Effect of substrate concentration in MFC operation

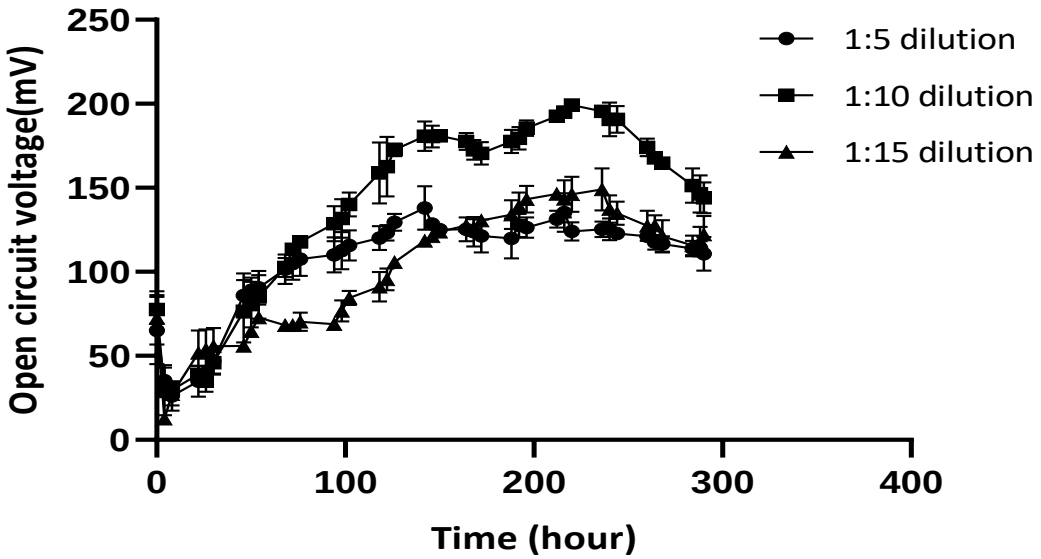

Figure 4.6: Effect of concentration of substrate on open circuit voltage at different time

Figure 4.6 shows that the curve obtained when different concentration of substrate were used in MFC operation. Maximum OCV obtained using 1:5, 1:10 and 1:15 dilution of substrate was found 450±36 V/m^3, 663±6 V/m^3 and 496±40 V/m^3 respectively. Error bars represents the standard error in triplicate experiment. The highest OCV value was shown in MFC using 1:10 dilution, which was used in further MFC operation.

4.4.2 Effect of different catholytes in MFC operation

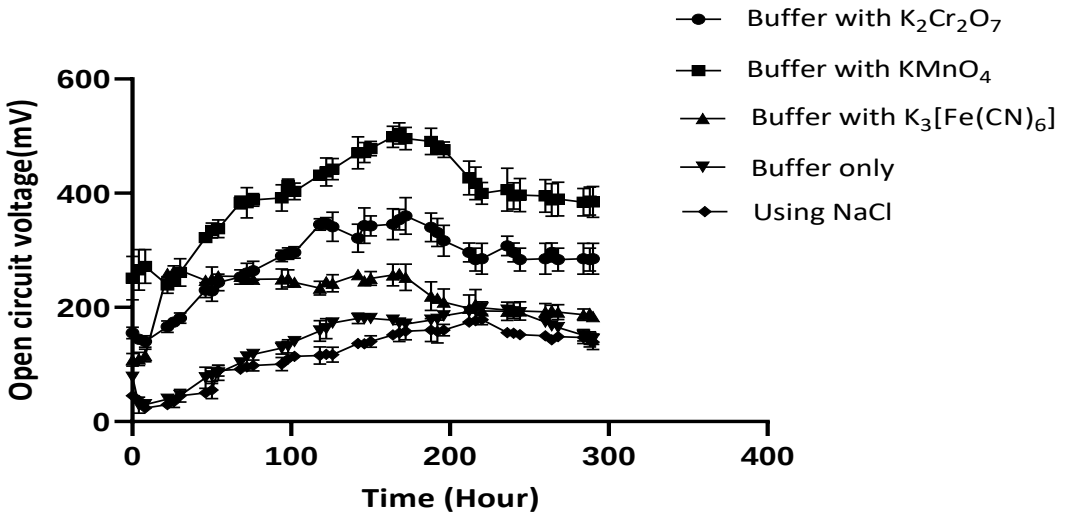

Figure 4.7: Effect of Different catholytes used in cathode on open circuit voltage at different time

The open circuit voltage reading of an MFC using various electron acceptor in the cathode is shown in the figure 4.7. According to this finding, OCV using normal buffer as control was found 663±6 V/m^3 which is nearly equal with using NaCl as catholyte 636±23 V/m^3. Three different electron acceptor used in catholyte $KMnO_4$ has the highest OCV i.e 1680±60 V/m^3 followed by $K_2Cr_2O_7$ i.e 1156±33 V/m^3 and then $K_3[Fe(CN)_6]$ i.e 863±26 V/m^3.

4.4.3 Effect of sterilized and unsterilized substrate in MFC Operation

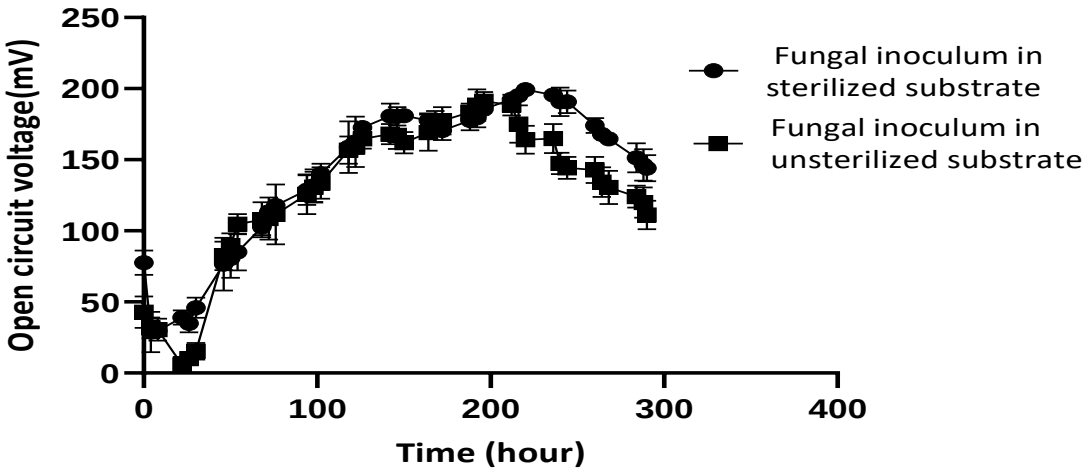

Figure 4.8: Effect of fungal inoculum in sterilized substrate and unsterilized substrate containing other microorganisms on open circuit voltage at different time

Figure 4.8 shows the time versus open circuit voltage curve obtained when fungus inoculum used in sterilized substrate and substrate containing other microorganism. When pure fungus culture was used as inoculum in sterilized anode sample the OCV value was found 663±6 V/m^3 whereas when unsterilized sample was used the OCV value was found to be 637±23 V/m^3.

4.4.4 Effect of Electrode modification in MFC Operation

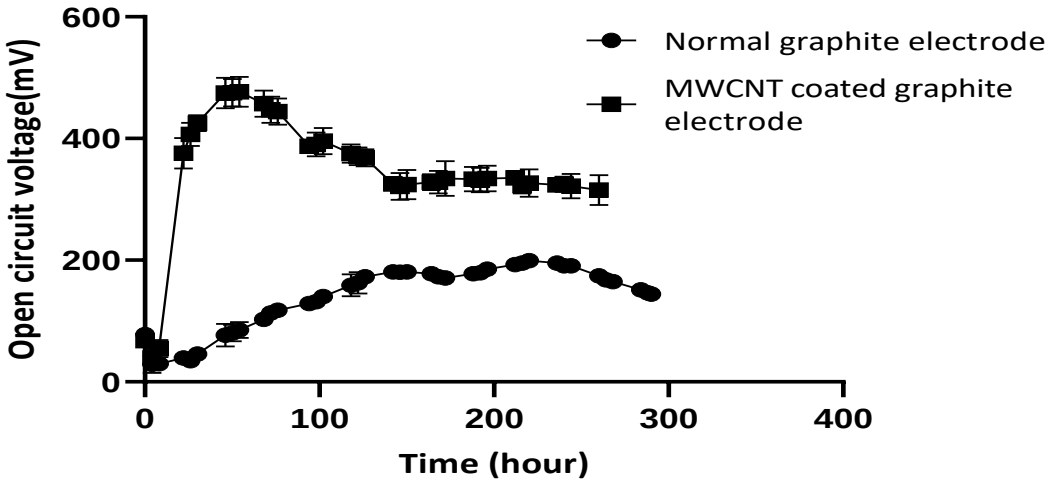

Figure 4.9: Effect of Electrode modification on open circuit voltage at different time

The time versus open circuit voltage of normal and MWCNT/PANI coated graphite electrode using acetate buffer in cathode is shown in figure 4.9. From this result we can concluded that the use of MWCNT coated anode clearly improve in open circuit voltage generation i.e. 1586±63 V/m^3 than using normal electrode i.e. 663±6 V/m^3. In MWCNT electrode maximum OCV was found in 2nd day of operation and final pH of sample after 12 days was found to be 3.5.

