ENHANCEMENT OF BIOGAS PRODUCTION FROM BOVINE MANURE USING ELECTROCHEMICAL CELL AND MOLECULAR CHARACTERIZATION OF ISOLATED MICROBES

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RECOMMENDATION

This is to certify that the research work entitled **"ENHANCEMENT OF BIOGAS PRODUCTION FROM BOVINE MANURE USING ELECTROCHEMICAL CELL AND MOLECUAR CHARACTERIZATION OF ISOLATED MICROBES"** has been carried out by **Ms. Pranita Poudyal** under my supervision.

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

This is to certify that this thesis entitled "**"ENHANCEMENT OF BIOGAS PRODUCTION FROM BOVINE MANURE USING ELECTROCHEMICAL CELL AND MOLECUAR CHARACTERIZATION OF ISOLATED MICROBES"**" presented to evaluation committee by **Ms. Pranita Poudyal** is found satisfactory for the partial fulfillment of Master of Science in Biotechnology.

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LIST OF ABBREVIATION

AcoD- Anaerobic Co-Digestion

AD- Anaerobic Digestion

As- Arsenic

ASB- Aeration stabilization basin

BSA- Bovine Serum Albumin

BSP- Biogas Support Programme

BSP-Nepal- Biogas Sector Programme

CGE- CNT treated graphite Electrode

CNTs- Carbon Nano tubes

DET- Direct Electron Transfer

DIET -Direct Interspecies Electron Transfer

DNS- 3, 5-dinitrosalicylic acid

EHT-Electron high tension

IEA-International energy Agency

IET -Indirect Electron Transfer

K- Potassium

LPG- Liquid Petroleum Gas

MES - Microbial Electrochemical Systems

MET -Mediated Electron Transfer

MTOE-Million Tons of Oil Equivalent

MWCNT- Multi walled carbon nanotubes

NAST- National Academy of Science and Technology

NEB- New England Bio labs

NMP - N-methyl Pyrollidone

P- Phosphorus

PANI/CNTs - Poly aniline coated multi-walled carbon nanotubes

P2G- Power-to-Gas

RECAST - Research Center for Applied Science and Technology

SEM- Scanning Electron Microscopy

SHE- Standard Hydrogen Electrode

TSS- Total Suspended Solids

TW- Terawatt (10^{12} watt)

UGE -Untreated Graphite Electrode

VFA- Volatile Fatty Acids

VSS- Volatile Suspended Solids

WD- Working distance

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ABSTRACT

Biogas program has long been in action as a household cooking fuel in Nepalese context. Nevertheless, it has potential of widespread energy utility beyond household cooking fuel. The increment in the methane content has a major role to broaden the application of biogas. Enhancement of the methane-generating metabolism of microorganisms known as methanogens is required to increase the methane production. In the anaerobic process of biogas plant, acetate utilizing methanogens are dominating the methane production process whereas H_2/CO_2 utilizing methanogen are often limited due to the restricted availability of hydrogen or other electron donors. Thus, large amount of CO_2 remained in the biogas. Research has shown that methanogens can use electricity to acquire the electrons for the production of methane from CO_2, the process is called Electromethanogenesis. In this process, methanogens reduce CO_2 to CH_4 utilizing electrons from electricity. One of the promising technologies to upgrade the methane content in biogas is to integrate electromethanogenesis in anaerobic digestion. In this research, Microbial electrochemical cells (MEC) was developed employing biocompatible electrodes. Lab-based investigation on the electrical energy input in anaerobic digestion process was performed. Cow dung was assessed for the use of anaerobic digestion with the objectives of treating the dung waste to decrease disposal costs and to upgrade the biogas at low temperature condition. The biogas produced contains mainly methane and carbon dioxide, and can be used as a source of renewable energy.The maximum gas production of 25cm^3 was obtained at 18˚C, which was later performed by mitigating carbon dioxide content and was found to be 20cm^3 in 1000 ml of production media. Five bacteria were isolated from the production media. Among them two distinct isolates (P1D and P2D) were subjected to 16s rRNA sequencing and they were found to be *Serratia liquefaciens and Zoballella taiwanensis* respectively.

Key Words: Biogas, methanogens, microbial electrochemical cell, anaerobic digestion, microbial ecology

CHAPTER 1. INTRODUCTION

1.1 Background

Handling of the livestock waste is considered as one of the major issue in many parts of the world as extends incessant environmental pressure. Livestock manure, like cow dung in the absence of appropriate disposal methods can cause adverse environmental and health problems such as: pathogen contamination, odour, air borne ammonia, greenhouse gases etc. (Harikishan *et al.,* 2003).

Production of biogas from animal manure, especially cow shows high prospective and has many advantages. Energy derived from it is very environmentally friendly since in addition to utilizing the waste from livestock, left over from the process (biogas slurry) can be used as organic fertilizer that is rich in the elements required by plants (Li *et al.,* 2009).

Anaerobic digestion has been considered as waste-to-energy technology, and is widely used in the treatment of different organic wastes, for example: organic fraction of municipal solid waste, sewage sludge, food waste, animal manure, etc. Anaerobic treatment comprises of decomposition of organic material in the absence of free oxygen and production of methane, carbon dioxide, ammonia and traces of other gases and organic acids of low molecular weight (Lopes *et al.,* 2004). The potential for the leaching of nitrates into groundwater, the potential release of nitrates and pathogens into surface waters, and the emission of odors from storage lagoons is significantly reduced with the use of anaerobic digestion. Elements of Anaerobic digester systems have been used for decades at municipal wastewater facilities, and more recently, have been used to process industrial and agricultural wastes (Burke, 2002). These systems are designed to optimize the growth of the methane-forming (methanogenic) bacteria that generate methane (CH_4). Typically, using organic wastes as the major input, the systems produce biogas that contains 55% to 70% methane and 30% to 45% carbon dioxide. (Feng *et al.,* 2015).

Several factors that affect the production of biogas are the condition of the digester, pH, nutrients, temperature, the ratio C / N, and starter (Lusk *et al.,* 1998). The condition in the anaerobic digester must be kept in equilibrium and dynamic. The degree of acidity is maintained in the range of 6.6 to 7.6 for bacteria methanogenic can only work in that range of pH (Metcalf *et al.,* 1978). Adequate levels of nutrients such as nitrogen and phosphorus must be added in the system to ensure the availability of nutrients for bacterial growth.

Recently, large volume of cow dung generated from feedlot farming increases annually, most of which are disposed into landfills or are applied to the land without treatment. Anaerobic digestion provides an alternative option for energy recovery and waste treatment.

Rapidly developing bioelectrochemical technology has been proved to be a promising platform for CO_2 capture and conversion comparing with other methods (Logan and Rabaey, 2012). With a small addition of voltage at the microbial electrolysis cell (MEC), electromethanogens can use electrons or hydrogen formed at cathode to convert CO_2 into CH_4 directly (Cheng *et al.,* 2009).

Microbial electrolysis cell (MEC), developed from microbial fuel cell (MFC), is a bio-electrochemical system for biological hydrogen production via the biodegradation of organic matter using exoelectrogenic microbes on anode biofilm (Rozendal *et al.,* 2006). This phenomena can briefly be explained since water addition is necessary to fulfil the need of water molecules to support the hydrolysis reaction and acetogenesis stage. At the stage of hydrolysis, hydrolytic microbes exist in the system which will degrade complex organic compounds in the form of polymers into monomers which is insoluble compounds and smaller molecular weights. (Putri *et al.,* 2012)

1.2 Current studies

1.2.1 Status of Biogas in Nepal

The present state of methane gas production in Nepal is performed using cattle manure, human excreta and vegetable wastes in anaerobic bioreactor. The methane gas is widely used to substitute the traditional forms of energy sources such as cattle dung cakes, fuelwood, agriculture residues, etc. This has greatly helped the nation in many ways such as income generation, litter management, life style improvement and cost savings. Although the biogas sector has helped the country in many ways, only 9% of the country’s biogas potential has been realized (Gautam *et al.,* 2009).Much work needs to be done to reach out to the people living in the remote areas and to provide the necessary financial and technical support to them. Also, the problems associated with the generation of methane in colder regions need to be addressed in order to make this technology more versatile throughout the country.

1.2.2 Advancement using Anaerobic Digestion

Biogas production using AcoD from different biodegradable organic materials is increasingly becoming a feasible renewable energy source. The AD process of biogas production has been growing into a fully matured and interesting broad discipline that is continually looking for challenging advanced technologies. AcoD technology is a promising method of AD for biogas production process technology in both lab-scale and large scale plants, and it also financially and environmentally feasible. However, AcoD system is challenging natural process. The challenging paradigms in biogas production technology are characterizing the organic materials, involving diversified microbial activities, biodegradability, accessibility, determining the exact limiting step and factors (Hagos *et al.,* 2017).

Anaerobic co-digestion of animal manures with lignocellulosic residues offers a promising route for efficient biogas production which is also in line with climate friendly farming practices. A major prerequisite for commercializing the technology of anaerobic co-digestion is to locate the livestock and agricultural farms as close as possible so that huge amount of lignocellulosic residues and animal manure will be available abundantly. Otherwise, the cost for collection of massive quantities of biomass resides, their transportation and delivery at the plant gate should be reasonable enough to make the process economically feasible. Combination of a treatment plant and combined heat and power generation unit will provide simultaneous waste management and power generation; the generated heat and electricity may then be utilized at the same plant. The number of full scale plants in recent years is increasing, but most of them are treating lignocellulosic materials and sewage sludge. (Neshat *et al.,* 2017)

Microbial electrochemical technologies (METs) use microorganisms to catalyze electrochemical reactions. These METs include microbial fuel cells (MFCs) that generate electrical power when oxygen is present at the cathode and microbial electrolysis cells (MECs) that generate hydrogen while consuming a small amount of electrical power, and are promising approaches for capturing the energy in waste biomass(Logan&Rabaey,2012).MECs can produce hydrogen gas with smaller voltages(as low as 0.2 V) than those required for electrolytic hydrogen production(1.23 V) Hydrogen can be produced in MECs from different substrates, including acetic acid, butyric acid, lactic acid, glucose, cellulose, and different types of wastewater(Logan *et al.,* 2008).

MECs can degrade organic matter while producing hydrogen at high yields using effluent from an ethanol-type reactor as the substrate (i.e., reducing sugars,ethanol, acetic acid, propionic acid, butyric acid, and valeric acid)(Lu *et al.,* 2009).

1.3 Rationale

A principal strategy associated with today is to develop an alternative energy source instead of fossil fuels in order to compensate present energy need in addition to reduce environmental concerns provoked by pollution and global warming. Biogas production with the evolution of flammable gas methane has been attracted promisingly since its lower production cost and lesser environmental impacts over the residual biofuels recognized. In many hilly areas of Nepal, the temperature drops below 10˚C, which is not suitable for biogas production. Due to this, the biogas technology has been found to be less feasible in hilly areas. Low temperature affects the production of biogas decreasing significantly so people are forced to choose other non-renewable sources, which in turn is expensive and have environmental hazards. Hence, enhancement of biogas by overcoming the prevalent problem i.e. temperature barrier is the utmost need at the present context.

1.4 Research Hypothesis

The use of electrochemical cell is supposed to enhance the production of biogas even at low temperature condition.

Null Hypothesis:

There will be no significant increase in biogas production with the use of electrochemical cell.

Alternative Hypothesis:

There will be significant increase in biogas production with the use of electrochemical cell.

1.5 Research Objectives

1.5.1 General Objective

- Use of the electrochemical cell for the enhancement of biogas production from cattle manure.

1.5.2 Specific Objectives

- Establish an efficient anaerobic digestion process for the production of CH_4.
- Develop and optimize the biological process to utilize electrical energy for methane production based on naturally occurring microorganisms.
- Investigation of the effectiveness and the performance characteristics of anaerobic digestion of cow dung for biogas production in batch operation.
- Identification of the most prevalent microbes.

1.6 Research Scope

Since climatic condition has remained an adverse factor for disrupting the continuous production of biogas all year round, an insight with the enhancement at even low temperature conditions using microbial electrochemical systems can broaden the idea of its use in critical temperatures too. Moreover, cheap and reliable option for household will not be a far cry. Shortage of cooking fuel can be met during adverse climatic condition with the cheapest and the most reliable means i.e. biogas. The prevailing disruption of the use of bovine manure during winter season and cold environmental condition can be overcame. Nation can have economic benefits over petroleum products. Renewable resources should always be kept under highest priority so that the sustainable use of the natural products can be maintained, the best example for this would be biogas enhancement. Nepal is an agricultural country and the main occupation of most of the Nepalese farmers is growing crops and cattle rearing. The by product, which is of least use if utilized can aid in meeting the scarcity of fuel. Biogas as a whole, reduces the dependence on forest product in the rural area and its enhancement would benefit the nation throughout the year irrespective of climatic fluctuations.

CHAPTER 2. LITERATURE REVIEW

2.1 World Energy Requirement

The world consumes about 13 TW of energy and approximately 80% of that comes from burning fossil fuels. The over dependence on the fossil fuels poses risks such as depletion of fossil fuel resources and global climatic changes caused by the net increase in the atmospheric CO_2 levels (Rittmann, 2008).However, the dependence on the bioenergy has increased from 1,023 MTOE to 1,354 MTOE in the last two decades. It is expected to reach 1801 MTOE by the year 2040 (IEA, 2018).

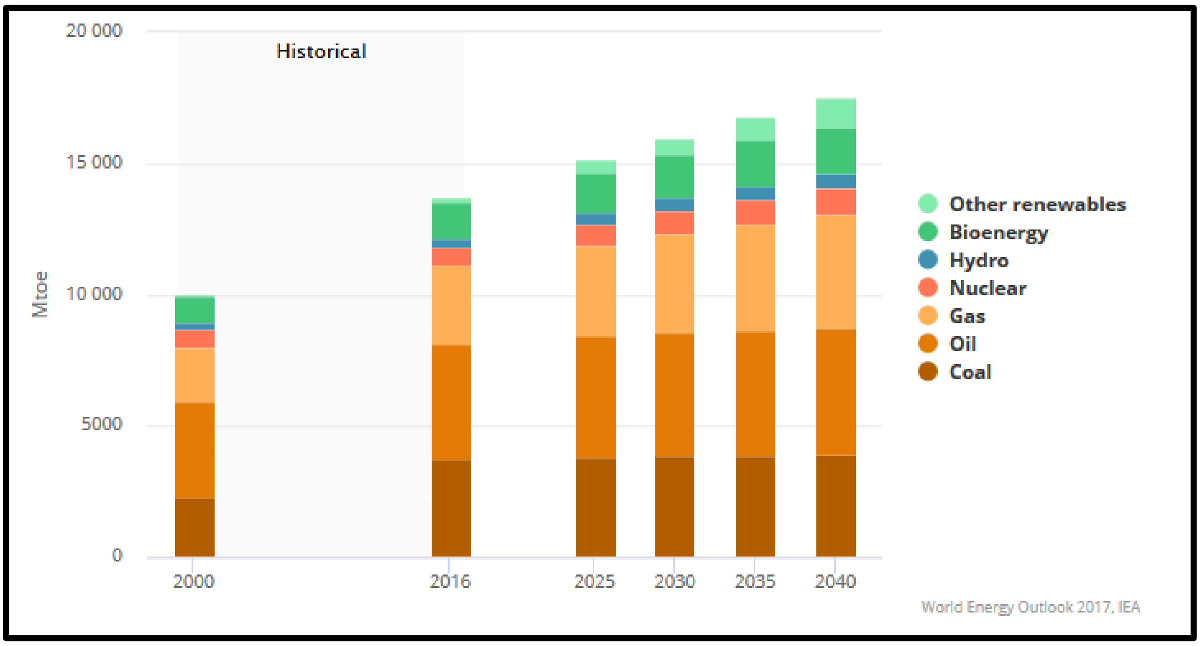

Figure 2.1 : Total primary energy demand (TPED) in the New Policies Scenario (NPS) (IEA, 2018)

2.2 Biomass as renewable energy option.

The basic concept of describing biomass as a renewable energy resource comprises the capture of solar energy and carbon from ambient CO_2 in growing biomass, which is converted to bioenergy or is used directly as a source of heat and power (Rao et al, 2010). When compared to renewable energy generation from biomass with the other sources such as hydro, solar and wind, the biomass option is economical as this requires less capital investment and per unit production cost. The potential for bioenergy which includes all the forms of conversion is understood to be very high (Schröder *et al.,* 2008).However it is necessary to ascertain the potential of converting the biomass to biogas by anaerobic digestion so as to completely utilize the biomass and meet the energy deficit and also to control the adverse effects on the environment because of its advantages over the other bioenergy conversion processes (Schröder *et al.,* 2008).