Enhancement of current generation in MFC was done using various experiments, in every steps the better condition was preferred for further experiments. Effect of different concentration of sample in anode was shown in figure 4.6, according to which 1:10 dilution of sample was better for further operation with highest open circuit voltage (OCV) followed by 1:15 and 1:5 dilution. MFC was operated up to 12 days and then final pH of sample was found to be 3.5. Thus, obtained data was compared with the data from Ghoreyshi et al., 2013, according to which as substrate concentration increases the OCV value was minimal. That was because the most of glucose remained unconsumed at high concentrations. However in lowest concentration up to 5 % the OCV value was minimum due to limitation of substrate after that OCV was decreases with increases substrate

concentration. According to this result 1:10 dilution generate highest value of OCV which was taken for further MFC operation.

The open circuit voltage reading of an MFC using various catholytes is shown in the figure 4.7. Using acetate buffer only in cathode was taken as control. According to this finding, out of three different electron acceptor $KMnO_4$ has the highest OCV followed by $K_2Cr_2O_7$ and $K_3[Fe(CN)_6]$. The concentration of these cathode electron acceptors were 10mM added in acetate buffer as catholyte. The final pH of sample in MFC after 12 days of operation was found 3.25-33.56. Similarly 5% NaCl was also used as catholyte instead of buffer, and the OCV generation using acetate buffer and NaCl was found in same range. Due to the reduction in oxygen transport into the anolyte, using a NaCl catholyte enhanced the CE to 43–60% (Ahn and Logan, 2013). The NaCl MFC had a maximum power density of 491 mW/m^2, which was only 17% lower than the MFC using 50 mM PBS. In comparison to using a normal buffer in the cathode, MFCs with various electron acceptors produce a higher voltage and using $KMnO_4$ generates highest value of OCV. According to Pandit et al., 2011 highest value of OCV was generated using the same concentration of potassium permagnate i.e 1110 mV followed by potassium ferricyanide and potassium dichromate both have nearly equal value of OCV i.e. 789mV and 769mV in 1 Liter of Sample.

Similarly figure 4.8 shows that the OCV obtained when sterilized sample and unsterilized sample used in anode. When sterilized sample was used only fungus was added as inoculum in anode sample and the OCV value was found 663±6 V/m^3, whereas when unsterilized sample was used the OCV value was found 637±23 V/m^3. According to Islam et al., (2020) co-culture of microorganism enhance the OCV generation in MFC than using pure culture. To evaluate the existence of mutualistic interactions between the microorganisms, co-culture and mixed culture inoculums were created using various combinations of targeted pure culture. In above result both condition had similar value of OCV. When sterilized sample with fungal culture was used the curve was going in a constant way while in microbial co-culture declined after reaching peak that might be due to acidic condition of media produced by other microorganism because the final pH was found very low i.e 2.8, or this is also might be due to the other microorganism present in sample decreased the degradation ability of fungal culture used as inoculum.

Depending on the type of modification, this method was an attempt at electrode modification, i.e. anode treatment, in order to create improved output. The comparison in OCV generation between using multiwalled carbon nanotubes (MWCNT) coated electrode and normal graphite electrode (not coated) using acetate buffer in both cases is shown in figure 4.9. From this result it can concluded that the use of MWCNT improve OCV generation than normal electrode. In MWCNT electrode maximum OCV was found

in 2nd day of operation and final pH of sample after 12 days was found to be 3.5. Thus obtained result might be due to changes in physical and chemical properties after the modification of electrodes and MWCNT increased the surface area of electrode. In MFCs, high conductivity and a wide unique surface area greatly improved charge transfer efficiency and biofilm formation on the electrode surface. The MWCNT nanocomposite was found to be a desired anode material for MFCs when the power density of the proposed MFC was compared to that of other MFCs in the literature. According to Fan M. et al., 2017, unmodified MFCs had the lower power density (375.58 W/m^3), followed by MFCs modified with MWCNTs (457.75 mW/m^3).

From all the above experiments, 1:10 dilution of sample concentration was used in anode. 50mM of acetate buffer was suitable with addition of $KMnO_4$ in cathode and was used for power generation. Error bars represents the standard error in triplicate experiment.

4.5 Open circuit voltage in MFC recycling substrate in 24 hours interval of time (Fed Batch)

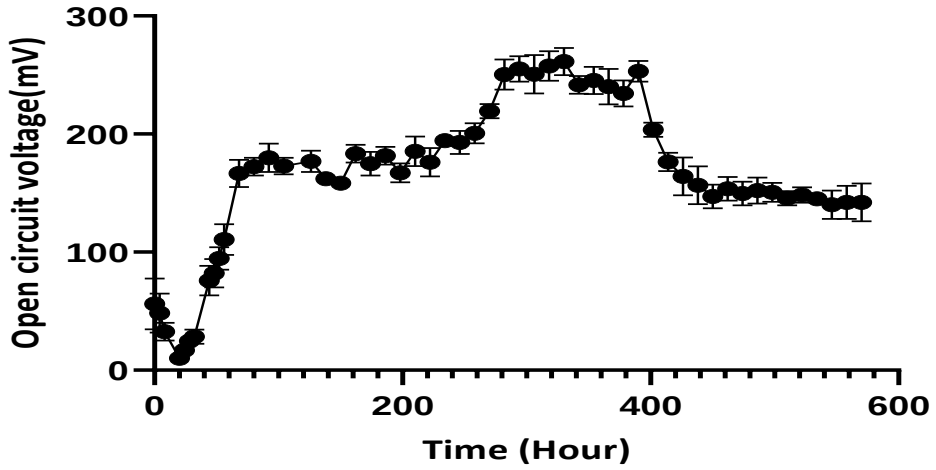

Figure 4.10: Effect on open circuit voltage observed on MFC with continuous addition of substrate at 24 hours interval of time

Figure 4.10 shows that the open circuit voltage (OCV) obtained from MFC operated in fed batch mode. After 72 h of operation sample 10% of initial sample was added in interval of every 24 h. Experiment was operated up to 25 days and maximum OCV was found 873±36 V/m^3 at 14th days of operation which is the clearly improved result that MFC operation without recycling the substrate. After 15 days, the level of OCV generation remain constant, it can be operated more and more days using buffer only in cathode because the pH was found 4.2 at the end of 25 days. According to Choudhury et al., 2020 in the beginning, 100 mL of sample solution containing 0.2 % milk powder was used in MFC with

minimal medium and run for 72 h. In addition, after 72 h of operation, 50 mL of sample was fed. The pH of the media was then increased to 7.4 and the sample concentration also increased. The results reveal that the fed-batch operation successfully caused changes in pH in the media, resulting in an increase in OCV. Within 3 h of fed-batch operation, OCV has climbed even more, reaching a maximum of 664 mV after 80 h of operation.

4.6 Power Generation in MFC using Different Resistors

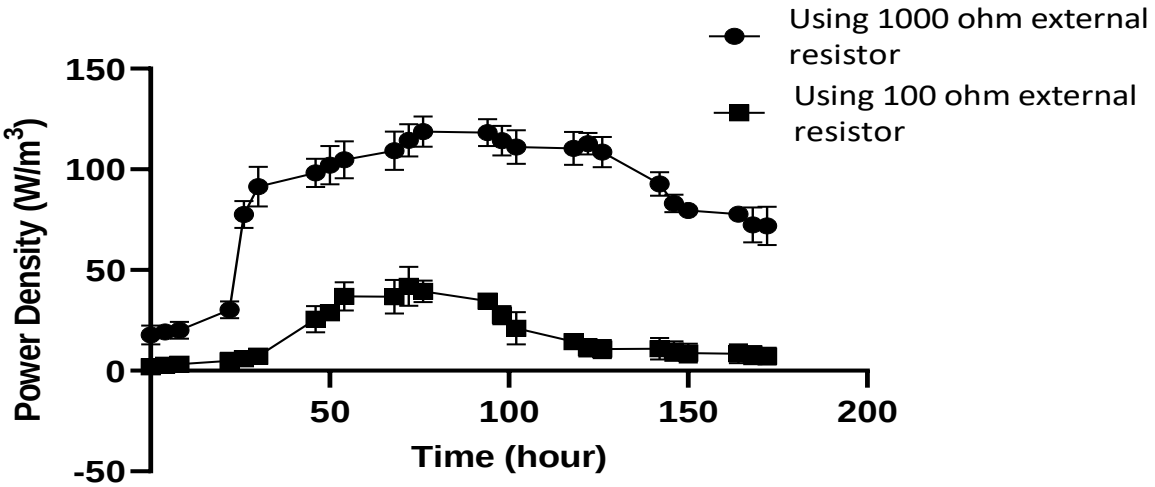

Figure 4.11: Effect of external resistor on power generation in Microbial fuel cell

[Note: p value = 0.0305, 95% level of confidence]

Power generation in MFC using Different two external resistors was calculated. This experiment was done to observe effect of external resistance on the overall performance of MFC. The maximum Power density generated using 1000 ohm resistance and 100 ohm resistance was found to be 396±23 W/m^3 and 140±30 W/m^3 respectively shown in figure 4.11. Error bars represent standard error based on measurements from triplicate operation. At 95 % level of confidence, using two tailed test p value was found 0.0305 (less than 0.5) between using 100 and 1000 ohm resistor. Thus the experiment was significant at 5% level of significance. According to Gonza'lez et al., 2014 in the first day, increasing the external resistance from 1 k to 33 k resulted in a power increase of 4.5× 10^{-3} mW to 1.386 ×10^{-3} mW. Similarly it found that reducing the external resistance from 2.7 k to 2.2 k resulted in a power reduction from 1.69 ×10^{-3} mW to 1.27 ×10^{-3} mW. In MFC external resistance corresponded to the lowest internal resistance and highest removal efficiency of organic matter in sediment (Tian-Shun Song et al., 2010). Similarly lower internal resistance favored both high power density and current density.

4.7 Analysis of different components reduction after 12 days in MFC operation

Table 4.5: Removal of COD with different modes of MFC Operation

	Initial concentration mg/g	**Final concentration mg/g**	**% Reduction**
Fungal inoculum in sterilized sample	10.24±0.12	6.97±0.13	31.93
Fungal inoculum in unsterilized sample	10.24±0.12	7.67±0.11	25.09
Using NaCl as catholyte	10.24±0.12	8.25±0.04	19.43
Using $KMnO_4$ in cathode	10.24±0.12	6.38±0.08	**37.69**
Using $K_2Cr_2O_7$ in cathode	10.24±0.12	6.44±0.11	37.10
Using KCN in cathode	10.24±0.12	6.81±0.12	33.49
Using MWCNT coated electrode	10.24±0.12	6.45±0.35	37.01
MFC with fed batch	10.24±0.12	6.95±0.47	32.13

The COD removal rate of different setup of MFC operation were determined using initial and final amount of COD in sample. Maximum COD removal was found to be 37.69% by using $KMnO_4$ as electron acceptor in cathode whereas minimum COD removal was found 19.43 % in using NaCl as catholyte shown in table 4.5.

Table 4.6: Removal of Ammonia-Nitrogen with different modes of MFC Operation

	Initial concentration mg/g	**Final concentration mg/g**	**% Reduction**
Fungal inoculum in sterilized sample	0.011±0.01	0.004±0.014	63.23
Fungal inoculum in unsterilized sample	0.011±0.01	0.0038±0.048	65.58
Using NaCl as catholyte	0.011±0.01	0.0039±0.014	64.54
Using $KMnO_4$ in cathode	0.011±0.01	0.0036±0.025	**67.72**
Using $K_2Cr_2O_7$ in cathode	0.011±0.01	0.0038±0.025	65.45
Using KCN in cathode	0.011±0.01	0.0037±0.011	66.36
Using MWCNT coated electrode	0.011±0.01	0.004±0.042	63.63
MFC with fed batch	0.011±0.01	0.005±0.01	54.54

Similarly, the removal of ammoniacal-nitrogen in waste sample after each MFC operation was determined. Maximum amount of ammoniacal-nitrogen reduction was found 67.72% in using $KMnO_4$ in cathode whereas minimum amount of ammoniacal nitrogen reduction was 54.54 % found in MFC with fed batch shown in table 4.6.

Table 4.7: Removal of reducing sugar with different modes of MFC Operation

	Initial concentration mg/g	**Final concentration mg/g**	**% Reduction**
Fungal inoculum in sterilized sample	1.061±0.64	0.29±0.003	72.64
Fungal inoculum in unsterilized sample	1.061±0.64	0.24±0.008	**77.16**
Using NaCl as catholyte	1.061±0.64	0.29±0.01	72.64
Using $KMnO_4$ in cathode	1.061±0.64	0.44±0.006	58.49
Using $K_2Cr_2O_7$ in cathode	1.061±0.64	0.55±0.02	48.11
Using KCN in cathode	1.061±0.64	0.50±0.007	52.83
Using MWCNT coated electrode	1.061±0.64	0.47±0.03	55.66
MFC with fed batch	1.061±0.64	0.52±0.12	50.98

Thus, removal of reducing sugar was determined from initial and final amount of reducing sugars. Maximum amount of reducing sugar removal was found 77.64 % in MFC operation using unsterilized sample and minimum amount was found 50.98 in MFC with fed batch shown in table 4.7. According to this result, 1mg/g of reducing sugar removal generate approximately 860 V/m^3 of open circuit voltage using pure culture of fungus in 1:10 dilution of sample.

Table 4.8: Removal of TSS with different modes of MFC Operation

	Initial concentration mg/g	**Final concentration mg/g**	**% Reduction**
Fungal inoculum in sterilized sample	405	145	64.19
Fungal inoculum in unsterilized sample	405	150	62.96
Using NaCl as catholyte	405	175	56.79
Using $KMnO_4$ in cathode	405	171	57.78
Using $K_2Cr_2O_7$ in cathode	405	180	55.55
Using KCN in cathode	405	168	58.52
Using MWCNT coated electrode	405	182	55.06
MFC with fed batch	405	138	**65.95**

Total suspended solid removal was also determined after each MFC operation. The maximum TSS removal was found in MFC with fed batch mode i.e 65.95% and minimum 55.55% using $K_2Cr_2O_7$ as electron acceptor.

Degradation of waste biomass and reduction in various parameters such as COD, reducing sugars, ammoniacal-nitrogen etc were calculated by determining initial and final concentration of theses parameters. Table 4.5 shows that the COD removal rate of different setup of MFC operation. Maximum COD removal was found in using $KMnO_4$ as

electron acceptor in cathode 37.69 % whereas minimum COD removal in using NaCl as catholyte. According to M.I. Din et al., 2020 COD removal rate is 40%, and the potato wastewater demonstrated its COD removal potential, indicating the role of microorganism in wastewaters in metabolizing carbon sources as electron suppliers. The observed COD reductions showed that the degradation rate was faster than the hydrolysis rate or that the hydrolysis reaction had already completed, and the COD reduction might possibly be attributable to active oxidation of substrate by microorganism present in MFC during treatment.

Similarly, the removal of ammoniacal-nitrogen in waste sample after MFC operation was determined. There was a huge reduction in ammoniacal-nitrogen by 54.54 to 67.72 % as given in table 4.6. With the passage of time, the amount of ammonia decreased linearly. Using a graphite electrode, the highest reduction after two weeks was approximately 55.9%. The amount of reduction differed depending on the type of material. The activated carbon sample produced a larger removal potential than any other anode material during the process (Alabiad et al., 2017).

Reducing sugars reduction was minimum at using $K_2Cr_2O_7$ i.e. 48.11% and maximum at using unsterilized sample in anode i.e. 77.16% shown in table 4.7. Unsterilized substrate might contains other microorganism so that these consumed more reducing sugars in comparison to another MFC operation which contains only fungal inoculum. According to this result, 1mg/g of reducing sugar removal generate approximately 860 V/m^3 of open circuit voltage using pure culture of fungus in 1:10 dilution of sample.

Total suspended solid removal was also determined which is shown in table 4.8. The maximum TSS removal was found in MFC with fed batch mode i.e 65.95% and minimum 55.55% using $K_2Cr_2O_7$ as electron acceptor. Maximum amount of TSS removal was 65.95%, this result indicates that pretreatment of sample might be helpful for more amount of TSS removal. According to Yushi Tian et al., 2017 MFCs fed with 10 g/L PPW removed 56.8% of total suspended solids, while MFCs fed with 20 g/L PPW removed 53.6 % of TSS. TSS removal was found higher in using fed batch in MFC.