2.3 Nepal LPG requirement

The per capita energy consumption in Nepal is very low (14.6 GJ) and most of the energy is being used for domestic purpose (Sigdel, 2007). In Nepal, the sources of energy are primarily conventional. The energy consumption in 2002, by percent is 75.78 percent fuelwood, 9.23 percent petroleum product, 1.47 percent electricity, 5.74 percent animal waste, 3.75 percent agricultural residue, 3.53 percent coal and 0.48 percent renewable energy (WECS, 2003). This indicates that the dependency on forests for energy in Nepal is very high and forests are being used beyond their capacity causing deforestation and environmental degradation. Due to many constraints of technology, finance, politics and many others; the country has failed to create a favourable environment to harness the high potential of water resource and other as well.

Nepal is an agricultural country and important component of Nepalese agricultural system is livestock farming. The number of households with cattle or buffaloes in Nepal is estimated to be 2,784,583 and the potential biogas households is 1,937,006 (Katuwal & Bohora, 2009). By the end of 2006, a total number of 157,675 plants have been installed which saves 305889 tons of fuelwood per year, 4894000 liters of kerosene and produce 260005 tons of bio-compost every year (Katuwal & Bohora, 2009). Nepal is a big importer of cooking gas or LPG for the home and for commercial use.

Table 2.1 : Annual Consumption of LPG in Nepal

S.N	Fiscal Year	LPG in MT
1.	2071/72	2,58,299
2.	2070/71	2,32,660
3.	2069/70	2,07,038
4.	2068/69	1,81,411
5.	2067/68	1,59,286

The above data shows that the requirement of LPG has been increasing 10-15 percent annually. Due to the lack of domestic cooking gas plant, people had to suffer for five months during the trade embargo imposed by India in September 2015.

2.4 Biogas

Biogas is an anaerobic decomposition technology in which biochemical reaction carried out by several types of microorganisms that require little or no oxygen to live. During this process a gas that is mainly composed of methane and carbon dioxide is produced. The amount of gas produced varies with biochemical characteristics of organic wastes, consortia of microorganisms, pH, temperature, etc. However, anaerobic digestion progresses with several complex sequential and parallel biological reactions with many intermittent products through action of microorganisms producing substrates for other compositional changes (Noykova *et al.,* 2002).Agricultural waste, municipal waste, plant material, sewage, manure, food waste can be utilized for the production of biogas. . Organic compounds are anaerobically used by microorganisms in biogas systems and convert them to methane, carbon dioxide, biomass, etc. (Pavlostathis and Giraldo-Gomez, 1991).

Electromethanogenesis is one of the most recent applications in this field, where methanogenic microorganisms of the Archaea domain can fix CO_2 to methane, under electrical stimulation. In other words, electricity can be efficiently converted into CH_4, i.e. one of the most commonly used fuels (Siegert *et al.,* 2014). Carbon felts are typically used in bioelectrochemical systems as porous carbon electrode for the development of electroactive bio-films (Bajracharya *et al.,* 2015). CO_2 can be metabolically reduced to multicarbon organic compounds by microbes using electrons or reducing equivalents derived from the cathode in microbial electrochemical systems (MES). An oxidation reaction at the anode produces protons and electrons for the cathodic reduction and an electric power source drives the electrons from anode to cathode through an external circuit.

2.5 Biogas plants in Nepal.

The Ministry of Agriculture observed the fiscal year 1975/76 as the ‘Agriculture year’, biogas was included as a special program for its effectiveness in controlling deforestation and preventing burning of animal dung which otherwise could be used as fertilizer (CES,2001).

Biogas Support Program (BSP) was initiated in July 1992 to develop and promote the use of biogas in Nepal (BSP, 2003) with the financial support of Netherlands Development Organization. After the establishment of BSP, the pace of biogas plant installation has increased in an accelerating rate. By the end of the year 2017, BSP alone installed over 260,899 biogas plants, in over 2,800 VDCs and all 75 districts. About 74 to 89% biogas users use slurry is one or another form and 63% after compositing (BSP, 2017).

2.6 Biogas Production

Biogas can be produced by anaerobic digestion with methanogen or anaerobic organisms, which digest material inside a closed system, or fermentation of biodegradable materials.(Price and Cheremisinoff, 1981).

Table 2.2 : Biogas production from various organic wastes

Substrate	**Biogas (ml/gVS)**	**Methane (ml/gVS)**	**Methane content (%)**	**References**
Food waste	784	518	66.1	(Liu *et al.,* 2009)
Green waste	631	357	56.5	(Liu *et al.,* 2009)
Bovine manure	150	40	46.5	(Fantozzi and Buratti , 2009)
Chicken manure	220	110	66.6	(Fantozzi and Buratti , 2009)
Pig manure	412	216	52	(Amon *et al.,* 2006)
Sugar beet	730	387	53	(Weiland, 2010)
Grass	211	150	71	(Yu *et al.,* 2002)
Maize	560	291	52	(Weiland, 2010)
Microalgae (*Chlamydomonas reinhardtii*)	784	518	66.1	(Mussgnug *et al.,*2010)
Microalgae (*Arthrospira platensis*)	631	357	56.5	(Mussgnug *et al.,*2010)

2.6.1 Anaerobic Digestion

Anaerobic digestion is a biochemical process where complex organic matter such as carbohydrates, proteins, and lipids degrade in the absence of oxygen and are converted into methane and carbon dioxide by the action of different groups of microorganisms. It is a sustainable waste management technology, which reduces and stabilizes organic wastes, recycles its nutrient and water content, while producing energy. Biogas reduces the demand for fossil fuels, since it can be used for the production of electric power. The degradation of organic material requires a synchronized action of different groups of microorganisms with different metabolic capabilities (Robles *et al.,* 2018).

Anaerobic degradation of organic matters in liquid wastes has long been employed to produce biogas (Weiland, 2010). Biogas produced from wastes, residues and energy crops is a reliable, flexible and renewable energy source (Holm-Nielsen *et al.,* 2009). The energy released during the combustion of methane and other combustible gases allows biogas to be used as a fuel. Biogas primarily composed of methane (CH4) and carbon dioxide (CO2). Raw biogas is composed of 40–75% CH4 and 25–60% CO2 but for biogas to work as an effective fuel source, the ratio of methane to CO2 needs to be increased. The methane content more than 70% can increase the widespread utility of biogas for multiple application. Biogas can replace fossil fuels needed for heat and power generation (Weiland, 2010); and, if properly upgraded, it can also be used as vehicle fuel and as a natural gas substitute (bio-natural gas) (Holm-Nielsen *et al.,* 2009; Appels *et al.,* 2011).

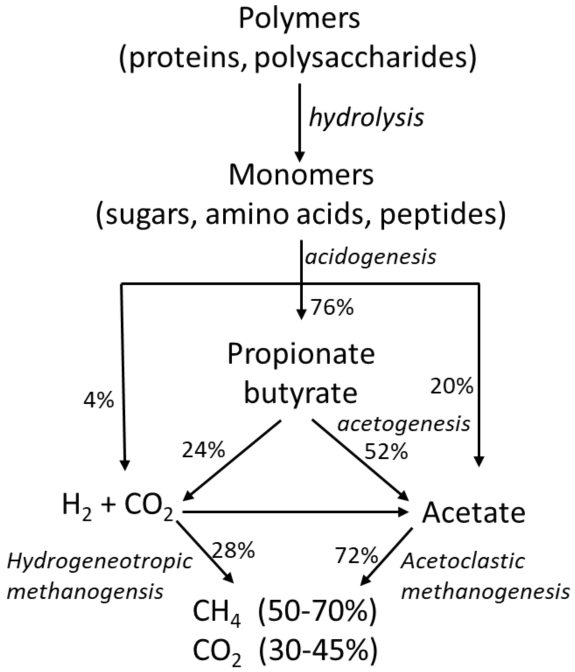

Figure 2.2 : Methane formation Pathway

In particular cases of biogas plants commonly operated in Nepal, the methane content reaches only upto 40-50% and CO2 remained the dominant gas. In anaerobic digestion process, methane generation is mainly contributed by the conversion of acetate (72% occurrence in figure 2.2) whereas only conversion of CO2 with H2 has only 28% occurrence. But due to insufficient production/availability of hydrogen or other reducing equivalents, CO2 reduction is limited.

2.6.1.1 Phases in Anaerobic Digestion

Methane fermentation is divided into four steps viz. hydrolysis, acidogenesis, acetogenesis/dehydrogenation, and methanogenesis.

2.6.1.1.1 Hydrolysis

Large organic polymers makes up the biomass. For the bacteria in anaerobic digesters to access the energy potential of the material, these chains must first be broken down into their smaller constituent parts. These constituent parts, or monomers, such as sugars, are readily available to other bacteria. The process of breaking these chains and dissolving the smaller molecules into solution is called hydrolysis. Therefore, hydrolysis of these high-molecular-weight polymeric components is the necessary first step in anaerobic digestion. Through hydrolysis the complex organic molecules are broken down into simple sugars, amino acids, and fatty acids.

Acetate and hydrogen produced in the first stages can be used directly by methanogens. Other molecules, such as volatile fatty acids (VFAs) with a chain length greater than that of acetate must first be catabolized into compounds that can be directly used by methanogens (Sun & Cheng, 2002).

2.6.1.1.2 Acidogenesis

The process of acidogenesis is similar to the way milk sours.The biological process of acidogenesis results in further breakdown of the remaining components by acidogenic (fermentative) bacteria. Here, VFAs are created, along with ammonia, carbon dioxide, and hydrogen sulfide, as well as other byproducts. (Shin *et al.,* 2004)

2.6.1.1.3 Acetogenesis

Acetogenesis is the third phase of anaerobic digestion. Here, simple molecules created through the acidogenesis phase are further digested by acetogens to produce largely acetic acid, as well as carbon dioxide and hydrogen (Mata-Alvarez *et al.,* 2000).

2.6.1.1.4 Methanogenesis

The biological process of methanogenesis is the terminal stage of anaerobic digestion. Here, methanogens use the intermediate products of the preceding stages and convert them into methane, carbon dioxide, and water. These components make up the majority of the biogas emitted from the system. Methanogenesis is sensitive to both high and low pHs and occurs between pH 6.5 and pH 8. The remaining, indigestible material the microbes cannot use and any dead bacterial remains constitute the digestate (Thauer, 1993).

2.6.1.2 Microbes involved in the phases

The individual degradation steps are carried out by different consortia of microorganisms, which partly stand in syntrophic interrelation and place different requirements on the environment (Angelidaki *et al.,* 1993). Hydrolyzing and fermenting microorganisms are responsible for the initial attack on polymers and monomers and produce mainly acetate and hydrogen and varying amounts of volatile fatty acids such as propionate and butyrate. Hydrolytic microorganisms excrete hydrolytic enzymes, e.g., cellulase, cellobiase, xylanase, amylase, lipase, and protease.

A complex consortium of microorganisms participates in the hydrolysis and fermentation of organic material. Most of the bacteria are strict anaerobes such as Bacteriocides, Clostridia, and Bifidobacteria. Furthermore, some facultative anaerobes such as Streptococci and Enterobacteriaceae take part. The higher volatile fatty acids are converted into acetate and hydrogen by obligate hydrogen-producing acetogenic bacteria. The hydrogen-producing acetogenic bacteria are not well characterized. Typical homoacetogenic bacteria are *Acetobacterium woodii* and *Clostridium aceticum*.

The accumulation of hydrogen can inhibit the metabolism of the acetogenic bacteria. The maintenance of an extremely low partial pressure of hydrogen is, therefore, essential for the acetogenic and H_2-producing bacteria. Although many microbial details of metabolic networks in a methanogenic consortium are not clear, present knowledge suggests that hydrogen may be a limiting substrate for methanogens (Bagi *et al.,* 2007). This assumption is based on the fact that addition of H_2 producing bacteria to the natural biogas-producing consortium increases the daily biogas production.

2.6.1.3 Role of the compounds in the substrate

At the end of the degradation chain, two groups of methanogenic bacteria produce methane from acetate or hydrogen and carbon dioxide. These bacteria are strict anaerobes and require a lower redox potential for growth than most other anaerobic bacteria. Only few species are able to degrade acetate into CH_4 and CO_2, e.g., *Methanosarcina barkeri*, *Metanonococcus mazei*, and *Methanotrix soehngenii*, whereas all methanogenic bacteria are able to use hydrogen to form methane. The first and second groups of microbes as well as the third and fourth groups are linked closely with each other (Schink, 1997). Therefore, the process can be accomplished in two stages.

A balanced anaerobic digestion process demands that in both stages the rates of degradation must be equal in size. If the first degradation step runs too fast, the acid concentration rises, and the pH drops below 7.0 which inhibits the methanogenic bacteria. If the second phase runs too fast, methane production is limited by the hydrolytic stage. Thus, the rate-limiting step depends on the compounds of the substrate which is used for biogas production. Undissolved compounds like cellulose, proteins, or fats are cracked slowly into monomers within several days whereas the hydrolysis of soluble carbohydrates takes place within few hours. Therefore, the process design must be well adapted to the substrate properties for achieving a complete degradation without process failure. It is difficult to describe the whole process by reliable kinetics since hydrolysis of complex insoluble substrate depends on many different parameters such as particle size, production of enzymes, pH, and temperature (Angelidaki *et al.,* 1999; Gavala *et al.,* 2003). For solid wastes, several kinetic models were developed for mesophilic and thermophilic digestion (Linke, 2006; Biswas *et al.,* 2007).

2.6.1.4 Molecular techniques involved in the characterization

Much is known about the basic metabolism in different types of anaerobic digestion processes, but little is known about the microbes responsible for these processes. Only few percent of bacteria and archaea have so far been isolated, but little is known about the dynamics and interactions between these microorganisms. The lack of knowledge results sometimes in malfunctions and unexplainable failures of biogas fermenters. With molecular techniques, more information can be received about the community structures in anaerobic processes (Elferink *et al.,* 1998; Karakashev *et al.,* 2005). Fluorescence in situ hybridization has been successfully used as a simple and rapid technique suitable for quantification of methanogens and the assessment of a wide range of samples from agricultural biogas plants (Stabnikova *et al.,* 2006). With 16SrDNA analysis technique, Klocke et al. (Klocke *et al.,* 2008) detected 68 taxonomic groups of methanogens in samples from ten agricultural biogas plants. He has shown that hydrogenotrophic methanogens dominate in most agricultural biogas plants. A high share of acetogenic methanogens can be found obviously only in biogas plants which are operated at low ammonia concentrations.

2.6.1.5 Role of temperature

The digestion process takes place at mesophilic (35– 42 °C) or thermophilic (45–60 °C) temperature conditions. It is important to keep a constant temperature during the digestion process, as temperature changes or fluctuations will affect the biogas production negatively. In most instances, methanogenic diversity is lower in plants operating at thermophilic temperatures (Karakashev *et al.,* 2005). Therefore, thermophilic processes are more sensitive to temperature fluctuations and require longer time to adapt to a new temperature. Mesophilic bacteria tolerate temperature fluctuations of +/−3 °C without significant reductions in methane production. The growth rate of methanogenic bacteria is higher at thermophilic process temperatures making the process faster and more efficient. Therefore, a well-functioning thermophilic digester can be loaded to a higher degree or operated at a lower hydraulic retention time (HRT) than at mesophilic conditions. But the thermophilic process temperature results in a larger degree of imbalance and a higher risk for ammonia inhibition.

2.6.1.5.1 Disadvantage of high temperature

Ammonia toxicity increases with increasing temperature, and washout of microbial population can occur (Angelidaki *et al.,* 2003). Especially the undissociated form of ammonia is considered to be responsible for process inhibition at concentrations above 80 mg/l . When the process is inhibited by ammonia, an increase in the concentration of volatile fatty acids (VFA) will lead to a decrease in pH which will partly counteract the effect of ammonia.

2.6.1.6 Dilution of reactor effluent for stable recovery

Strategies for recovery of the biogas efficiency following ammonia inhibition were studied and evaluated for anaerobic digestion of manure together with organic industrial waste (Angelidaki *et al.,* 1999). The most stable recovery process was observed when biomass was diluted with reactor effluent.

2.6.1.7 Optimum pH for methane production

Methane formation takes place within a relatively narrow pH interval, from about 6.5 to 8.5 with an optimum interval between 7.0 and 8.0. The process is severely inhibited if the pH decreases below 6.0 or rises above 8.5. The pH value increases by ammonia accumulation during degradation of proteins, while the accumulation of VFA decreases the pH value. The accumulation of VFA will often not always result in a pH drop, due to the buffer capacity of the substrate. Animal manure has a surplus of alkalinity which stabilizes the pH value at VFA accumulation. VFA are a key intermediate in the process and are capable of inhibiting methanogenesis in high concentrations. Acetic acid is usually present in higher concentration than other fatty acids, but propionic and butyric acids have more inhibitory effect to methanogens (Wang *et al.,* 1999). The inhibition is clearly associated with the undissociated form. Therefore, the inhibiting effect of VFAs is much higher in systems of low pH value.

2.6.1.8 Need of Macronutrient, micronutrient and trace element.

For the growth and survival of the specific groups of microorganisms, several macro- and micronutrients are necessary. Macronutrients are carbon, phosphor, and sulfur. The need of nutrients is very low due to the fact that not much biomass is developed, so that a nutrient ratio of C: N: P : S=600:15:5:1 is sufficient. Trace elements like iron, nickel, cobalt, selenium, molybdenum, and tungsten are important for the growth rate of microorganisms and must be added if, e.g., energy crops are used for biogas production as the only substrate (Abdoun and Weiland., 2009). Nickel is generally required for all methanogenic bacteria because it is necessary for the synthesis of the cell component cofactor F430, which is involved in the methane formation. For optimal growth, the cells require cobalt to build up the Co-containing corrinoid factor III. The function of selenium, molybdenum, and tungsten is not completely clear, and the growth of only few methanogens depends on these trace elements. The necessary concentration for the micronutrients is very low and in the range between 0.05 und 0.06 mg/l. Only iron is necessary in higher concentration between 1 and 10 mg/l. For monofermentation of energy crops, addition of micronutrients is absolutely necessary in order to achieve stable process conditions and high loadings. Addition of manure reduces the lack of micronutrients, but even in processes with a share of 50% manure, the adding of micronutrients can increase the anaerobic conversion rate (Mösche and Jördening., 1999).

2.6.1.9 Digestate utilization

The anaerobic digestion process results in a mineralization of organically bounded nutrients, in particular nitrogen and in a lowering of the C/N ratio. Both effects increase the short-term N fertilization effect. The digestate allows an accurate dosage and integration in a fertilization plan with a reduced application of additional mineral nitrogen fertilizers. The ammonia nitrogen content increases in some cases by a factor of three if energy crops are used as the only substrate .Due to the improved flow properties, the digestate can penetrate faster in the soil which reduces the risk for nitrogen losses by ammonia emissions. Anaerobic digestion results also in a significant reduction of odors and in a positive change in the composition of odors. Measurements have shown that up to 80% of the odors in the feedstock can be reduced. The anaerobic digestion process is able to inactivate weed seeds, bacteria (e.g., Salmonella, Escherichia coli, Listeria), viruses, fungi, and parasites in the feedstock which is of great importance if the digestate is used as fertilizer. The decay rate is dependent on temperature, treatment time, pH, and volatile fatty acids concentration. Temperature is the most important factor concerning survival of pathogens during anaerobic digestion. The best sanitation effect is obtained at thermophilic temperatures above 50 °C and long retention times. A 90% reduction of a Salmonella population is achieved at thermophilic temperature (53 °C) within only 0.7 h whereas at mesophilic conditions (35 °C) at least 2.4 days are necessary. For specific wastes, a separate pasteurization before or after anaerobic digestion at 70 °C for 60 min is stipulated by the European Union Animal By-Products Regulation (EC 1774/2002).

Pasteurization is an effective way of heat treatment; however, bacterial spores are not reduced. Pasteurization after digestion is more effective but the digestate is particularly prone to recontamination (Bendixen, 1999).

2.7 Biogas composition and process optimizations

The composition of biogas does not only include methane, but also up to 40% CO_2, water, hydrogen sulfide and other trace gases. Biogas is usually flammable due to the high yield of methane (40–75%), but for the use in engines or for injection into the natural gas grid it has to be purified and upgraded in methane content. This leads to higher calorific values of the biogas and avoids the presence of corrosive gases like hydrogen sulfide, which could cause damages to engines and pipes if remaining in the biogas (Ryckebosch *et al.,* 2011). There are several upgrading techniques, which take place after digestion (extensively reviewed by Bauer *et al.,* 2013). Process optimization can influence the biogas composition already during the process, lowering the costs of after-process purification. Numerous investigations on improvement of the biogas process have been undertaken, either to increase the overall amount of biogas, or to increase the methane content of the biogas.

It has turned out that careful pretreatment of the organic substrates leads to higher percentages of methane in the biogas. Several pretreatment methods such as chopping, alkali treatment and thermal treatment are present. (Andriani *et al.,* 2014).

2.7.1.1 Biological Pretreatment

From a biotechnological point of view, biological pretreatment of substrate is especially interesting. Biological pretreatment can increase the biogas production; this method was described by Zhong et al. (2011) which led to a 33% increase of biogas production (Zhong *et al.,* 2011). The substrates were exposed to a microbial agent including yeasts, celluleutic bacteria and lactic acid bacteria, which degraded the substrate before the actual start of the anaerobic digestion. A reduction in lignin, cellulose and hemicelluloses content could be observed after 15 days of pretreatment. The following anaerobic digestion showed an increase of biogas yield and methane content (Zhong *et al.,* 2011).

2.7.1.2 Co-digestion

Apart from the pretreatment of the single substrates, a mixture of different substrates (co-digestion) or a back mixing of digester effluent can lead to a better performance of the system (Weiland, 2010; Sosnowski *et al.,* 2003). Co-digestions can be carried out with mixtures of manure and energy plants or sewage slug and solid wastes and increase the methane production because of stabilizing the C : N ratio within the digester (Ward *et al.,* 2008; Sosnowski *et al.,* 2003). Another optimization method is addition of inorganic particles to the fermentation medium. Addition of nanoparticles of zero-valent iron could enhance the methane production by 28% (Carpenter *et al.,* 2015). An increase in biogas formation could also be observed with magnetic iron oxide particles. Other particles include charcoal, silica and mineral salts were investigated. The improvement in biogas yield could be due to aggregation of bacteria and methanogens around the particles, leading to a lower washout and higher culture densities; it is also possible that metal particles release electrons to the surrounding medium, which can be used for methane formation, but the exact mechanism remains unclear (Carpenter *et al.,* 2015).

2.7.1.3 Hydrogenotropic Methanogens

One promising method for biological biogas upgrading in methane content is the conversion of the residual CO_2 to additional methane using hydrogenothrophic methanogens, which are capable of producing methane solely out of CO_2 and H_2 (Bassani *et al.,* 2015). H_2 can either be injected into the anaerobic digester (Luo *et al.,* 2012), or H_2 and biogas can be mixed in a second reactor containing methanogens (Bassani *et al.,* 2015; Luo and Angelidaki, 2012). Hydrogen when introduced to the anaerobic digester, there may be a shift within the methanogenic community: acetoclastic methanogens may decrease, while hydrogenotrophic methanogens (especially *Methanoculleus*) are enriched; also, hydrolyzing and acidifying bacteria may decrease, while synthrophic bacteria producing acetate increase (Bassani *et al.,* 2015). Technical concepts for the integration of H_2 into existing biogas plants and effective new means of process control are necessary to make this process commercially attractive. Therefore, experiments have to be carried out under industrial conditions, i.e. under fluctuating substrate compositions, in reactors with zones of different substrate concentrations, changing microbial consortium and different pressure zones according to a larger reactor height; these conditions will usually not appear in lab scale, unless they are particularly tested.

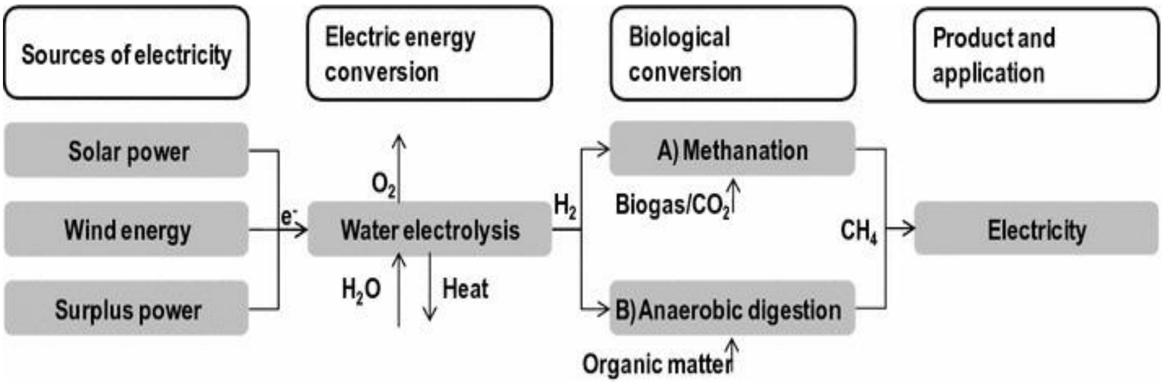

Figure 2.3 : Increasing methane yield by hydrogen addition (Enzmann *et al.,* 2018) .

H_2 is produced via water electrolysis and fed into the second reactor for the conversion of CO_2 into methane, or feed directly to the anaerobic digester for in situ methane production.

H_2 is usually produced by water electrolysis, a process in which electricity is used to split water and generate oxygen and hydrogen. To couple water electrolysis to anaerobic methanogenesis and provide a constant level of H_2 within the digester, methanogenic bioelectrochemical systems were invented.

2.8 Biochemical and microbial background

2.8.1 Methanogens

Methanogens are biocatalysts, which have the potential to contribute to a solution for future energy problems by producing methane as storable energy carrier. The very diverse archaeal group of methanogens is characterized by the ability of methane production (Balch *et al.,* 1979). The flammable gas methane is considered to be a suitable future replacement for fossil oil, which is about to be depleted during the next decades (Ren *et al.,* 2008). Methane can be used as a storable energy carrier, as fuel for vehicles, for the production of electricity, or as base chemical for synthesis and many countries do already have well developed natural gas grids (Ren *et al.,* 2008). In terms of the necessary transition from chemical to biological processes, methanotrophic bacteria can use methane as a carbon and energy source to produce biomass, enzymes, PHB or methanol (Strong et al. 2015; Ge et al. 2014). The biological methanation is the main industrial process involving methanogens. These archaea use CO_2 and H_2 and/or small organic molecules, such as acetate, formate, and methylamine and convert it to methane. Although the electrochemical production of methane is still more energy efficient than the biological production [below 0.3 kWh/cubic meter of methane](Bär *et al.,* 2015), the biological conversion may be advantageous due to its higher tolerance against impurities (H_2S and NH_3) within the educt streams, especially if CO_2 rich waste gas streams shall be used (Bär et al. 2015). Apart from that, research is going on to increase the energy efficiency of the biological process, so that it might be the preferred way of methane production in the future (Bär *et al.,* 2015). Biological methanation occurs naturally in swamps, digestive systems of animals, oil fields and other environments (Garcia *et al.,* 2000) and is already commonly used in sewage water plants and biogas plants. New applications for methanogens such as electromethanogenesis are on the rise, and yet, there is still a lot of basic research, such as strain characterization and development of basic genetic tools, going on about the very diverse, unique group of methanogens (Blasco-Gómez *et al.,* 2017).Methanogens are the only group of microorganisms on earth producing significant amounts of methane. They are unique in terms of metabolism and energy conservation, are widespread in different habitats and show a high diversity in morphology and physiological parameters.

2.8.2 Phylogeny and habitats of methanogens

For decades known methanogenic archaea belonged exclusively to the phylum *Euryarchaeota*. There, methanogens were classified first into five orders, namely *Methanococcales*, *Methanobacteriales*, *Methanosarcinales*, *Methanomicrobiales* and *Methanopyrales* (Balch *et al.,* 1979; Stadtman and Barker, 1951; Kurr *et al.,* 1991). Between the years 2008 and 2012 another two orders of methanogens, namely *Methanocellales* (Sakai *et al.,* 2008) and *Methanomassiliicoccales* (Dridi *et al.,* 2012; Iino *et al.,* 2013), were added to the phylum *Euryarchaeota*. Hydrogenotrophic methanogenesis from H_2 and CO_2 is found in almost all methanogenic orders with the exception of the *Methanomassiliicoccales*. Due to its broad distribution it is postulated that this type of methanogenesis is the ancestral form of methane production (Bapteste *et al.,* 2005). Methane formation from acetate, called aceticlastic methanogenesis, can be found only in the order *Methanosarcinales*. In contrast to that, methylotrophic methanogenesis, which is the methane formation from different methylated compounds such as methanol, methylamines or methylated thiols, is found in the orders *Methanomassiliicoccales*, *Methanobacteriales* and *Methanosarcinales*. Extensive recent metagenomic analyses suggested that methanogens may no longer restricted to the *Euryarchaeota*. Two new phyla, namely the *Bathyarchaeota* (Evans *et al.*, 2015) and the *Verstraetearchaeota* (Vanwonterghem *et al.,* 2016) were postulated. Genome sequences from both phyla indicate a methylotrophic methane metabolism in these -as of yet uncultivated- potential methanogens.

Methanogens are a relative diverse group of archaea and can be found in various anoxic habitats (Garcia *et al.,* 2000). For example, they can be cultured from extreme environments such as hydrothermal vents or saline lakes. *Methanocaldococcus jannaschii* was isolated from a white smoker chimney of the East Pacific Rise at a depth of 2600 m (Jones *et al.,* 1983) and *Methanopyrus kandleri* from a black smoker chimney from the Gulf of California in a depth of 2000 m (Kurr *et al.,* 1991). From a saline lake in Egypt the halophilic methanogen *Methanohalophilus zhilinae* was cultured (Mathrani *et al.,* 1988). But methanogens also colonize non-extreme environments. They can be isolated from anoxic soil sediments such as rice fields, peat bogs, and marshland or wet lands. For example, *Methanoregula boonei* was obtained from an acidic peat bog (Bräuer *et al.,* 2006) and several strains of *Methanobacterium* as well as *Methanosarcina mazei* TMA and *Methanobrevibacter arboriphilus* were isolated from rice fields (Asakawa *et al.,* 1995).

Some methanogens can also associate with plants, animals and could be found in the human body. *Methanobacterium arbophilicum* could be isolated from a tree wetwood tissue and uses the H_2 resulting from pectin and cellulose degradation by *Clostridium butyricum* for methanogenesis (Schink *et al.,* 1981; Zeikus and Henning, 1975). From the feces of cattle, horse, sheep and goose the methanogens *Methanobrevibacter thaueri*, *Methanobrevibacter gottschalkii*, *Methanobrevibacter wolinii* and *Methanobrevibacter woesei* have been isolated, respectively (Miller and Lin, 2002). In addition, different *Methanobrevibacter* species could be found in the intestinal tract of insects such as termites (Leadbetter and Breznak, 1996). Beside the intestinal tract of herbivorous mammals also the rumen contains methanogens. One of the major species here is *Methanobrevibacter ruminantium* (Hook *et al.,* 2010). Methanogenic archaea are also present in the human body. *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* as well as *Methanomassiliicoccus luminyensis* could be detected in human feces (Dridi *et al.,* 2009, 2012; Miller *et al.,* 2002). Further *Methanosarcina* sp., *Methanosphaera* sp. and *Methanobrevibacter oralis* were discovered in human dental plaque (Robichaux *et al.,* 2003).

Methanogens can be also found in non-natural habitats such as landfills, digesters or biogas plants. There, the microbial community varies with the substrate. In biogas plants, due to hydrolysis of complex polymers to sugars and amino acids, followed by fermentation and acetogenesis, acetate, H_2 and CO_2 is produced as substrates for methanogenesis. Therefore, hydrogenotrophic and aceticlastic methanogens are prevalent in mesophilic biogas plants, often dominated by species of *Methanosarcina*

(*Methanothrix* at low acetate concentrations) or *Methanoculleus* (Kern *et al.,* 2016; Karakashev *et al.,* 2005; Lucas et al., 2015; Sundberg *et al.,* 2013). However, under certain conditions syntrophic acetate oxidation may be the dominant path towards methane (Schnürer and Nordberg, 2008; Westerholm *et al.,* 2016).

2.8.3 Electromethanogenesis

One of the promising approach for biogas upgrading involves the use of bioelectrochemical systems to provide electrons or reducing equivalents to the hydrogenotrophic methanogens which results in the conversion of CO_2 to CH_4 (Lovley and Nevin, 2013).

The anaerobic conversion of CO_2 and H_2 to acetate by acetogenic bacteria has been described to follow the reductive acetyl-CoA pathway (Ljungdahl, 1986). In the case of MES, reduction at the biocathode occurs with the application of electric energy without the external supply of reductants such as hydrogen. The microbes involved in the biocathode can receive the electrons either directly from the cathode or indirectly via mediators or via H_2 produced by water electrolysis (Nevin *et al.,* 2010) described the first proof of principle of CO_2 reduction in microbial electrosynthesis using the acetogen

Sporomusa ovata, which can use electrons directly from solid graphite electrodes for the reduction of CO_2 to produce acetate and small amounts of 2-oxobutyrate at 0.6 V/Ag/AgCl cathode potential.

Methane content in the biogas can be increased by stimulating the hydrogenotrophic methanogens to convert CO_2 to methane. In electromethanogenesis, CO_2 is reduced by methanogens, either directly taking the electrons from electricity at the cathode or some mediator or hydrogen (produced from biological activity or due to water splitting) facilitates the electron transfer from cathode to the microbes.

The process is called ''electromethanogenesis,'' in which methanogens can directly accept electrons from electrodes for CO_2 reduction ($CO_2 + 8H^+ + 8e^- {>} CH_4 + 2H_2O$) (Villano *et al.,* 2010).