4.8 Cyclic Voltammetry

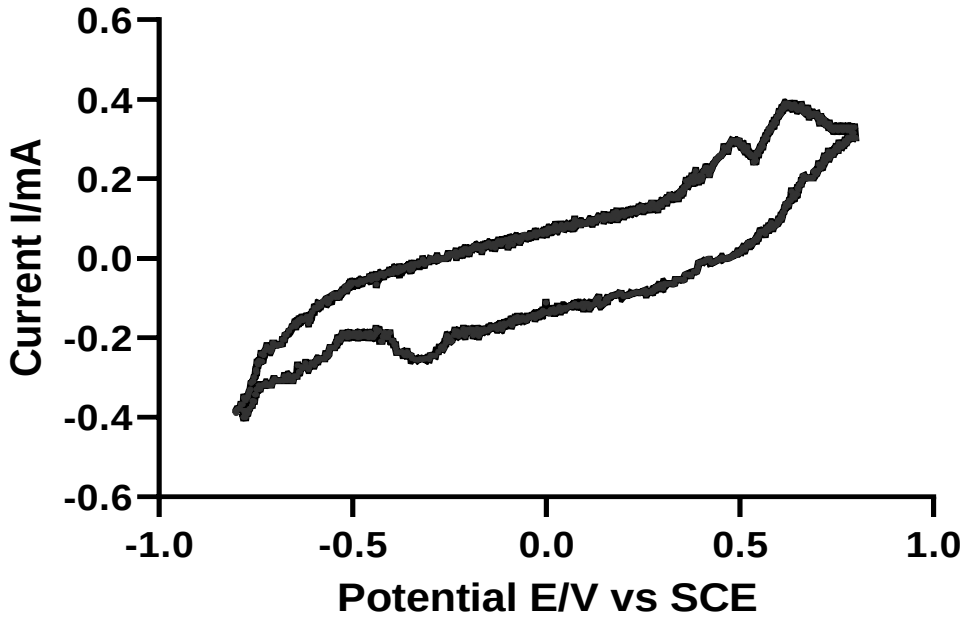

Figure 4.12: Cyclic voltammogram of graphite sheet used as electrode in the MFC at the fourth day of setup

During metabolic processes, protons (H+) and electrons (e^-) are created and consumed continually in the cell. A potential difference was created between the cell and the surrounding medium when an external potential was applied, causing the e^- to travel towards the working electrode and deposit, resulting in a voltammogram. The cyclic voltammogram consists of 2 anodic peaks and a cathodic peak shown in figure 4.12. The peak observed between 0.6-0.7 was probably metal oxide, that potato peels sample contained metals such as iron, copper and zinc. According to Martin et al., 2011 Fe_2O_3 oxidation peak was found around 0.7 V and peak of carbon was found around 0.55 V. Thus the first anodic peak was probably of iron and another peak at 0.5 V was the typical carbon electrode shows the peak of electroactive oxide/hydroxide. The Cathodic peak of the voltammograms was observed at -0.35 V was probably oxygen reduction peak. During oxidation in anodic peak maximum current produced was found 1.26A / m^3 of sample volume.

4.9 Analysis of Sugars and byproduct after MFC operation by HPLC

4.9.1 Sugar Analysis in potato peels sample by HPLC

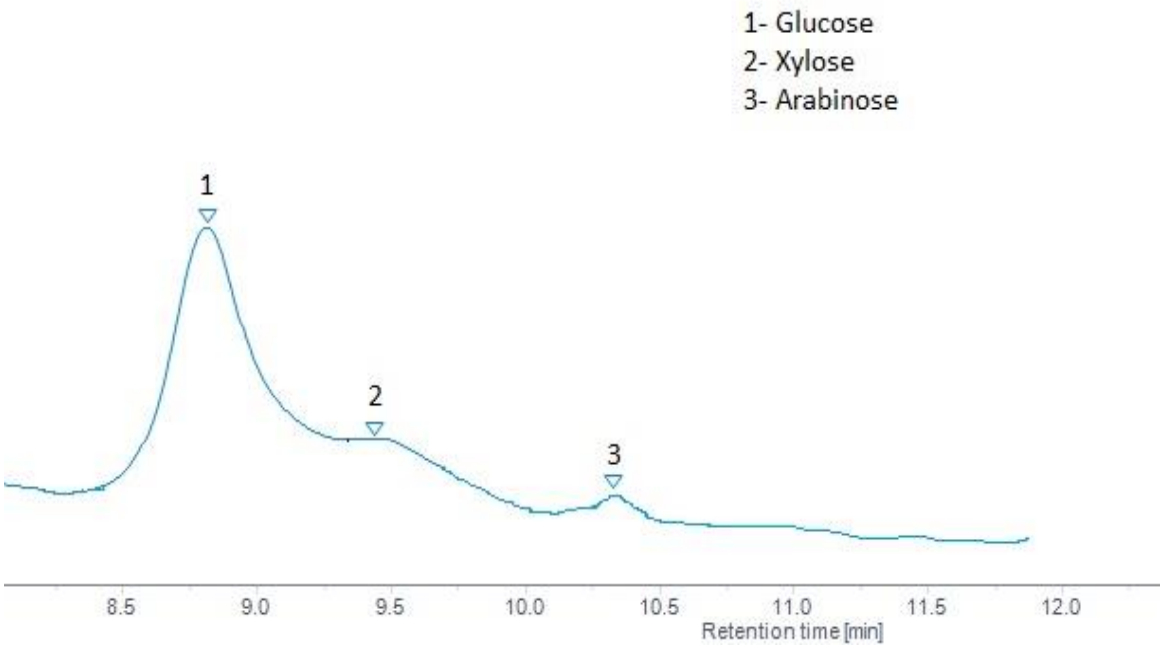

Figure 4.13: Chromatogram of sugar analysis of potato peel sample using RI detector

Sugar analysis in potato peels sample was done in HPLC using Zorbax carbohydrates specific column. Calibration standard were analyzed in HPLC according to which different peaks in chromatogram were identified on the basis of retention time. Three different sugars glucose, xylose and arabinose were identified in chromatogram on the basis of retention time, shown in figure 4.13.

Table 4.9: Concentration of different sugars determined by HPLC analysis

Sugar	concentration (mg/g)
Glucose	3.34±0.2
Xylose	0.467±0.17
Arabinose	0.02±0.05

4.8.2 Byproducts Analysis after MFC operation by HPLC

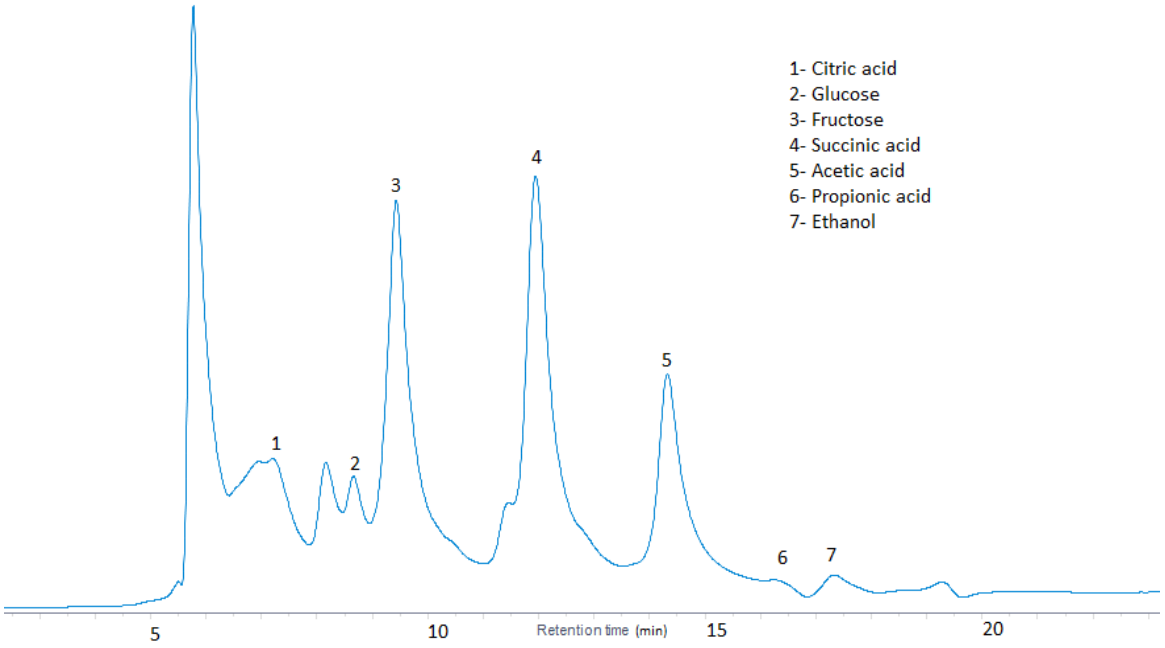

Figure 4.14: Chromatogram of MFC byproduct analysis using RI detector

The chromatogram of MFC byproducts shown in the figure 4.14, and the identified peaks were labeled. Organic acids such as citric acid, succinic acid, acetic acid, and propionic acid were detected. Similarly glucose, fructose and ethanol also found in the sample after MFC operation. These components were separated on the basis of refractive index and detected by RI (Refractive Index) detector.