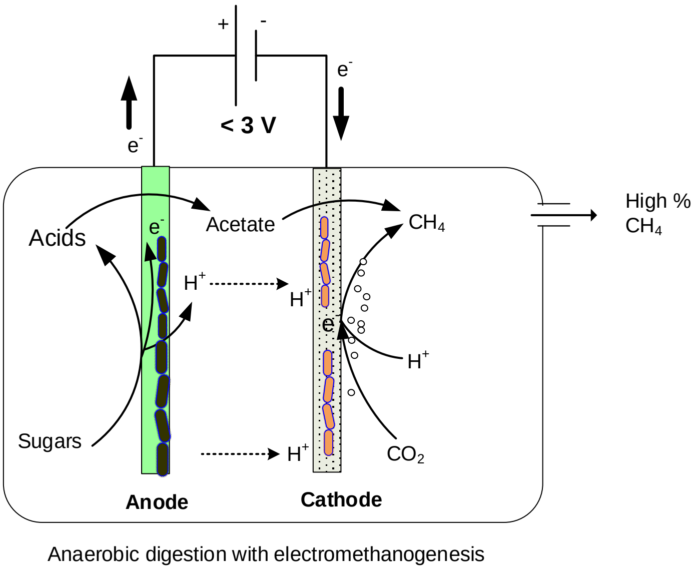

Figure 2.4 : Anaerobic Digestion with electromethanogenesis (Bajracharya *et al.,* 2015)

At anode,

$C_6H_{12}O_6 + 12H_2O \rightarrow 6HCO_3^- + 30H^+ + 24\ e^-$

anode potential E°' = -0.41 V (Rozendal *et al.*, 2006)

At cathode,

$HCO_3^- + 8e^- + 9H^+ \rightarrow CH_4 + 3H_2O$

cathode potential E°' = -0.24 V (Rozendal *et al.*, 2006)

An alternative option for more realistic applications is power-to-methane. The conversion of electrical current into methane (i.e. CO_2 methanation) has been for long proposed as alternative, considering that many Countries already count on a capillary methane distribution grid (Zoss *et al.,* 2016). High-temperature and pressure catalytic power-to-methane conversions are the state of the art, but they encounter serious constraints in power-to-gas P2G applications: a) relatively small-scale plants are too expensive;

intermittent use, as needed for day-night grid variations, are not viable; and large amounts of high purity CO_2 gas streams are required to avoid hindering the metal catalysts (Götz *et al.,* 2016). Also, the territorially distributed availability of concentrated and pure CO_2 streams is not guaranteed, if we exclude fossil-based power plants.

Biological methanation has been proposed as an alternative for smaller-scale applications (Götz *et al.,* 2016). Several methanogenic microbes of the domain Archaea are known to catalyze the reduction of of CO_2 to methane (autotrophic methanogenesis), in strictly anaerobic environments (Bapteste *et al.,* 2005). Hydrogenotrophic methanogenesis, for example, has been longely known and it is widely used in full-scale anaerobic digestion (AD) facilities as one of the two main metabolic routes towards methane production from biomass (Premier *et al.,* 2013). Electro-trophic methanogenesis (or electro-methanogenesis) was also recently discovered as an alternative path towards CO2 reduction (Cheng *et al.,* 2009). Different innovative biotechnological applications of these biochemical pathway were proposed as options for methane generations by reduction of CO_2 through electrostimulation of methanogenic microbial communities in bioelectrochemical systems (BES) (Blasco-Gómez *et al.,* 2017).

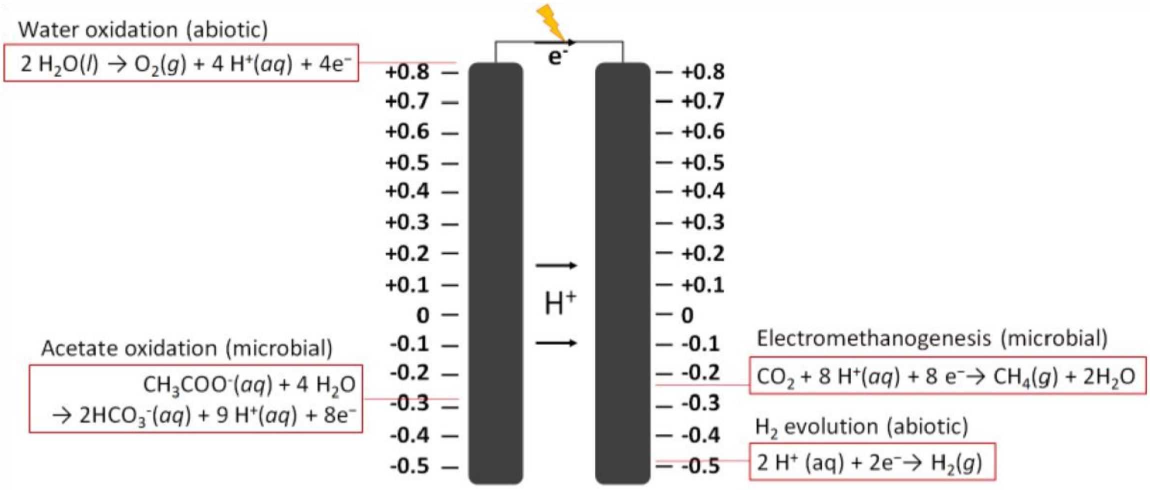

Figure 2.5 : Electrochemical reaction standard potentials (E0, V vs standard hydrogen electrode, pH 7) for anodic and cathodic main reactions.

Among a range of possible technological solutions, power-to-gas technologies have attracted attention. Water electrolysis to produce gaseous hydrogen as chemical energy vector (power-to-gas, P2G) has been proposed as the solution, to be coupled to fuel cell to reconvert H_2 into electricity. The abiotic electrocatalysis of the reaction $2H^+ + 2e^- \rightarrow H_2$ works theoretically at a cathodic potential of -0.410 V vs Standard Hydrogen Electrode (SHE, pH=7). However, in applications with high current-densities, according to the catalyst and the electrode properties, this reaction may require much lower potentials (-0.7 – 1 V vs SHE), due to consistent overpotentials of both cathodic and anodic reactions (Zoulias and Varkaraki, 2004).

Unfortunately, the first step of this transformation chain (i.e. water electrolysis in electrochemical cells, based on abiotic catalysts) is a relatively inefficient process. Due to the high overpotentials of the cathodic electrocatalysis at such high current densities (Zoulias and Varkaraki, 2004). Even considering a stoichiometric conversion of this H_2 to methane: $4H_2 + CO_2 \rightarrow 2H_2O + CH_4$ the electricity-CH_4 conversion efficiency would result of 16-20 kWh/Nm^3 CH_4. Additionally, the final conversion efficiency would be even lower due to the low solubility in water of H_2 (Bo *et al.,* 2014).

Also, water electrolysis suffers of poor efficiency in the counter reactions, at the anode. The use of water as electron donor, with O_2 evolution, is definitely not a thermodynamically favorable reaction.To overcome these constraints, a new generation of biological methanation process was introduced and called Electromethanogenesis (Blasco-Gómez *et al.,* 2017; Cheng *et al.,* 2009). It results from the integration of electrochemical systems and microbial autotrophic methanogenesis (hydrogenotrophic route). Electroactive microbial communities, grown as biofilms on solid electrodes, were demonstrated to be able of direct electron transfer towards the fixation of inorganic carbon to methane (Cheng *et al.,* 2009), following the reaction:

$CO_2 + 8H^+ + 8e^- \rightarrow CH_4 + 2H_2O$

This reaction theoretically happens at a cathodic potential of -0.224 V vs SHE, i.e. it takes half of the energy, with respect to water electrolysis.The most common microbial species able to perform this reaction belong to the Archaea domain and are normally found in regular anaerobic sludge in biogas-producing facilities. Direct electron transfers towards inorganic carbon fixation to methane, in fact, was demonstrated as a mechanism that happens in natural anaerobic environments, between different microbial species. The so-called DIET (direct interspecies electron transfer), mediated by membrane bound proteins and conductive extracellular filaments (called e-pili), was demonstrated between acetoclasts (e.g. Geobacter sp., Shewanella sp.) and methanogens (e.g. Methanobacterium sp., Methanosaeta sp., Methanosarcina sp.). In few words, microbes create a network of nanowires to exchange electrons among different species. Where conductive solid materials are present, this connection is favored, as compared to water-suspended cells (Chen *et al.,* 2014).

Additionally, if the electron flow is forced from externally imposed electrochemical potentials, methane formation can be favored, even in absence of favorable electron donors, such as organic molecules (e.g. acetate) (Bajracharya *et al.,* 2015). However, the thermodynamics of the system are favored when the electron donor at the anode is an organic molecule (Jadhav *et al.,* 2017; Logan and Rabaey, 2012). In this case, the oxidation reaction is mediated by acetoclastic electro-active microbes that discharge electrons to the conductive surface of the anode (You *et al.,* 2014). Acetoclastic methanogens were often found in anodic biofilms of bioelectrochemical systems (Rago *et al.,* 2017; Rago *et al.,* 2018) where they compete with electro-active microorganisms for the same electron donor (acetate). For this reason, several studies focused on different strategies for methanogenisis inibition in recent years (Chae *et al.,* 2010, Rago *et al.,* 2015).

2.8.4 Electroactivity of methanogens

2.8.4.1 Electron transfer

When electrodes are inserted into a reactor with methanogens, these electrodes can eventually be used by the organisms to produce methane. An external potential leads to the electrolysis of water at the anode; oxygen and protons are produced, electrons are transferred to the anode. Otherwise, excess electrons out of metabolic reactions can be transferred to the anode, like it would happen in a microbial fuel cell. The electrons migrate to the cathode through an external circuit. At the cathode surface, the electrons are transferred to the methanogens, which can use them to produce methane. The complete mechanism is not yet elucidated, but mainly, three possibilities are suggested (Fig. 2.6) (Sydow *et al.,* 2014; Geppert *et al.,* 2016). Probably, more than one of these mechanisms contributes to the electron transfer (Zhen *et al.,* 2015).

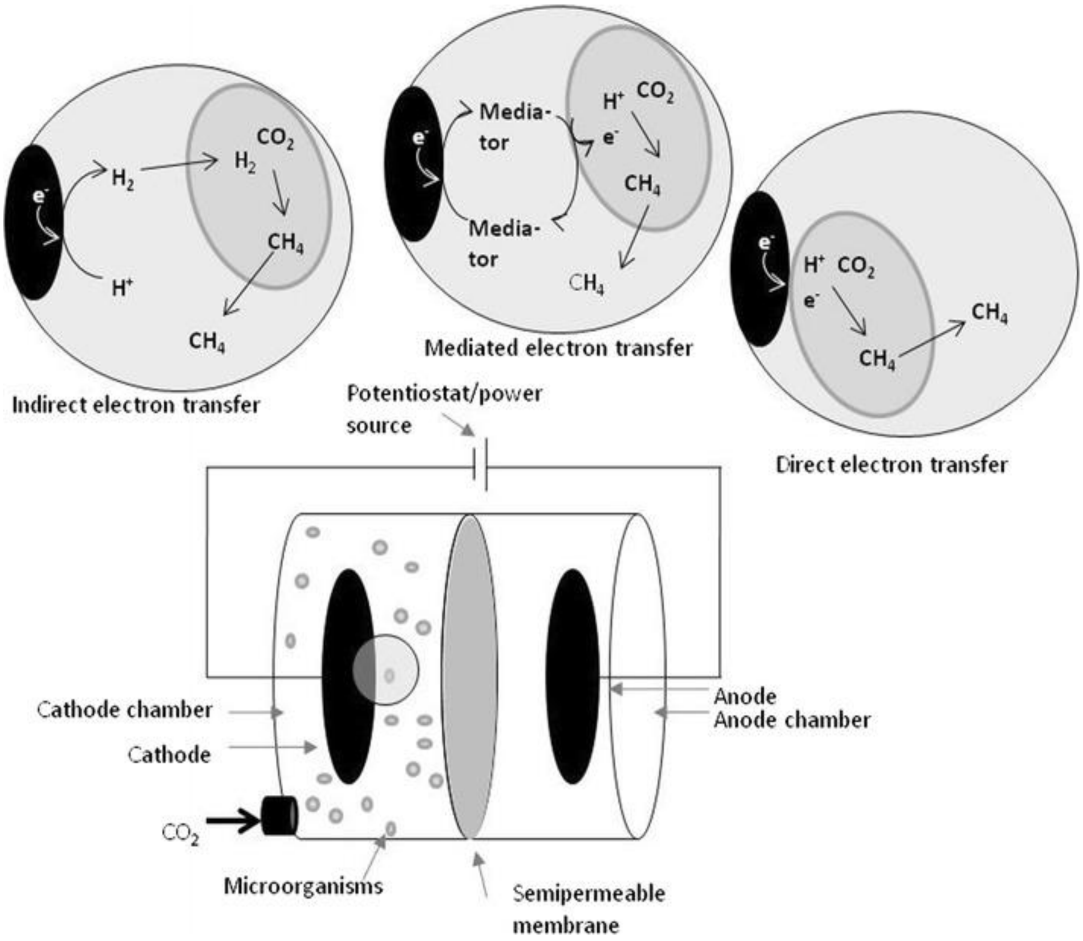

Figure 2.6: Extracellular electron transfer (Enzmann *et al.,* 2018)

Means of electron transfer within a separated, electromethanogenic system at the cathode: indirect electron transfer (IET), mediated electron transfer (MET) and direct electron transfer (DET)

One possible way would be the transfer of electrons from the cathode to protons, which have been produced at the anode and migrated through the membrane between anodic and cathodic chamber. Thereby, hydrogen is produced at the cathode, which is then consumed by the methanogens. This indirect electron transfer (IET) would allow the production of methane out of hydrogen and CO_2 (Villano *et al.,* 2010). As an example, IET was observed in *M. thermautotrophicus* (Hara *et al.,* 2013). It has also been shown that some *Methanococcus maripaludis* secrete hydrogenases and probably formate dehydrogenases which catalyze the formation of hydrogen and formate directly at the electrode surface; the produced hydrogen and formate is then metabolized by the cells (Deutzmann *et al.,* 2015). This has to be seen as an indirect electron transfer, since the cells were not directly attached to the electrode; from the experimental results, it may be mistaken for a direct electron transport, since the abiotically (without catalyzing hydrogenases) produced amounts of hydrogen and formate cannot explain the amount of methane produced (Deutzmann *et al.,* 2015).

Another possibility suggests that mediator molecules could accept the electrons at the cathode surface, shuttle it to the methanogens and donate it to the microorganisms. This mediated electron transfer (MET) would imply that the methanogens take up electrons, protons and CO_2 to form methane (Choi and Sang, 2016). Flavins, phenazines or quinones can serve as mediator, either naturally secreted by the organisms or added to the reaction medium (Sydow *et al.,* 2014; Patil *et al.,* 2012). A natural secretion of mediators with a redox potential suitable for microbial electrosynthesis (should be < -0.4 V vs. SHE) has not been observed yet (Sydow *et al.,* 2014). In methanogens, MET could be performed by using neutral red as an electron shuttle (Park *et al.,* 1999).

The third option would be the direct electron transfer (DET) from the cathode surface to the methanogens, e.g. via surface proteins or conductive filaments (so-called nanowires). To generate methane, the microorganisms would use electrons, protons and CO_2 (Cheng et al. 2009). Several studies suggest that direct electron transfer indeed occurs in methanogens (Zhen *et al.,* 2016; Lohner *et al.,* 2014). For a hydrogenase-deficient strain of *M. maripaludis* hydrogenase-independent electron uptake was demonstrated (Lohner *et al.,* 2014), ruling out IET.

In a mixed microbial consortium, direct interspecies electron transfer (DIET) is another possible way of electron transfer. There, one microbial strain takes up electrons at the cathode surface and transfers it to another strain. This may happen, e.g. via conductive filaments (Gorby *et al.,* 2006). It has been reported that this (syntrophic) electron transfer can be very specific between two species, e.g. based on conductive filaments between *M. thermautotrophicus* and *Pelotomaculum thermopropionicum* (Gorby *et al.,* 2006) or between *M. barkeri* and *Geobacter metallireducens* (Rotaru *et al.,* 2014). Apart from this direct interspecies electron transfer, an interspecies hydrogen transfer can occur. Here, one organisms takes up electrons, produces hydrogen as an intermediate and transfers them to a second organism that forms another product. An example is the defined co-culture between the iron-corroding, sulfate-reducing bacterium 'Desulfopila corrodens' IS4 (former name: *Desulfobacterium corrodens*) for electron uptake and *M. maripaludis* for methane production (Deutzmann and Spormann, 2017).