Table 4.10: Concentration of different byproducts determined from HPLC analysis

Components	Concentration (mg/g)
Citric acid	3.04± 0.11
Glucose	0.87± 0.06
Fructose	10.11± 0.81
Succinic acid	12.03± 0.56
Acetic acid	2.51± 0.09
Propionic acid	0.021± 0.0015
Ethanol	0.81± 0.079

Sugar analysis in potato peels sample was done in HPLC using carbohydrates analysis specific column. Calibration standard were analyzed in HPLC according to which different peaks in chromatogram were identified on the basis of retention time. Three different sugars were identified in chromatogram: glucose, xylose and arabinose shown in figure 4.13. The concentration of glucose, xylose and arabinose were found 3.34±0.2 mg/g, 0.467±0.17 and 0.02±0.05 mg/g respectively shown in table 4.9. According to Kaaber et al., 2001 potatoes mostly comprise non-starch polysaccharides (NSP), methyl groups, glucose, starch, and amylose. Rhamnose, galactose, arabinose, xylose, mannose, and glucose were found in the insoluble NSP; the first three sugars decreased significantly after the three weeks of storage. Thus concentration of arabinose was very low might decreased because of storage of sample.

Similarly, figure 4.14 shows that the byproduct analysis after MFC operation using Aminex HPX-87H column. Quantitative analysis of HPLC was shown in table 4.10 on the basis of different concentration of various calibration standard (appendix II). Thus the concentration of citric acid, acetic acid, propionic acid and succinic acid were found 3.04±0.11 mg/g, 2.51±0.09 mg/g, 0.021±0.0015 mg/g and 12.03±0.56 mg/g respectively shown in table 4.10. According to Robbert Kleerebezem et al., 2015, anaerobic digestion of organic waste produces acetic acid, propionic acid, succinic acid, lactic acid and butyric acid. In our study lactic acid and butyric acid were not determined, it might be due very low amount which could not detected by HPLC.

Similarly concentration of glucose and fructose was found to be 0.87±0.06 mg/g and 10.11±0.81 mg/g respectively shown in table 4.10. Potato peels contains high amount of starch which oxidized in glucose and fructose by the activity of amylase. The concentration of fructose was found higher than glucose after MFC operation this might be due to that organism used glucose easily than fructose. Finally the concentration of ethanol was found 0.81±0.079 mg/g also shown in table 4.10. Ethanol can be produced from the anaerobic digestion of compounds which contains carbohydrates (Moraes et al., 2015).

CHAPTER 5: SUMMARY

MFC is a renewable and sustainable technology that produces electric energy using enzymes produced by fungus for oxidation of biodegradable substrate. This work was based on the fact that microorganism and enzymes can oxidize the substrate to produce electricity and other valuable byproducts makes MFCs an ideal solution for waste treatment and energy production.

General introduction of this work was described in chapter first. According to which solid waste management is the problem for every society and this becomes serious problem in future. This is the most environmentally friendly method of managing organic waste while also producing energy. This is pure, renewable energy that may be used in our daily lives. Apart from bioremediation, this total process also contributes to the generation of electricity.

Second chapter describe about literatures reviewed related to this work. The review found that the content and type of biomass, as well as the microbial species involved in the production process, all influence the production of electricity from potato peels waste. Similarly the optimization is carried out for enhancement of electricity from MFC. Selection of better strain which produces amylase and pectinase and resist in normal room temperature is important for better electricity production.

The third chapter describe about materials and methods of research work. Potato peels waste was used as sample. Dual chambered MFC was constructed using graphite electrodes. Anode and cathode chamber was separated by proton exchange membrane. Anode contained 300 mL sample and cathode contained 300 mL acetate buffer, isolated fungal inoculum was used in anode sample. Similarly optimization for the enhancement of OCV was done using 10mM of various electron acceptors such as $KMnO_4$, $K_2Cr_2O_7$ and $K_3[Fe(CN)_6]$ were used in cathode buffer. NaCl was also used instead of acetate buffer in cathode. Electrode modification was done by coating MWCNT in anode which also enhance the OCV generation. Finally after all operation two different resistor were used for determination of power density. During MFC operation byproducts generated were analyzed by HPLC analysis.

Similarly, chapter four described about the finding of this research work. Physical and analytic parameters of the potato peel waste were determined. Screening of fungal strain for production amylase and pectinase enzymes was done which was used in MFC operation for degradation of waste sample. Construction of MFC was done using appropriate electrode and PEM. Optimization of MFC was done using various electron

acceptors and electrode modification for enhancement power generation. Among all of them using $KMnO_4$ in cathode produces highest OCV. Similarly anode modification by coating with MWCNT/PANI composite also generated high OCV. Two different external resistors 100 ohm and 1000 ohm were used for determination of power density. Among them, 1000 ohm resistor produced higher power density. After every MFC operation, all analytic parameters were determined, from which the removal rate was calculated. Cyclic voltammetry was also measured for observation of oxidation and reduction process in MFC. Sugar analysis of waste sample and byproduct analysis in anodic sample after MFC operation was done in HPLC using specific column. Various organic acids; citric acid, acetic acid, propionic acid and succinic acid were determined from the waste sample after MFC operation. Ethanol is an important byproduct of anaerobic digestion, was also determined.

CHAPTER 6: CONCLUSION

Environmental analysis of potato peel waste was determined as its pH, moisture content, ash content, TSS, VSS, Chemical Oxygen Demand (COD), Reducing sugar, Nitrogen, Phosphorus contents and different metal ions such as iron, copper, zinc and manganese. Isolated fungal strain shows both amylase and pectinase producing ability. Molecular characterization signifies that the isolate resembled to *Aspergillus niger.* Operation of microbial fuel cell for enhancement of electricity generation using sample in anode and acetate buffer as catholyte, graphite sheets used as anode and cathode. Organic waste degradation status using different concentration of sample on OCV generation was determined and among all concentration 1:10 dilution showed better production of OCV which was used in further MFC operation. Various MFC operation were done for enhancement of OCV, using electron acceptor in catholyte, electrode modification, using sterilized and unsterilized substrate etc. After all these operation highest OCV production is observe in using $KMnO_4$ as an electron acceptor in buffer as catholyte. The powerful oxidant $KMnO_4$ exerts pressure on overall reactor performance by increasing proton consumption, which accelerates MFC performance. Determination of power generation was done using two different external resistors. COD removal, Ammoniacal-nitrogen removal, reducing removal and TSS removal were determined. From this result the TSS removal after MFC operation was found 55.55 - 65.95% which indicated that more hydrolysis of sample is needed for better production of electricity. Current generation from oxidation of sample in anode is found 1.26A / m^3 of sample volume. Finally various compounds generated during MFC operation were analyzed using HPLC and concentration determined using calibration standard. The main purpose of this research work was the management of organic waste and generation of alternative energy from the waste.

CHAPTER 7: RECOMMENDATION

This research can be recommended to numerous industries for further improvement, production, and/or policy implementation based on yield and process efficiency. Some of them are listed below.

1. This research could be useful to the potato chips industry in terms of waste management and production electricity.
2. In further research it is recommended to recirculate the sample in anode for improvements in yield.
3. It is recommended to perform scale up of this process prior to industrial application.

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APPENDICES

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

A Culture Media

Potato Dextrose Agar

Dextrose	20 g
Potato extract	4 g
Agar	15 g
Distilled water	1 L

Modified Starch Agar Media

Potassium dihydrogen phosphate	1.4 g
Ammonium nitrate	10 g
Ammonium oxalate	12.5 g
Potassium chloride	5 g
Magnessium sulfate	0.1 g
Ferric sulphate	0.01 g
Soluble starch	20 g
Distilled water	1 L
Agar	15 g

Pectinase Production Media

Pectin	25 g
Ammonium oxalate	12.5 g
0.1 M Sodium Acetate buffer	1 L
Agar	15 g

B Reagents

1.Digestion Mixture for phosphorus

100 mL of 96% sulfuric acid mixed with 18 mL water. Then 6 g of salicylic acid powder was added to the mixture.

2. Stock solution of PO_4

1.432 g potassium dihydrogen phosphate was dissolved in 900 ml of distilled water in volumetric flask and volume was made 1 L by adding distilled water.

3. Ascorbic Acid Solution

1.76 g of ascorbic acid was dissolved in 100 ml ultrapure water and mixed (used freshly prepared).

4. Ammonium Molybdate Solution

40 g of ammonium molybdate tetrahydrate [$(NH_4)_6MO_7O_{24}.4H_2O$] in 900 ml ultrapure water and final volume was made 1 L.

5. Potassium Antimony Tartarate Solution

0.274 g Potassium Antimony Tartarate was dissolved in 100 ml of ultrapure water.

6. Sulphuric Acid solution (2.5 mol/L)

140 ml of conc. Sulfuric acid was diluted in 500 ml of ultrapure water, allowed to cool and final vilume was made 1 L.

7. Mixed Reagent

50 ml of sulfuric acid, 15 ml ammonium molybdate solution, 30 ml ascorbic acid and 5 ml potassium antimony tartarate were mixed in a reagent bottle. Then 80 ml of mixed solution was diluted with 300 ml ultrapure water.

8. Hydrogen Phthalate stock solution

0.085 g of KHP was mixed in 80 ml of ultrapure water in volumetric flask and volume was made up to 100 ml. Stock solution of 1gm/L was prepared.

9. Digestion Solution

1.02 g potassium dichromate, 16.7 ml sulfuric acid and 3.32 g mercury sulfate were dissolved in 50 ml of distilled water and final volume was made 100 ml with water.

10. Catalyst solution

0.09 g silver sulfate was mixed in 100 ml of conc sulfuric acid and left overnight.

11. Ammonia nitrogen stock solution

3.819 g of ammonium chloride was dissolved in 900 ml of distilled water and volume was made up to 1 L by adding water.

12. Ammonia nitrogen working solution

10 ml stock solution was diluted up to 1000 ml with distilled water.

13. Borate Buffer (pH 9.5)

88 ml of 0.1mol/L NaOH was added to 500 ml of 0.025mol/L sodium tetraborate solution and final volume was made 1000 ml with distilled water.

14. Boric acid solution

20 g of boric acid was mixed with 900 ml of distilled water and final volume was made 1000 ml.

15. Nessler's Reagent

100 g of mercuric iodide and 70 g of potassium iodide in small amount of water. Then mixture was added to a cooled solution of 160 g NaOH in 500 ml of distilled water and dilute up to mark with distilled water in a 1000 ml volumetric flask.

16. DNS Reagent

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

17. Acetate Buffer

50 mM of acetate buffer was prepared by dissolving 1.933 mg of sodium acetate in 300 ml of water. pH 5 was maintained by using acetic acid and final volume was made up to 350 ml using distilled water

Appendix II: Standard Curves

1. Standard Curve of Spectrophotometer analysis

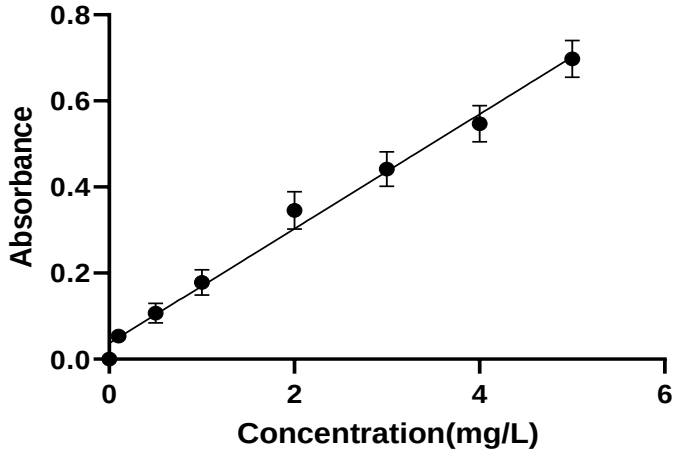

Figure 1: Standard curve of Phosphorus (y=0.1436x+0000)

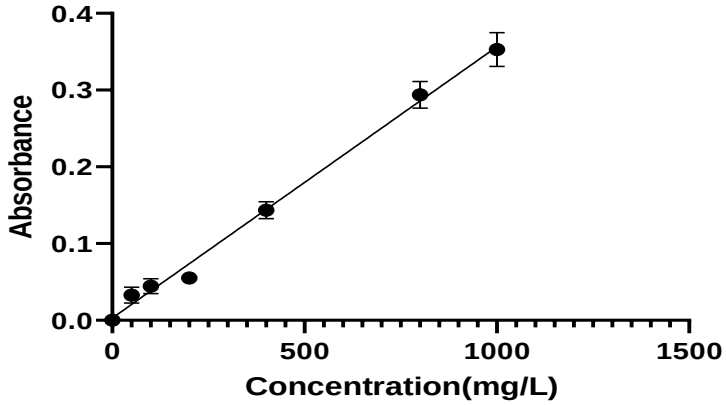

Figure 2: Standard curve of chemical oxidation demand (y=0.004x+0.004)

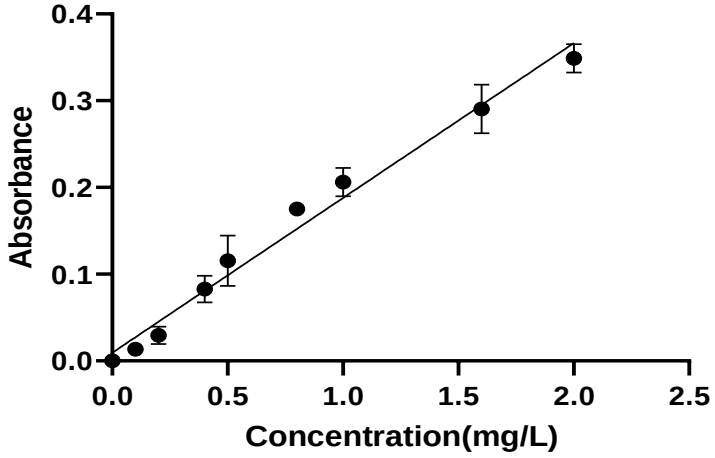

Figure 3: Standard curve of Ammoniacal-nitrogen (y=0.1712x + 0.0010)

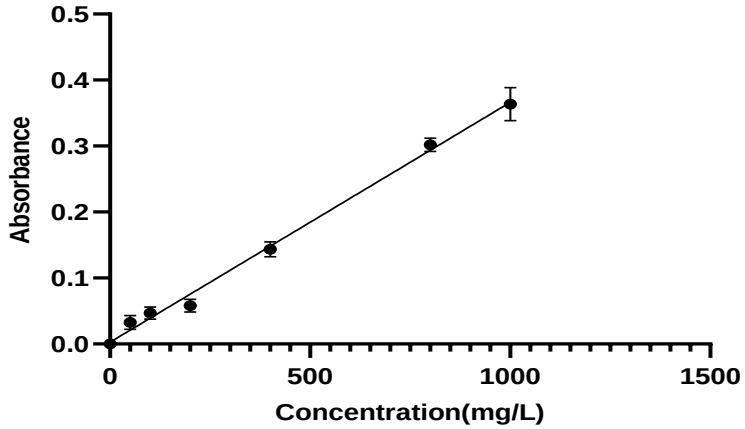

Figure 4: Standard curve of reducing sugars (y=0.009x+0.000)

2. Standard curve of sugar analysis in HPLC

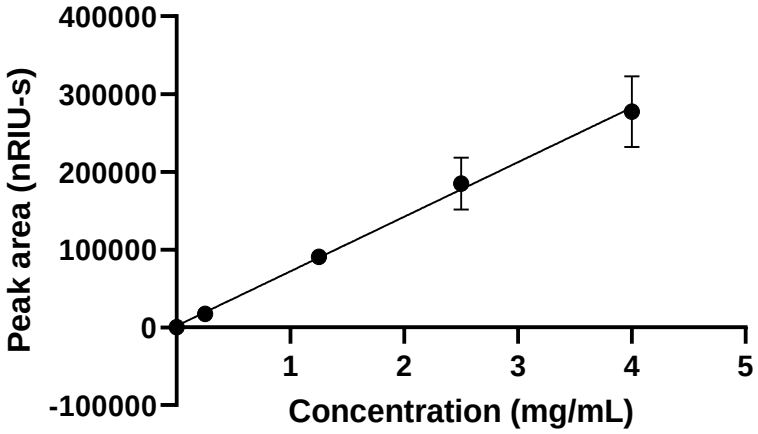

Figure 1: Standard curve of glucose (y=67094x+00000)

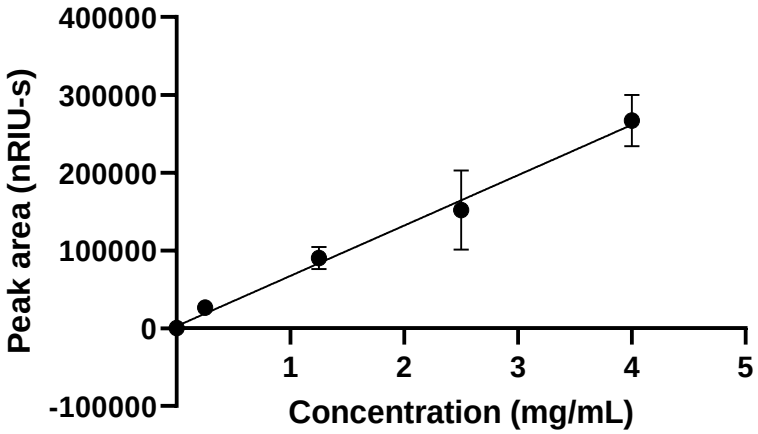

Figure 2: Standard curve of xylose (y=108195x+00000)