2.9 Carbon Nanotubes in Electrochemical Devices

Carbon nanotubes (CNTs) because of the high electrochemically accessible surface area of porous nanotube arrays, combined with their high electronic conductivity and useful mechanical properties, these materials are attractive as electrodes for devices that use electrochemical double-layer charge injection.(Baughman *et al.,* 2002)

2.9.1 Carbon nanotubes in electrochemical catalysis

In recent years, one dimensional nanomaterials like nanotubes and nanofibers have attracted much attention from researchers due to their one dimensional nature and nanometer structure. Moreover, these nanomaterials have unique electronic, optical and magnetic properties that can be used in many applications (Ghasemi *et al.,* 2013). CNTs have a cylindrical structure with a high length to diameter ratio of around 28,000,000:1, which is considerably larger than any other known material (Iijma *et al.,* 1999) CNTs have shown very promising properties as a catalyst support because of their unique electrical (high conductivity), and structural properties(Che *et al.,* 1998).CNTs have also been shown to have ability to enhance direct electron transfer (DET) (Zhang *et al.,* 1993) One of the main problems of synthesizing CNTs in electronic devices is the insolubility of unmodified CNTs in all organic solvents and water, which limits the use of solvent based techniques (Ishihara *et al.,* 2001). The poor stability of CNTs in most solvents prevents the facile fabrication of homogeneously dispersed CNT device. Sprayed thin film of CNTs for example, is not stable for long. CNTs must be modified chemically to make them soluble or to integrate them into inorganic, organic and biological systems. (Ghasemi *et al.,* 2013).

Carbon materials have been reported to facilitate direct interspecies electron transfer (DIET) between bacteria and methanogens improving methane production in anaerobic processes. The effect of increasing concentrations of carbon nanotubes (CNT) on the activity of pure cultures of methanogens and on typical fatty acid-degrading syntrophic methanogenic coculture was evaluated. CNT affected methane production by methanogenic cultures, although acceleration was higher for hydrogenotrophic methanogens than for acetoclastic methanogens or syntrophic coculture. (Salvador *et al.,* 2017).

2.10 Application of biogas

The most common method of using biogas for energy production is through converting it into heat and electricity in combined heat and power (CHP) plants. During the biogas production process, roughly an equivalent amount of carbon dioxide (CO_2) is generated as is absorbed by the energy crops during their growth cycle, or as would be released naturally in the rotting of waste. This makes power and heat from biogas plants particularly climate-friendly.

2.10.1 Heat and power from one energy source

Biogas production also has an indirect impact on climate protection. This is because the controlled fermentation of liquid manure and compostable waste prevents the release of harmful gases such as methane, which has a far more influence on the greenhouse effect than CO_2.

2.10.2 Micro gas grids: from biogas plant to combined heat and power plant

If purchasers of heat are located at too great a distance from the biogas plant, transporting this heat is extremely complex and costly. In these cases, the CHP plant can be constructed where the heat is needed. Here, the biogas is simply transported via dedicated pipes to satellite CHP plants in the town, or directly to heat consumers, and then converted into heat and power on site. The waste heat can then be cheaply distributed where it is required.

2.10.3 Biomethane in the natural gas grid

After processing, biogas can also be injected directly into the existing natural gas grid. In this scenario, it exploits the infrastructure available and finds application as a natural gas equivalent (known as biomethane).

2.10.4 Biogas as a fuel

Biogas can also be used as a replacement for natural gas in vehicle fuel.

2.10.5 Energy that can be stored and used at the drop of a hat

Biogas can be stored without difficulty, meaning that it can be used at any time in any location irrespective of where it is produced. Hybrid power plants, for example, are able to take advantage of these qualities. They combine different renewable energy sources to complement one another, such that the amount of energy required is always at hand.

Owing to its aptitude for storage, biogas is particularly valuable here, as it is able to compensate for fluctuations in other renewable sources such as wind and solar energy.

2.11 Prevalent problem in existing biogas plant

Single household biogas plants have successfully contributed to improve the health and economic situation for thousands of Nepalese families and is continuing to do so at an increased rate. Several users' survey confirm that the development has been a success and 97 percent of the digesters were estimated to be operational in 2013 (Sigdel, 2007; Bajgain & Shakya, 2005).However, two key parameters are limiting the beneficial outcome of biogas:

1. Technical limitations. Temperature is by far the most important parameter in terms of plant performance. Seasonal variances can reduce biogas production by 50 percent (Finlay, *et al.,* 2013), and is the reason for why almost one third of the users receive sufficient gas in the summer months only. Climate is also limiting expansion of biogas to the mountain regions of Nepal, concentrating the technology to the southern districts (Bajgain & Shakya, 2005).
2. It is expensive to build and maintain a digester. The wealth requirement to build and properly maintain a digester is relatively high. The farmer needs sufficient cattle, water and economic strength to apply for a subsidy and build a digester. This is preventing biogas from benefiting the poorer families and has been a point of critique of the technology (BSP-Nepal, 2005).

2.12 Methods implemented to overcome the problem

2.12.1 Increasing digester temperature

Variance in temperature is the most important parameter for fluctuation in gas production. Reducing temperature has severe implications on the gas production rate and virtually no gas is being produced in temperatures less than 15 °C. One solution practiced in parts of northern China, is to shut down gas production during winter and only operate the plant during the 6-8 months period when gas production is at its peak (Sasse, 1988). Most biogas users still utilize traditional fuels to some degree (BSP-Nepal, 2005), but is not ideal since most of the benefits related to using biogas rely on not using firewood. There are many techniques to increase digester temperature, but they are not widespread and often require more knowledge, materials and dedication.

2.12.2 Insulation

Insulating the digester is the most energy effective method to maintain digester temperature by reducing energy losses to the environment. This can be done relatively cheap, since the materials used for insulation in conventional biogas plants are replaceable by local materials with similar properties. Cereal straws, rice husks, sawdust and shavings have good thermal conducting properties that are comparable to industrial materials, as shown in table 7 (Finlay, *et al.,* 2013).

Table 2.3 : Typical values for the thermal conductivity of some materials

Material	**Thermal conductivity, W m^{-1} K^{-1}**
Concrete	1,0
Soil (fairly dry)	1,4
Saturated soil	2,4
Saw dust (loose)	0,06
Shavings (loose)	0,06
Sugar cane fiber	0,05
Insulite (wood pulp)	0,05
Glass wool	0,04

The materials proposed in *table* 2.2 are organic and turn into compost, which releases heat during decomposition and further increases the temperature of the system. A study covering the digester with compost, has reported more than a 50 percent increase in biogas production during winter months (Finlay, *et al.,* 2013). The compost pile was 0.7-0.8 m high and was covered by a plastic sheet, giving a similar effect to a greenhouse. The disadvantages of this method is that the composting is not permanent and can give an unpleasant odor.

2.12.3 External heating

For simple digesters, the main source of external heating is solar radiation. The incoming energy should be maximized by placing the digester on a cleared area and facing the sun in the case of a hilly side. Preheating the feed material by mixing and leaving it in the sun is also possible. One study found an increase of influent temperature by 4.5 to 9 °C depending on daily conditions (Finlay *et al.,* 2013). Solar radiation does not penetrate far into the slurry, so the mixing tank was made with a large surface area, shallow and covered with a transparent plastic sheet. Solar reflectors or constructing a greenhouse above the digester has also been reported to give positive results (MinErgy Pvt. Ltd., 2014).

Most simple biogas systems operate without any complex heating system. However, it is possible to increase the temperature of the digester by using the heat loss of an engine or generator with a heat exchanger. This can also have a positive impact on the engine as it releases heat to the digester. The heat exchanger can be used to either heat up the feeding material as a form of preheating or it can be used inside the digester at the risk of corrosion damages (MinErgy Pvt. Ltd., 2014).

Other preheating methods are often expensive and underdeveloped for simple digesters. Some industrial plants use a fraction of the biogas produced to heat the same digester. However, using 20-30 percent of the produced gas to heat the digester is not viable for small-scale plants.

CHAPTER 3. MATERIALS AND METHODS

3.1 Laboratory Setting

This study was conducted in the laboratory of Central Department of Biotechnology,

Tribhuvan University. All the chemicals were of reagent grade. All the experiments were done in triplicate.

3.2 Collection of cattle manure

Cattle manure was obtained from Rana Kumari Khadka's cow shed in Kirtipur, Nepal. The manure collected was fresh and formed. Cows were fed with grass, hay, grains and legumes.

3.3 Analysis

The samples were subjected for analysis of pH, soluble protein, reducing sugar and total soluble solid (TSS), volatile soluble solid (VSS), chemical oxygen demand (COD). The phosphorus content were determined according to Standard Methods for the Examination of Water and Wastewater (1998).

3.3.1 Determination of total suspended solid and volatile suspended solids

In order to test the total suspended solids (TSS), cow dung sample was weighed .Sample was dried to a constant weight at a temperature between 105±1 °C and was left to dry overnight. After the total suspended solids value was determined a volatile suspended solids (VSS) test was performed in order to determine the concentration of volatile suspended solids in the sample. The sample used for total suspended solids testing was transferred to a crucible and it was ignited at 550 °C for 1.5 hrs in muffle furnace (Valo *et al.,* 2004). The VSS determination was performed at Research Center for Applied Science and Technology, Nepal (RECAST).

3.3.2 Estimation of soluble protein

Proteins were measured with Lowry's method using bovine serum albumin as a standard solution. Different dilutions of BSA solutions were prepared by mixing stock BSA solution (100μg/ml) and water to make the BSA solution of concentration range is 20-100 μg/ml. In each solution 5 ml of alkaline copper sulphate reagent (analytical reagent) was added. The solutions was mixed well. Then the solution was incubated at room temperature for 10 mins. Then 0.5 ml of reagent Folin Ciocalteau solution (reagent solutions) was added to each tube and incubated for 30 min. The absorbance was recorded at 660nm with blank. The absorbance against protein concentration to get a standard calibration curve was plotted. Hence the absorbance of the sampe was taken and the concentration of protein was determined using the standard curve (Sawhney and Singh, 2000).

3.3.3 Determination of Reducing Sugar

Firstly stock solution of Glucose was prepared (1000 µg/mL). Standard of glucose concentration from 10 µg/mL to 500 µg/mL was prepared. Similarly, 0.02 ml of sample was taken along with 0.18ml of water and final volume was made 200 µl .Then 200 µl of each standard and sample was taken into which 0.2ml DNS reagent was added. After the addition of DNS it was kept in boiling water for 10 minutes. It was allowed to cool for a while and later 2 ml of distilled water was added. Absorbance was read in 540nm for both sample and standard. (Miller *et al.,* 1961)

3.3.4 Determination of Chemical Oxygen Demand

A series of concentration ranging from 20mg/L,50mg/L,100mg/L,200mg/L,400mg/L,600mg/L, and 900mg/L of phthalate working solution was prepared, by pipetting suitable volumes of the phthalate stock solution(1000mg/L) in 50-mL volumetric flasks. Then 10 ml of the reference solutions were added to the culture tubes. After then 6 ml of digestion solution (Appendix-I) was added to each of the references and mix thoroughly. To the tubes, 14 ml of catalyst solution was added to each of the references, down the side of the culture tube. It was capped tightly and was shake to mix the layers .The culture tubes were placed in an oven at 150°C for 2 hours. The tubes were cooled and any precipitate if was allowed to settle. Background correction was performed with the blank solution (without sample) and the absorbance of the solutions was taken at 600 nm using the cuvette. (Henze *et al.,* 2001)

Finally 2 ml of the sample was taken into a culture tube and the same procedure was followed as in the standard curve preparation with appropriate ratio and COD was determined by using the linear regression equation of the standard.

3.3.5 Digestion of sample in flask with H_2SO_4- Salicylic acid- H_2O_2

0.6 g of the cow dung sample was weighed in a digital weighing balance and the sample was trasferred to a 50-mL volumetric flask. All the sample was allowed to come below the neck of the flask. Exactly 3.3 mL of the digestion mixture (Appendix-I) was added and 4 carborundum beads was introduced and swirlled carefully until all the sample was moistened. It was then allowed to stand overnight. In addition, two blank digestions was prepared. The flask was heated on a hot plate at 180 ˚C for about 1 h. The flask was removed from the plate, cooled down, and 5 drops of hydrogen peroxide was added. The flask was plated on the hot plate and the temperature was increased to about 280 ˚C. The flask was heated for 10 min until the water had evaporated. The flask was removed from the plate, allowed to cool down, again 5 drops of hydrogen peroxide was added, and heated again for 10 min that resulted in the appearance of white vapours. 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; swirlled until most of the precipitate had dissolved. The mark was made up with water, mix well and filtered over coarse filter paper. The digest was filtered to remove any SiO_2 that will otherwise dissolve gradually and then interfere in the determinations. The calibration solutions for analysis was prepared in the same final medium as the samples in order to get a matrix, which was the same as in the samples. The final medium was 0.8 M H_2SO_4. (Temminghoff and Houba 2004)

3.3.6 Determination of Potassium and Arsenic

The prepared digested solution was subjected to K and As estimation at National Academy of Science and Technology (NAST).

3.3.7 Determination of Phosphorus

3.3.7.1 Standard curve of Phosphorus

A series of reference solution ranging from 0.01 mg/L,0.03mg/L,0.05mg/L,0.1mg/L,0.2mg/L, 0.3mg/L,0.4mg/L,0.5mg/L of phosphorus working solution was prepared by pipetting suitable volumes of the phosphorus stock solution.

3.3.7.2 Determination of Phosphorus

The blanks and all digests were mixed in the ratio of 1:9 (v/v) with ultra-pure water. Pipette Exactly 1 mL of, the diluted blanks and the diluted sample digests was pipetted into test tubes.Later, 3.8 mL of the diluted mixed reagent(Appendix-I) was added and mix. It was allowed to stand for 1 hour. The absorbance was measured in a 1-cm cuvette at a wavelength of 880 nm. (Temminghoff and Houba 2004)

3.4 Enrichment of the collected sample in methanobacterium II medium (MMII).

An enrichment of culture towards methane production was carried out providing sugars through DSMZ 825 methanogen enhancement media (Appendix-I) in anaerobic state in air tight bottles and N_2 gas was bubbled in it. The culture was allowed to acclimatize. Exactly 25 gram of cowdung was mixed in 5000ml of the MMII media. (Atlas, 2005)

3.4.1 Preparation of the inoculum.

The culture after 2-3 culture transfers was used as an inoculum for the electromethanogenesis in the MEC. The inoculum was incubated for 5 days.

3.4.2 Optimization of apparatus for enhancement of gas.

Several types of culture bottles were used to assist both anaerobic condition and continuous sampling. The shift was done from the reagent bottle, saline bottle, MFC set to L shaped cork using reactor.

3.5 Experimental Design

A pair of graphite electrode (Nippon Electrode Co Ltd, Japan) of dimension 10cm×3cm ×1cm was inserted into a aspirator bottle that served as anaerobic reactor to form an electric-biological reactor (referred to as MEC-anaerobic reactor). The working volume of the reactor was 1.0 L. The voltage supplied was fixed at 1, 2, 3 and 4 V. The control experiments were conducted in a common reactor that was the same as MEC-anaerobic reactor but without electrodes. Before digestion, oxygen was removed from the headspace by exchanging it with nitrogen gas for 10 min, and then sealed the reactor. Silica was applied across the caps (cork) of reactor and a syringe was inserted. During the digestion, the biogas produced from the reactor was collected in the syringe. The biogas in syringe was monitored for measuring volume. The reactors were operated as a batch mode and the digestion was lasted for 5 days. The experiments were operated at 18 ˚C, 28 ˚C , 37˚C respectively.

3.6 Monitoring methane production with or without supply of electricity.

After start-up phase, the catholyte was renewed with fresh mineral medium, N_2 gas bubbling in the catholyte was carried out by providing step wise increasing in the cell voltage from 1-4 V. Control operation without electrical voltage application was also simultaneously carried out as control. Methane was measured excluding CO_2 where the amount of CO_2 was absorbed using KOH.

3.7 Liquid sampling and analysis

Samples of liquid in the reactors was taken every day for the analysis of Chemical oxygen demand (COD). The pH of sample was measured using pH strip because of the minimal volume of the sample. Gas production measurement and liquid sampling for analysis was carried to measure the methane production.

3.8 Pretreatment of Carbon natotubes

First of all, 10mg of CNT was mixed in 35ml of 2:5 v/v of HNO_3/H_2SO_4.The mixture was then ultrasonicated for 6 hours and centrifuged at 4000 rpm for 15 minutes. The rpm was slightly increased if the settlement wasn’t proper. The CNT was washed with deionized water several times until the pH reached close to 7. Finally the CNT was washed with 100%

ethanol. The solid black powder was dried in vacuum at 80˚C for about 8-12 hours (Cabezas, 2013).

3.8.1 In situ oxidative polymerization of aniline

The reaction was done in an ice bath. The reaction reactor containing 1M HCl (9.55 ml) and Multiwalled carbon nanotubes (MWCNT) was kept at 4 ˚C with constant stirring. First aniline monomer was added to nanotube solution and then a solution of 1M HCl containing APS (Aniline: APS molar ratio) was added dropwise to the reaction reactor. Finally the mixture was further stirred for 2 hours at 4 ˚C to ensure a good polymerization. Thus, the composite was formed in ES form (Cabezas, 2013).