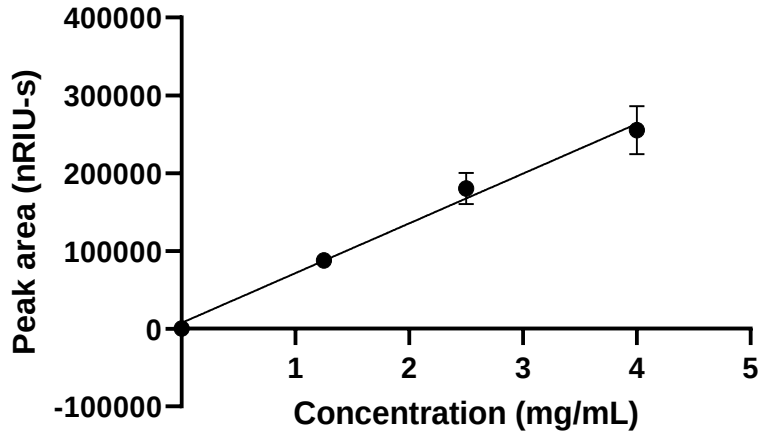

Figure 3: Standard curve of arabinose (y=65501x+00000)

3. Standard curve of End product analysis in HPLC

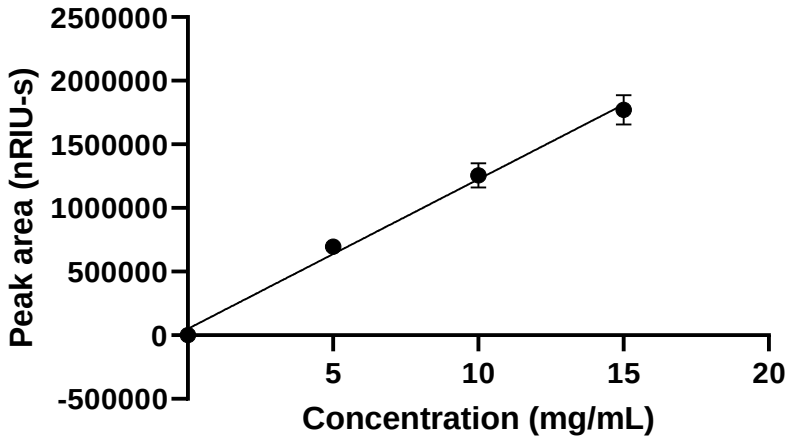

Figure 1: Standard curve of citric acid (y=122934x+00000)

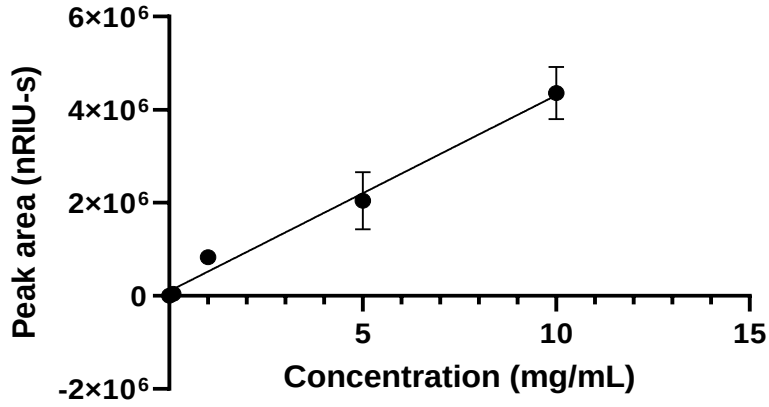

Figure 2: Standard curve of acetic acid (y=498937x+00000)

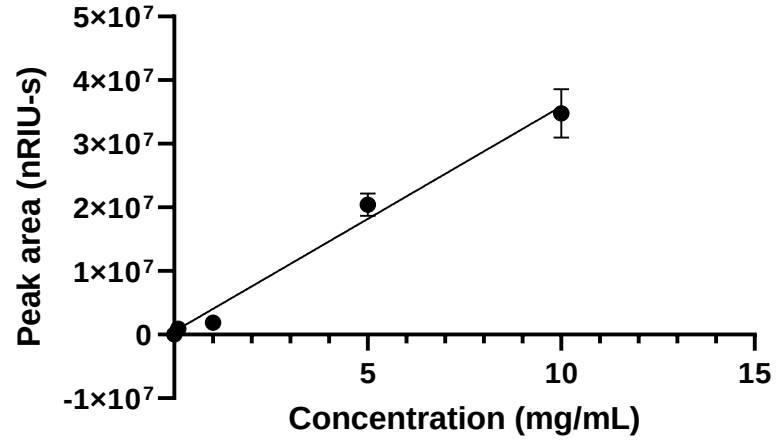

Figure 3: Standard curve of propionic acid (y= 2958601x+00000)

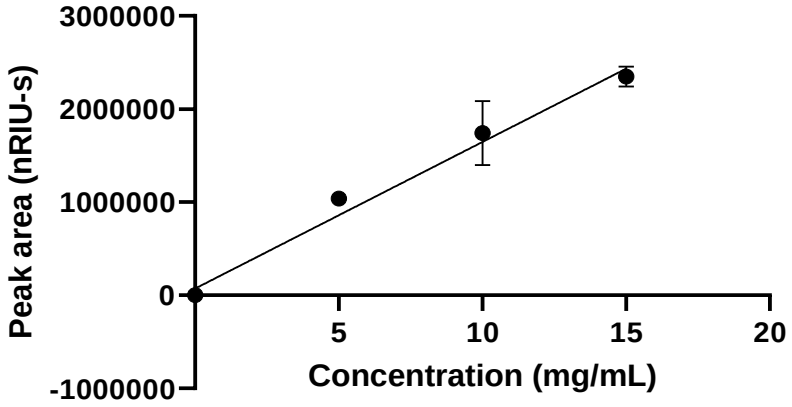

Figure 4: Standard curve of succinic acid (y= 165150x+00000)

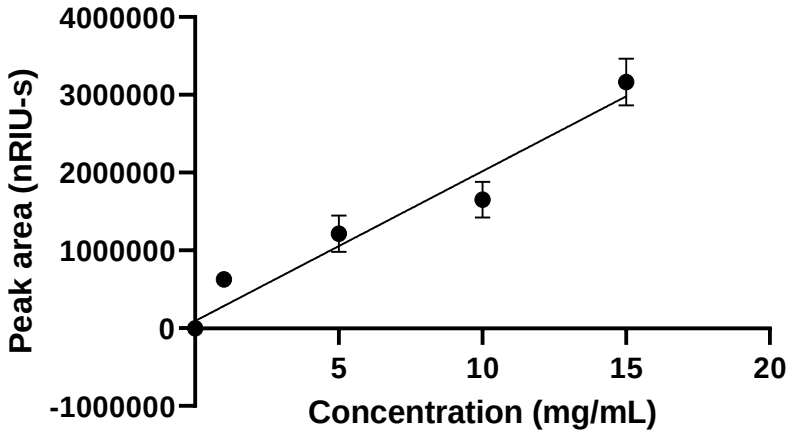

Figure 5: Standard curve of glucose (y= 200933x+00000)

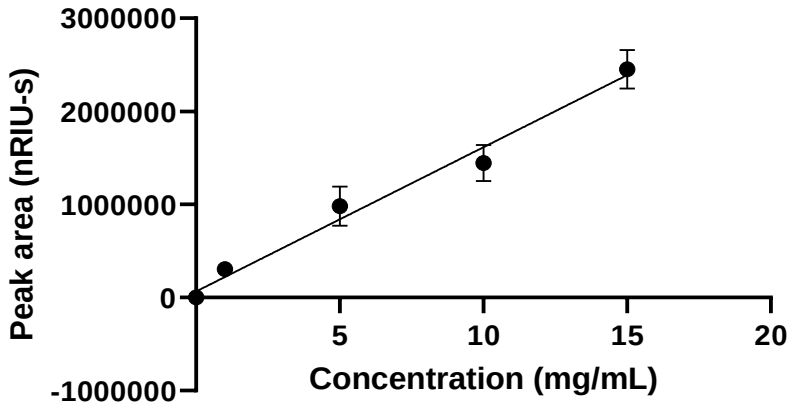

Figure 6: Standard curve of fructose (y= 157960x+00000)

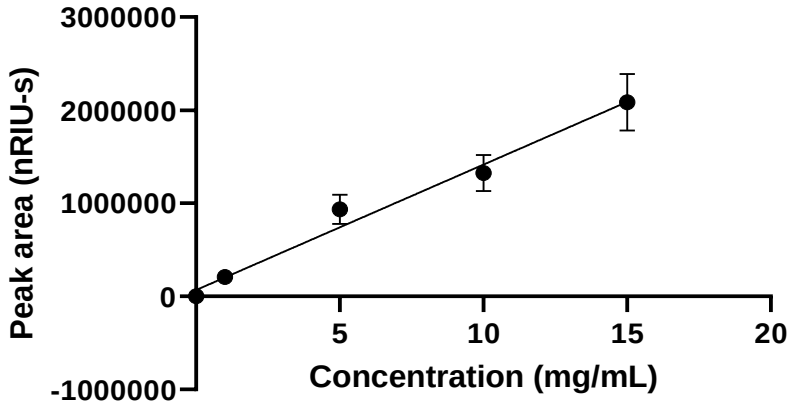

Figure 7: Standard curve of ethanol (y= 138436x+00000)