3.8.2 Composite Washing and dedoping process

Composite was collected as solid with a Buchner funnel and resulting cake was carefully washed, first with distilled water , until the final filtrate was neutral and then with methanol. To the mixture of carbon nanotubes (CNT) and poly aniline (PANI) 50mg of N-Methyl-2-Pyrrolidone (NMP) was put 100ml of methanol. In order to transform the emeraldine salt (ES) into emeraldine base (EB), the composite was dedoped by stirring for 2 hours with 3 wt % ammonium hydroxide (NH_4OH), filtered and washed with water, ethanol, water and this was followed by vacuum drying at room temperature for 24 hours. The dedoped composite was dissolved in 50mg of NMP per 100ml of methanol. Sonication was done in an ice bath for 15 minutes. The graphite electrode was dipped in the NMP solution. Further vacuum dry was done at 70 ˚C for 24 hours. Finally, oven dry was done at 55 ˚C for 8-12 hours. (Saint *et al.,* 2005; Cabezas, 2013)

3.8.3 Anode material coated with Carbon nanotube composite

The treated CNT was mixed with absolute ethanol and vortexed with the help of magnetic stirrer for about 15 minutes. The treated graphite electrodes was dipped in the mixture and sonicated for about 15 minutes. Then vacuum dry was done for about 12 hours and dried in an oven for 12 hours at 60 ˚C (Wang et al., 2009).

3.9 Treatment of Graphite electrodes

The graphite electrodes were firstly treated with 70% methanol and sonicated for 15 minutes at the temperature of 25 ˚C. This was followed by treatment with distilled water and ultrasonication for 15 mins. Finally the graphite electrodes were treated with 70% acetone and ultrasonicated for 15 mins followed by treatment with distilled water and ultrasonication for minutes.The electrodes were then dried in the oven at 60 ˚C for 1 day. Before the use, these electrodes were treated with UV for about 15 mins (Liu et al., 2017).

3.10 Cyclic Voltammetry

Hokuto-Denko HA151 potentiostat was used in combination with the National Instrument Lab View work station. Three electrode arrangement were used to perform the measurement. The counter electrode employed was made of platinum. The pretreatment consisted of washing with distilled water. The reference electrode was used as calomel (mercury chloride). The measurements were done between -0.8 V to +0.4 V. The characterization by means of CV was done at scan rate of 1 mV/sec. The data were collected at the interval of 1 mV to obtain the stable current values. The cyclic voltammetry was done for 1 cycle only (Mohina *et al.,* 2011). The cyclic voltammetry was performed in Central Department of Chemistry, Tribhuvan University (CDC, TU).

3.11 SEM analysis of the electrode

The Untreated Graphite Electrode (UGE), CNT treated graphite electrode (CGE) and CNT treated graphite Electrode with mixed culture (CGE+M) was sent for SEM analysis to Advanced Instrumentation Research Faculty, JNU, Delhi.

3.12 Isolation and identification of Bacteria

Exactly 0.1 ml of the inoculum prepared in DSMZ 825 methanogen enhancement media was spread plate on the agar plate enriched with DSMZ 825 methanogen enhancement media. The plates was sealed with parafilm, labelled and kept in the anaerobic jar and placed in 37°C kept in incubator for 24hrs.The different colonies of bacteria on the plate were chosen and pure culture was isolated. By using aseptic technique, bacteria were streaked on the agar plate (containing DSMZ 825 methanogen enhancement media). The streak agar plate was sealed and kept in the anaerobic jar and it was placed in 37°C incubator for growing. Besides that, from the petriplate, gram staining was done. Samples were observed under microscope and characterized (Lozano *et al.,* 2009).

3.12.1 gDNA extraction of screened organism.

3 ml of liquid culture was spun down and the media was removed. 567 µl of TE buffer was added to the pelleted cells. The pellet was re-suspended by repeated pipetting with the solution. 30 µl of 10 % SDS and 3 µl of 20 mg/ml solution of proteinase was added. After proper mixing it was incubated for 1 hour at 37˚C. Then, 100 µl of 5M NaCl was mixed. Later 80 µl of NaCl solution (0.7 M NaCl) was added. The solution was incubated for 65 ˚C for 10 mins. After incubation, equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed properly. It was centrifuged for 5 mins and aqueous solution was transferred into a new tube. The interface was kept undisturbed. Another equal volume of PCl was added and mixed well. The solution was then centrifuged at 14,000 rpm for 5 mins and the supernatant was transferred to a new tube. The first extraction was

repeated again (Chloroform: Isoamyl alcohol alone).0.6 volume of isopropanol was added and mixed until precipitation of DNA was observed. It was subjected to centrifugation and removal of isopropanol. 1ml of 70% ethanol was added to wash the salt away from the DNA. After centrifuging the ethanol was discarded and dried on the bench top at room temperature. The pellet was re-suspended in 50 µl of TE buffer and kept on -20˚C for further use.

3.12.2 PCR amplification of gDNA

The gDNA was amplified by using 16s rRNA primers from NEB. The sequence of the forward primer and reverse primer were 5’-AACGCGAAGAACCTTAC-3’ and 5’-CGGTGTGTACAAGGCCCGGGAACG-3’ respectively. The PCR mixture was prepared in PCR tubes with the following components.

Table 3.1 : PCR components.

S.N	**Components**	**Volume(µl)**
1.	Master mix (2X)	12.5
2.	Forward Primer	2.5
3.	Reverse Primer	2.5
4.	Nuclease Free water	5
5.	Template	2.5

[Note: Reaction conditions at 1X (Working Mix) contained 2 U of DNA polymerase.]

Then after mixing the component (1, 2, 3 and 4) well and performing the short spin, 2.5 µl template was added in each tube. The PCR mixtures which contained the respective templates was mixed well and centrifuged. It was placed in the PCR machine that was previously set with the following condition.

Table 3.2 : PCR condition for 16s rRNA amplification

S.N	Step	Temperature	Time
1.	Intial Denaturation	95°C	2 min
2.	Denaturation	95°C	30 sec
3.	Annealing	56.7°C	30sec (decreases 0.5°C per cycle)
4.	Extention	72°C	40 sec
5.	Repeat steps 2-4 (14 ×)		
6.	Denaturation	95°C	30 sec
7.	Annealing	49.7°C	30 sec
8.	Extention	72°C	40 sec
9.	Repeat steps 6-8 (19 ×)		
10.	Final Extension	72°C	10min
11.	Hold	4°C	Forever

After completion of PCR, gDNA was run in 1% gel-electrophoresis at 50 V for 1 hour and then it was visualized in UV transilluminator.

3.12.3 Sequence analysis of the amplicons

The amplicons were sent to Xcelris Labs Limited, Gujarat, India for sequencing.

3.12.4 Sequence Editing and Alignment

The chromatograms obtained for each region were base called using Phred quality score (Ewing & Green, 1998b). To estimate the quality of generated sequence traces, the original forward and reverse raw sequences were assembled and edited in Sequencer v. 4.1.4 (GeneCodes Corporation, USA). Sequences were assembled based on the parameters minimum match percentage 70 and minimum overlap 20. Each contig were viewed and manually edited (removal of gaps and dealing with ambiguous nucleotides). The aligned sequences were also edited by comparing with the reference sequence (www.ncbi.nlm.nih.gov/blast) by closely inspecting the peaks of chromatograms of forward and reverse sequence. The assembled consensus contigs were exported in text format and imported in Bioedit v.7. All candidate sequences were aligned by ClusterW, (multiple sequence alignment tools) in Bioedit using default parameters. The both primer end was delineated from the alignment matrix. Primer excluded barcode sequences were exported for further analysis.

3.12.5 Construction of phylogenetic tree

Phylogeny tree was reconstructed by Neighbor-joining (NJ) in MEGA v.7.0.14. NJ tree was constructed using K2P distance as genetic measure and setting negative branch length to zero with uniform distribution rates applied. Typically 1000 replicates of bootstrap were used to estimate tree reliability.

CHAPTER 4. RESULTS

4.1 Determination of environmental parameters of the collected cow dung.

The table 4.1 shows the values of total soluble protein, COD, reducing sugar, phosphorus, potassium and arsenic content in the cow dung. The pH of the sample was also determined along with the TSS and VSS. The amount of protein was approximately 116 mg/L and COD accounted for 438 mg/L. Similarly, the amount of phosphorus, potassium and arsenic was found to be 0.015 mg/L, 0.015 mg/L, and 1.3 ×10-7 mg/L respectively. The pH initially was 7 and the value of TSS, VSS and moisture content were 19.7, 10.5 and 66.78% respectively.

Table 4.1 : Determination of chemical components of collected cow dung

Analytical parameters of cow dung	**Concentration**
Total Soluble Protein	116.25±4.340 mg/L
Chemical Oxygen Demand	438.75±15.110 mg/L
Total phosphorus	0.015 ±0.007 mg/L
Reducing sugar	0.515±0.033 mg/L
Potassium	0.015±0.002 mg/L
Arsenic	1.3×10^{-7} mg/L
pH	7.35±0.15
TSS	19.7±1.5%
VSS	10.5±0.5%
Moisture content	66.78±2.5%

4.2 Optimization of apparatus for enhancement of gas

Several attempts were made to choose the correct apparatus that would maintain the anaerobic state as well as facilitated daily sampling to monitor the changes in the organic matter of the media. Reagent bottle was used with rubber tubes in the cover so that syringe could be inserted and anaerobic environment would be maintained, but upon several trials we found that it could not serve the expected outcome. Neither it could maintain the anaerobic state nor could we perform daily sampling. Another alternative used was Saline bottle which promoted anaerobic condition but we couldn’t perform regular sampling due to the chance of escape of the accumulated gas. Later, H- shaped two-chamber reactors was used and was connected to peristaltic pump for maintaining the flow. In spite of maintaining both the anaerobic state and making sampling possible this apparatus couldn't be used because of its small size. The volume of the media used was comparatively only 600ml.

After several trials we could find aspirator bottle as our perfect choice as it could hold about 1000ml of the media and made daily sampling also maintained anaerobic state too. For supplying the appropriate voltage too, aspirator bottle served well.

Table 4.2 : Selection of the most suitable apparatus

S.N	**Type of Apparatus**	**Anaerobic State**	**Sampling Possibility**	**Volume Extension(upto 1000ml)**	**Application of electrodes**
1	Reagent Bottle	±	-	-	-
2	Saline Bottle	+	-	-	-
3	H- shaped two-chamber reactors	+	+	-	+
4	**Aspirator Bottle**	**+**	**+**	**+**	**+**

4.3 Optimization of production media

Figure 4.1 : Aspirator bottle used for the anaerobic digestion which served feasible not only for sampling and maintenance of anaerobic state but also for conduction of microbial electrochemical cell.

The production of biogas was significant i.e. 4cm^3 at 37˚C among 18˚C, 28˚C and 37˚C without the supply of voltage but our intention was to generate production at low temperature condition. So, alteration in the temperature condition and amount of voltage supplied was carried out.

4.3.1 Voltage optimization

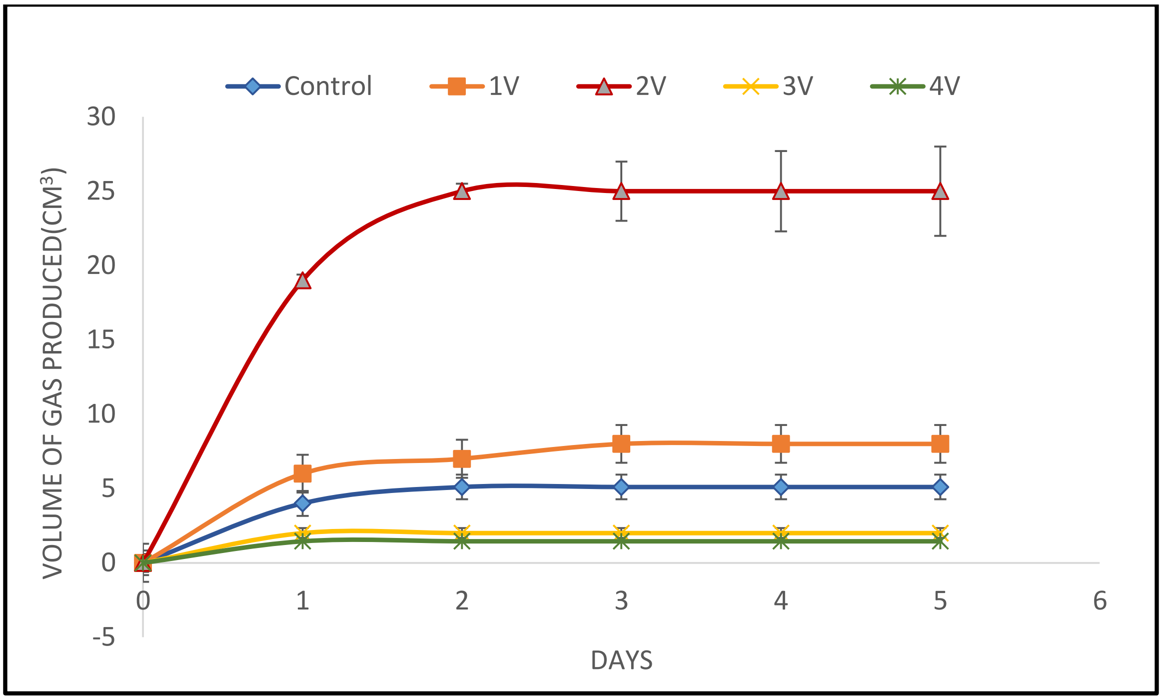

Figure 4.2 : Effect of Voltage in production of biogas at 37 ˚C , where the supply of 2V potential indicated highest production in comparison to control, 1V, 3V among 4V supplement.

When voltage was supplied varying from 1-4 V following change was observed in the amount of production of gas at 37˚C. At 2V the highest biogas yield was achieved. The amount of gas produced at the end of the fifth day was 5.1 cm^3, 8 cm^3, 25 cm^3, 2 .2 cm^3 and 2 cm^3 on control , supply of potential of 1V, 2V, 3V and 4V respectively.

4.3.2 Temperature optimization

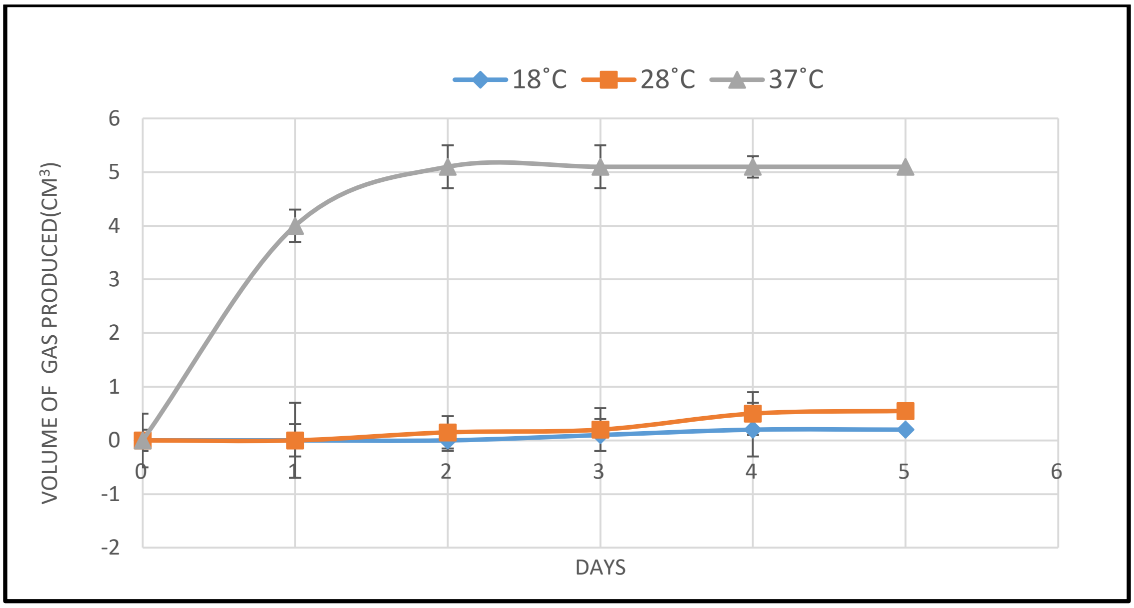

Figure 4.3 : Gas production at different temperature conditions without the supply of voltage

Optimum temperature for the production was found to be at 37˚C among 18˚C, 28˚C and 37˚C but as the purpose of the study was to find the least temperature at which the production was possible.

4.3.3 Temperature optimization using electrochemical cell.

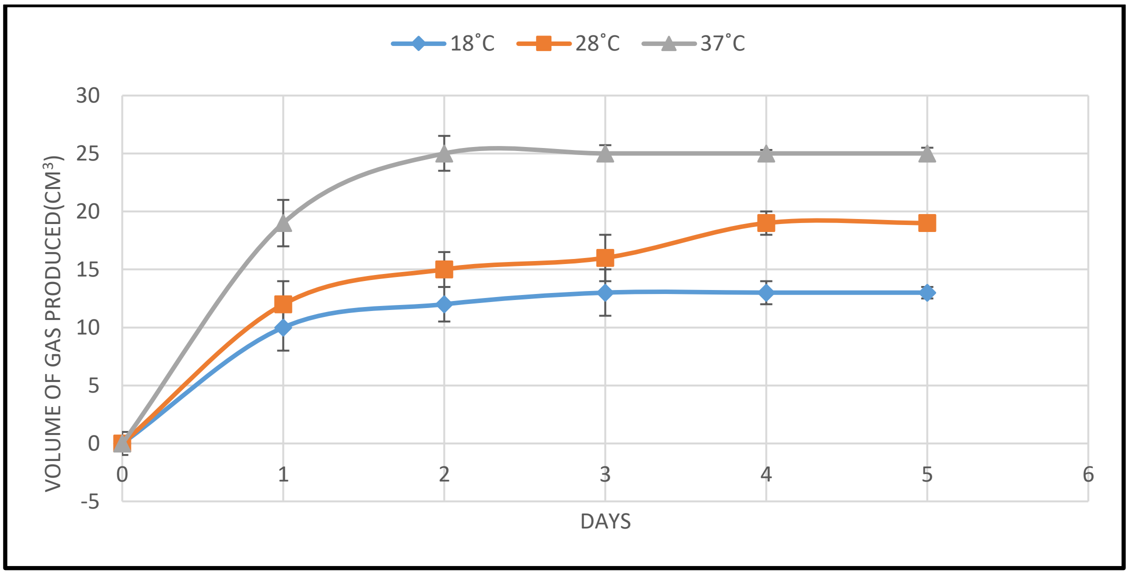

Figure 4.4 : Gas production using electrochemical cell with the supplement of 2V potential. At different temperature conditions the production was highest at 37˚C among 18˚C , 28˚C and 37˚C .

The gas produced at the end of 5days at 18˚C , 28˚C and 37˚C was 13cm^3,19 cm^{3}and 25 cm^3 respectively. The gas production was seen even at 18˚C upon supplying the 2V potential.

4.3.4 Comparison of the gas production at 18˚C

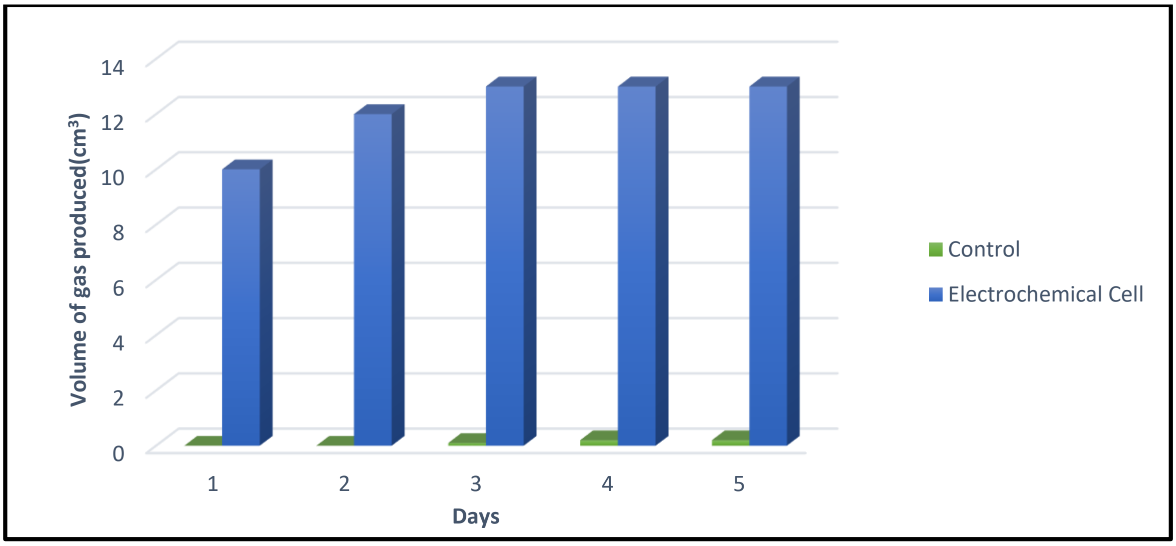

Figure 4.5 : Comparison of gas production at 18˚C between electrochemical cell and control. In the electrochemical cell voltage supplied was 2 V where production was significantly high.

There was a considerable rise in the amount of gas production after the supply of the voltage. Without the voltage supply at 18˚C the volume of gas was only 0.2cm^3 but later after the supply of voltage the amount was 65 times higher than the initial.

4.4 Variation in biogas production with MWCNT coated electrodes

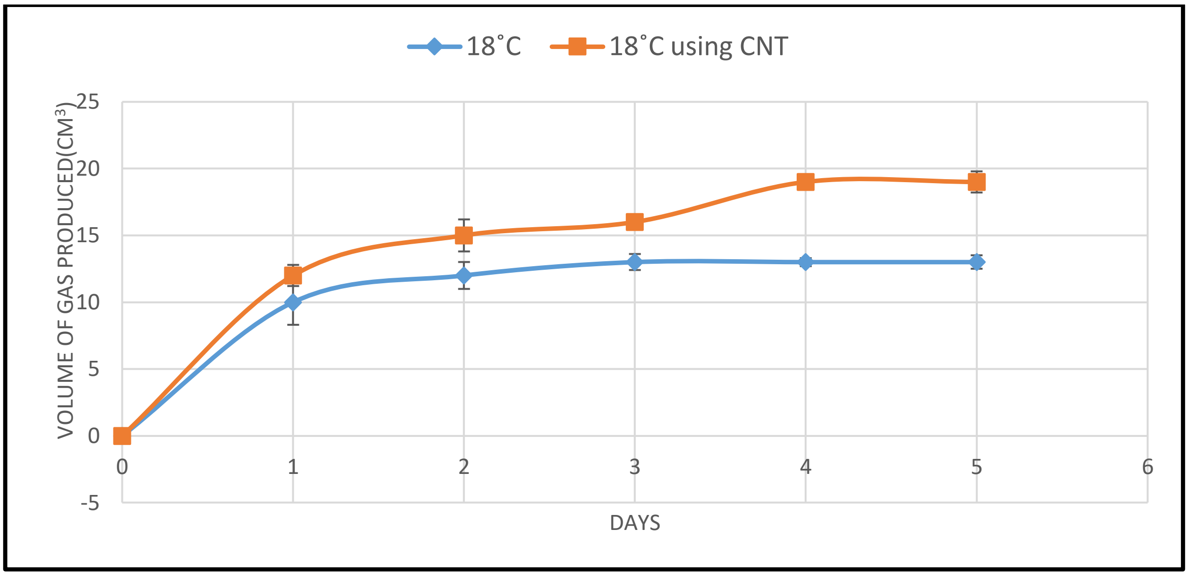

Figure 4.6 : Gas production using electrochemical with MWCNT coating electrode was high at 18˚C.

Compared to the use of graphite and MWCNT coated graphite as electrodes, marginal increase was observed in the amount of gas produced upon using MWCNT coated electrode.

4.5 Gas collected after absorption of CO_2

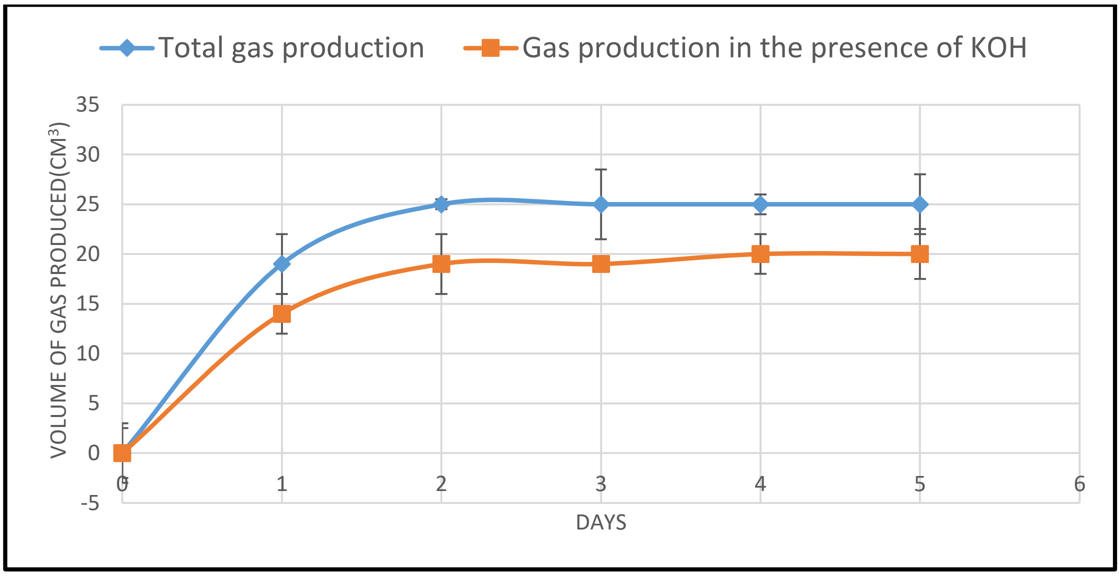

Figure 4.7 : Gas collection with and without the presence of KOH denoted significant difference.

CO_2 was physically absorbed in the liquid solution where it reacts with the alkaline compound producing carbonate (CO_3) and bicarbonate ions (HCO_3) (chemical absorption)(Corti,2004).The amount of gas produced in the presence of KOH was 20cm^3 which indicated that $C0_2$ produced was only about 20% of the total gas accumulated.

4.6 Removal of COD

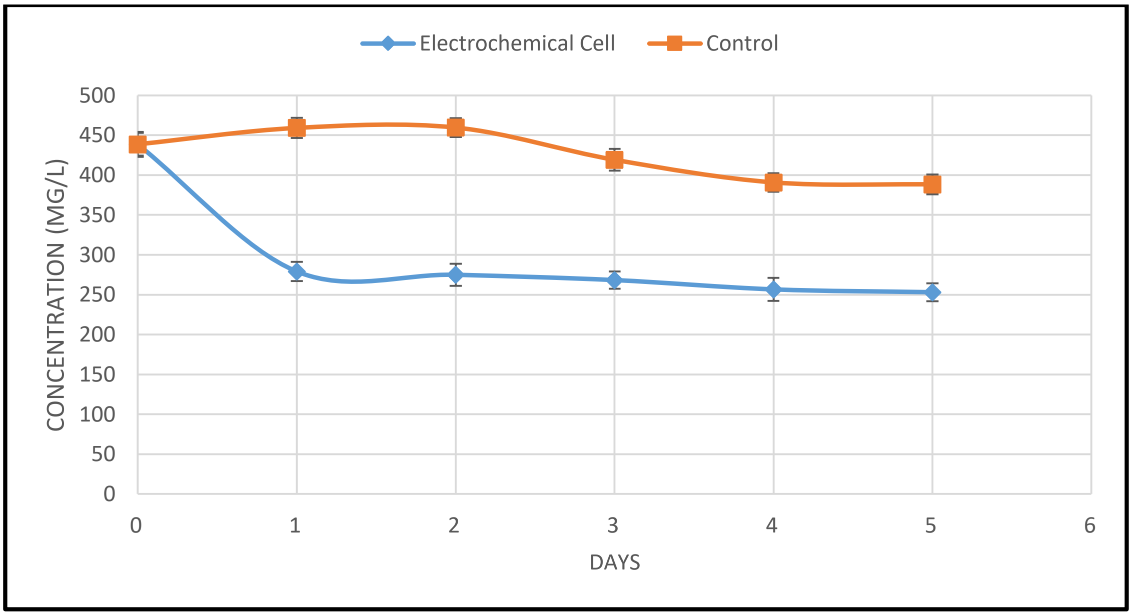

Figure 4.8 : Estimation of COD revealed drastic decline in COD using Electrochemical cell.

The MEC was operated for five days along with the control and the absorbance of solution was noted daily. The COD of media was found to be reduced from 438.75 mg/L to 256.67 mg/L on the fifth day which was a decreased by 41.55%.

4.7 Removal of reducing sugar

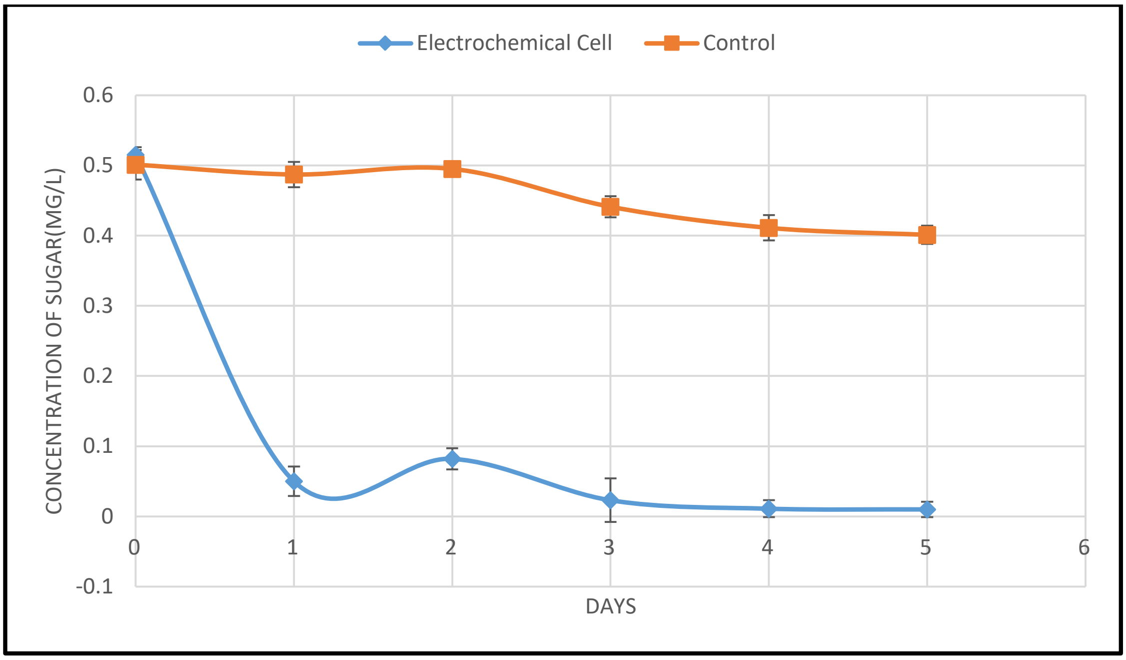

Figure 4.9: Determination of reducing sugar content in the control and electrochemical cell altered considerably at the end of five days of anaerobic digestion.

Similarly, the MEC was operated for five days along with the control and the absorbance of solution was noted daily after performing the DNS test in both experimental and control bottles. The concentration of reducing sugar was found to gradually decrease from 0.515mg/L to 0.013mg/L on the fifth the day and remained approximately 0.00 on the sixth day in the electrochemical cell whereas the reducing sugar content didn’t vary significantly in the control. The concentration of reducing sugar was lowered by 97.85% using MEC.

4.8 Change observed in the pH of the anaerobic reactor

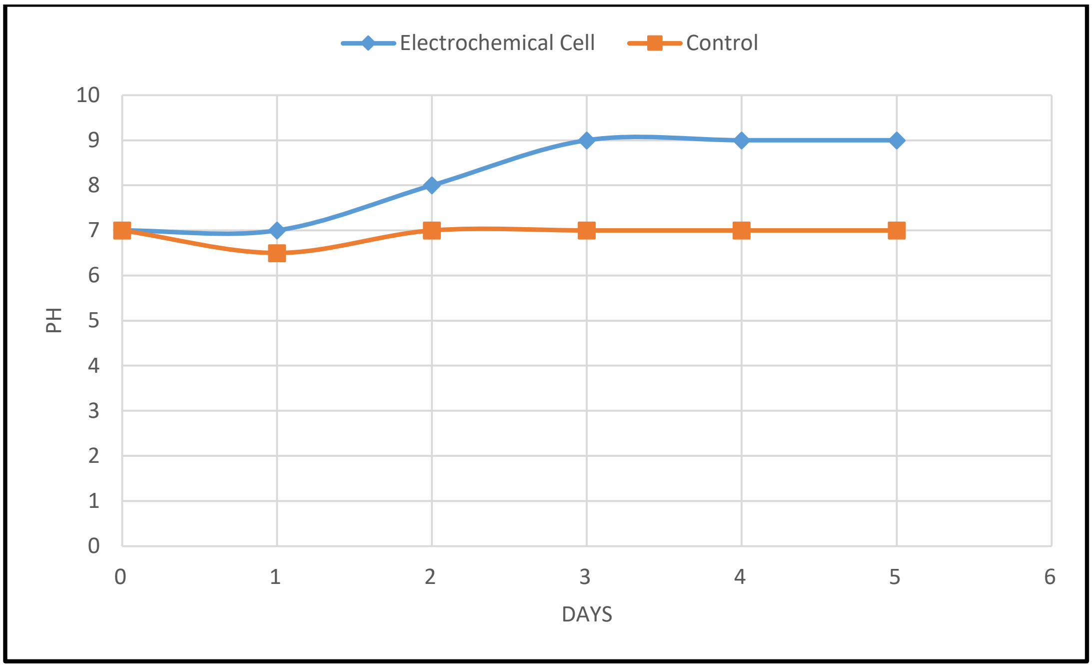

Figure 4.10 : Change in the pH of the anaerobic digester with the supply of 2V potential in 5 days indicated alkaline shift in pH.