Appendix III: Fungal isolate sequence

>F

CGTGGTTTTCTTTAATTGGAATGAGTACAATCTAAATCCCTTAACGAGGA
ACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCA
ATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGAACCTTG
GGTCTGGCTGGCCGGTCCGCCTCACCGCGAGTACTGGTCCGGCTGGACCT
TTCCTTCTGGGGAATCTCATGGCCTTCACTGGCTGTGGGGGGAACCAGGA
CTTTTACTGTGAAAAAATTAGAGTGTTCAAAGCAGGCCTTTGCTCGAATA
CATTAGCATGGAATAATAGAATAGGACGTGCGGTTCTATTTTGTTGGTTT
CTAGGACCGCCGTAATGATTAATAGGGATAGTCGGGGGCGTCAGTATTCA
GCTGTCAGAGGTGAAATTCTTGGATTTGCTGAAGACTAACTACTGCGAAA
GCATTCGCCAAGGATGTTTTCATTAATCAGGGAACGAAAGTTAGGGGATC
GAAGACGATCAGATACCGTCGGAGTCTTAACCATAAACTATGCCGACTAG
GGATCGGACGGTGTTTCTATTATGACCCGTTCGGCGCCTTACGAGAAATC
AAAGTTGTTGGGTTCTGGGGGGGGGGATGGGGGGTAAAGATGGAACTTAT
CGAAATGAGACAACACAAG

Appendix IV: Photographs of Laboratory works

Figure 1: Potato peels waste sample

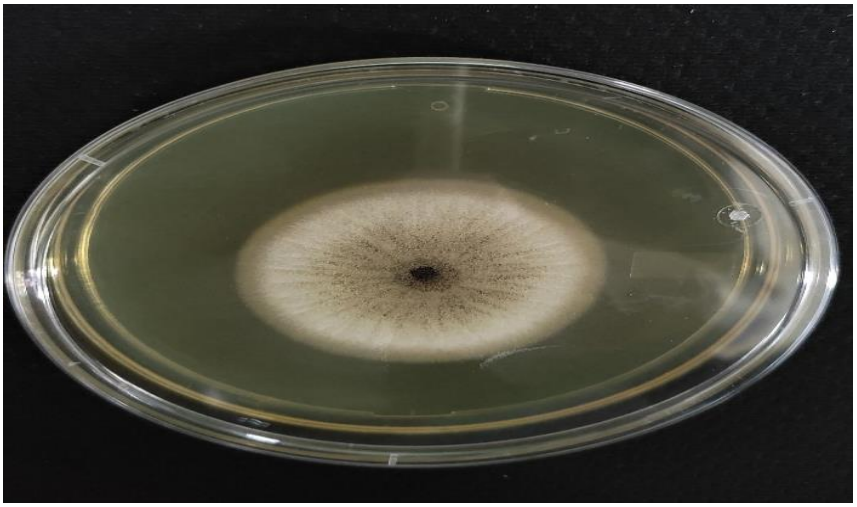

Figure 2: Fungal isolate on PDA plate after 48 h culture

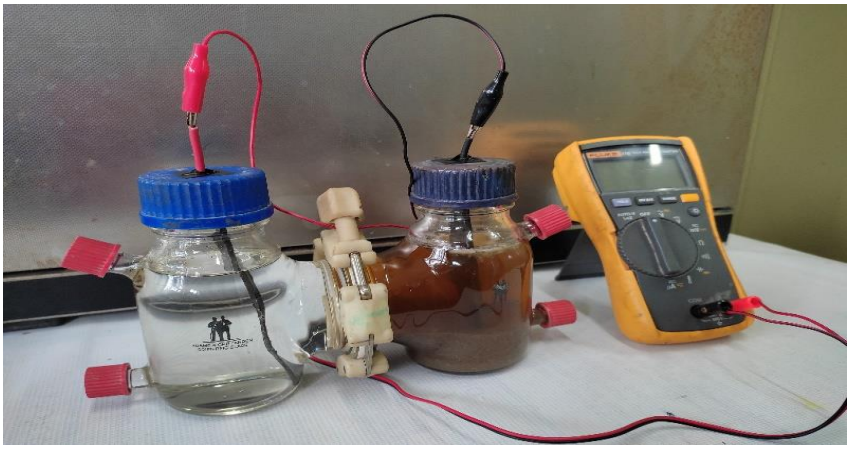

Figure 3: Dual-chambered MFC setup at Biofuel lab of CDBT

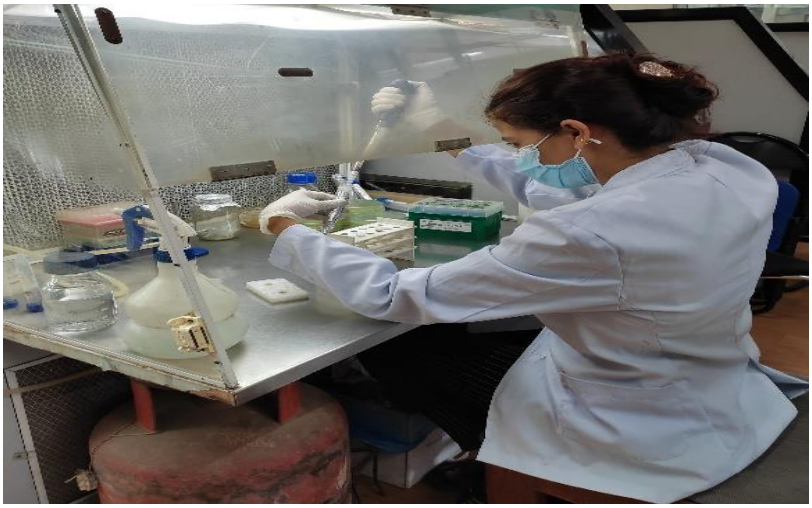

Figure 4: Working on Biosafety cabinet

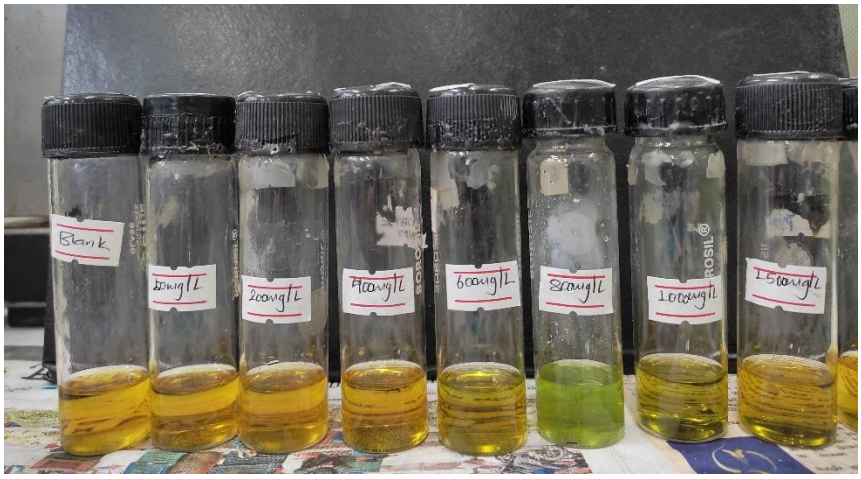

Figure 5: color observed of digested standard during COD determination

Appendix V: Publication

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Characterization of Ethanol Producing Yeasts for their Efficiency in Ethanol Production, Salt Tolerance, and Utilization of Glucose and Xylose

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Abstract

Yeast is the mainstay in ethanol production industry. Search for efficient salt tolerant as well as hexose and pentose utilizing yeast strains is important in fermentation industry. In this regard, 12 yeast strains, *viz.,* CDBT1-12, were isolated from various sources and characterized. Molecular characterization of the yeast strains was done by sequencing their D1D2 region of 26S rRNA gene. Out of 12, 10 were found to be *Saccharomyces cerevisiae*, 1 was *Wikerhamomyces anomalous* (CDBT7), and the other was *Cyberlindnera fabianii* (CDBT8). All of the strains were found to be good ethanol producers. CDBT2 was found to have tolerance for high salt (up to 15%) and ethanol (up to 16%) concentrations. CDBT7 was both salt tolerant (up to 15%) as well as utilizes glucose and xylose without compromising on ethanol production efficiency. CDBT2's ethanol production efficiency was further enhanced by application of low voltage. Under such conditions alcohol dehydrogenase (ADH1) and pyruvate decarboxylase (PDC1) mRNA levels were increased by 2.78 ± 0.80 and 1.12 ± 0.37 fold, respectively, in CDBT2. This observation is novel, it has not been reported previously.

Keywords: Yeast, Molecular Characterization, Alcohol Dehydrogenase, Pyruvate Decarboxylase, External Voltage.

Introduction

Yeast strains are the common dwellers of most of nutrient rich media/sources such as fruits, tree bark, soils etc. [1]. They form one of the important classes of microorganisms that are more complex than

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