The figure shows the pH variation during the fermentation. The pH of the electrochemical cell increased and was finally up to alkali pH ranges at the end of the fifth day. However within that interval there was no significant change in the pH of the control. The pH reached about 7 and 9 in the control and electrochemical cell respectively.

4.9 Scanning Electron Microscopy (SEM) analysis

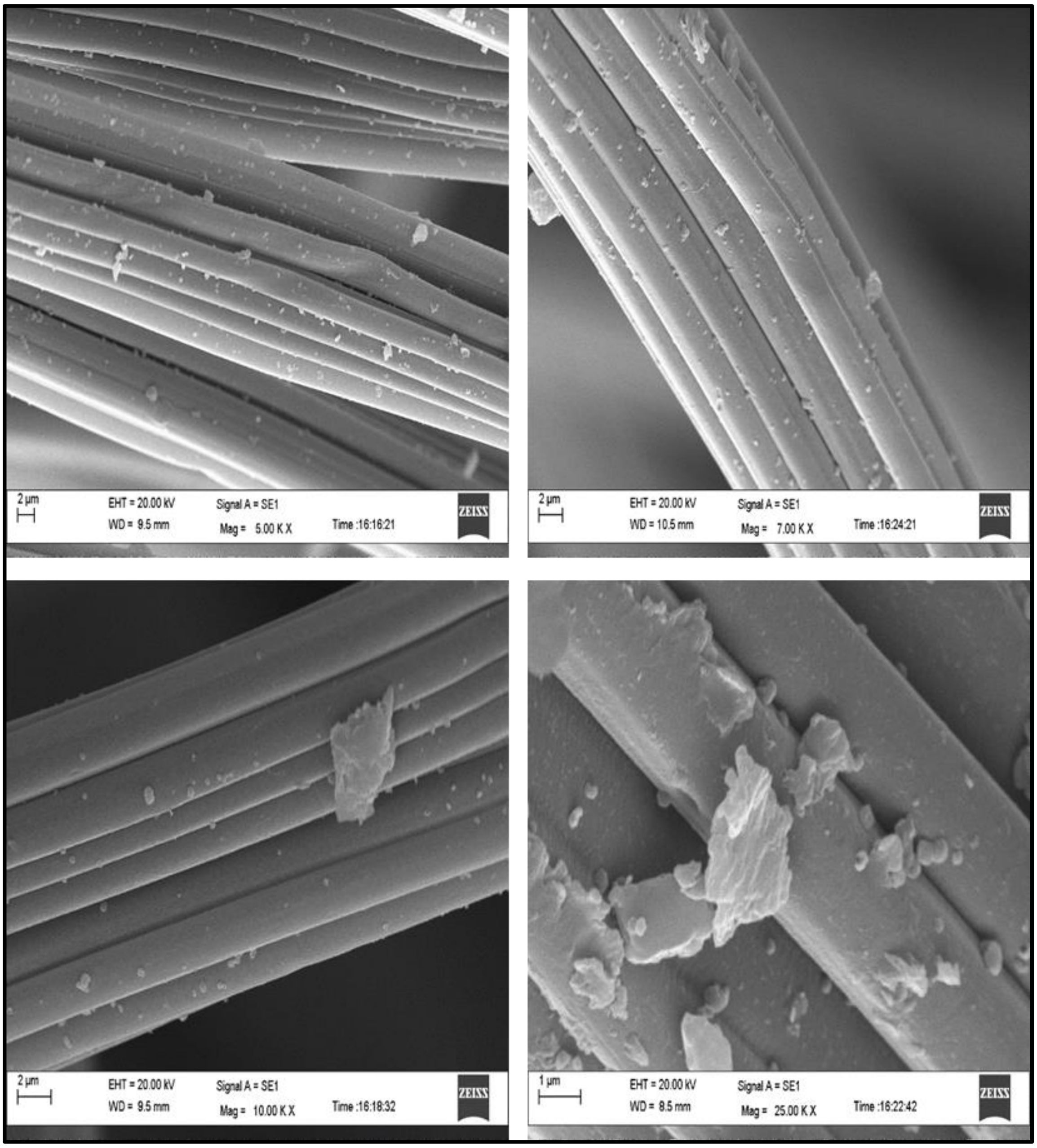

Figure 4.11 : Surface morphologies of Untreated Graphite Electrode (UGE) by SEM

The surface morphologies of Untreated Graphite Electrode (UGE) are analyzed by SEM under 5 K,7K,10K and 25 K X magnification.

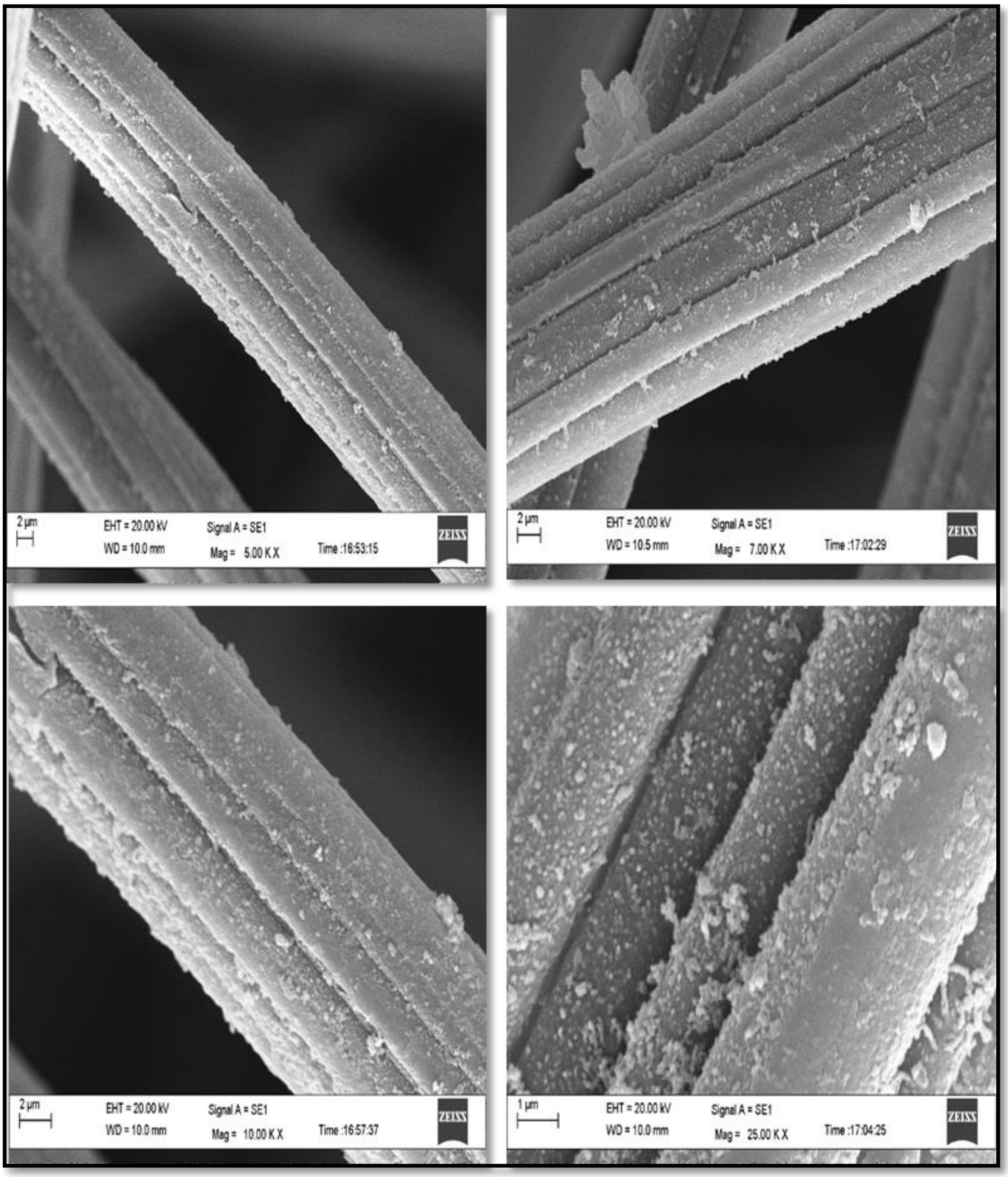

Figure 4.12 : Surface morphologies of CNT treated graphite Electrode (CGE) by SEM

The surface morphologies of CNT treated graphite Electrode (CGE) are analyzed by SEM under 5 K, 7K, 10K and 25 K X magnification. The SEM images clearly show the differences of surfaces between UGE and CGE.

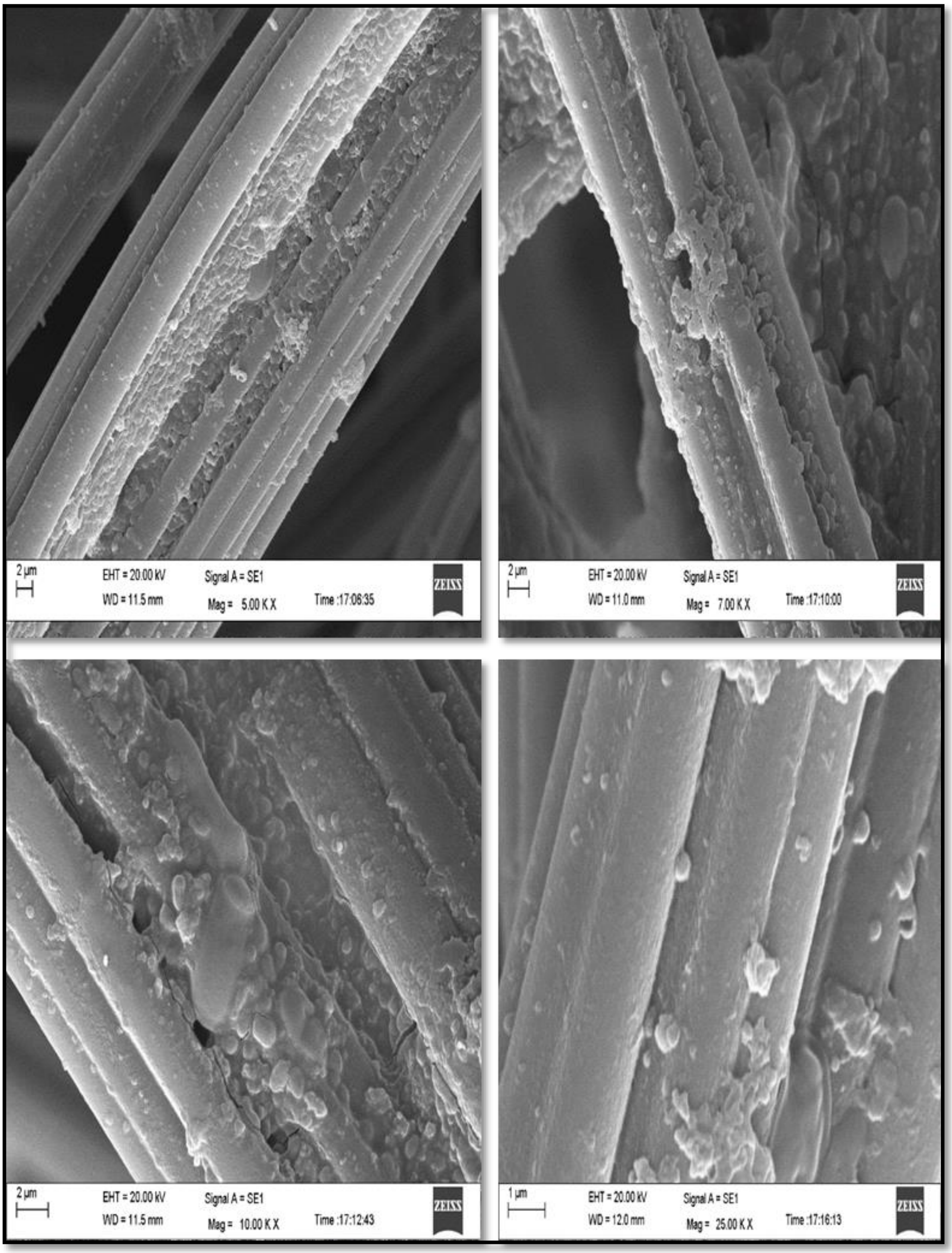

Figure 4.13 : Surface morphologies of CNT treated graphite Electrode with mixed culture (CGE+M) by SEM

The surface morphologies of CNT treated graphite Electrode with mixed culture (CGE+M) are analyzed by SEM under 5 K,7K,10K and 25 K X.

4.10 Cyclic voltammeter measurement

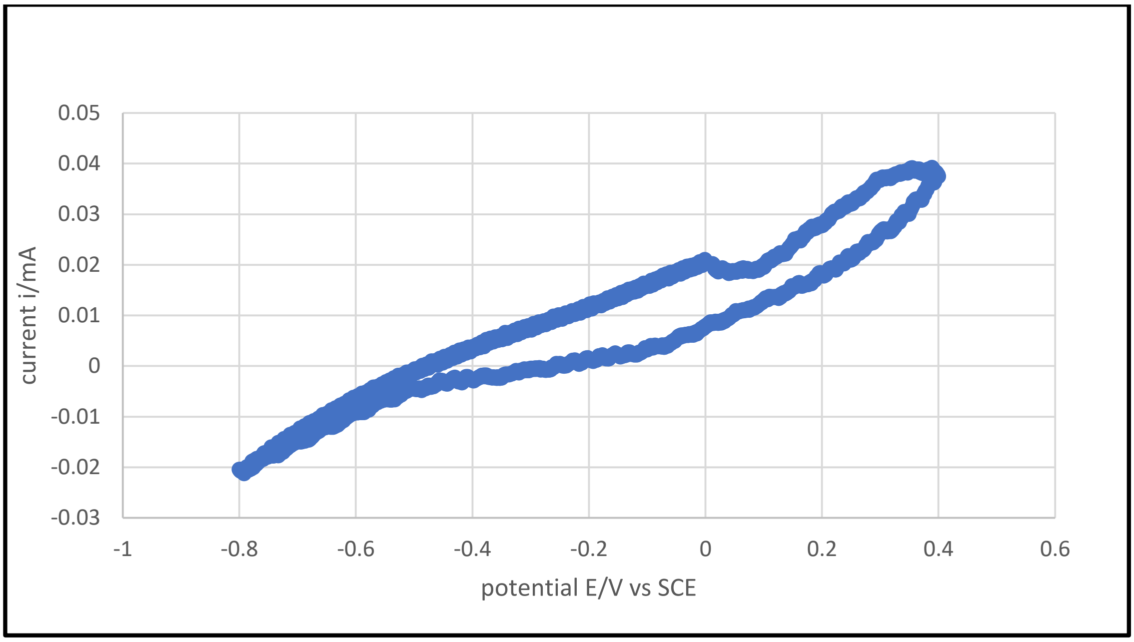

Figure 4.14 : A typical cyclic voltammogram of graphite felt used as electrode in the MEC at the second day of setup in 2 V

Cyclic voltammetry was conducted in the potential range from -0.8 to 0.4 V at low scan rate of 1mV/s which illustrated a typical sigmoidal shape. -1 to -0.5V represented reduction while the range above 0 V represented oxidation. The range of -0.4V to 0 V depicted double layer charging. Anodic Peak was observed in between 0.02 A-0.03 A by applying 0.01- 0.2 V electric potential.

4.11 Isolation of microbes from the inoculum

The inoculum was taken and spread in the agar plate enriched with DSMZ 825 methanogen enhancement media. Five different bacteria were isolated.

4.11.1 Colony Morphology of strains

Table 4.3 : Colony morphology of the isolated strains

S.N	**Characters**	**P1D**	**P2D**	**P3D**	**P4D**	**P5D**
1.	Shape	Round	Irregular	Round	Round	Irregular
2.	Size	Small	Small	Small	Small	Small
3.	Color	Creamy White	Greyish White	Orange	White	White
4.	Consistency	Sticky	Sticky	Sticky	Sticky	Sticky
5.	Margin	uniform	Non-uniform	uniform	uniform	Uniform
6.	Elevation	Convex	Convex	Convex	Convex	Convex

The isolated bacterial strains were subjected to observation of their morphological characteristics in which its shape, size, color, consistency, margin and elevation was noted.

4.11.2 Microscopic Structure of the isolated strains

Table 4.4 : Microscopic structure of isolated strains

S.N	**Sample**	**Gram staining**	**Shape**	**Appearance**
1.	P1D	-ve	Rod	Fixed pattern
2.	P2D	-ve	Rod	Scattered
3.	P3D	-ve	Rod	Fixed pattern
4.	P4D	+ve	Rod	Scattered
5.	P5D	-ve	Rod	Fixed pattern

Bacterial stain was observed under microscope as shown in appendix. When the gram stain was performed and observed under microscope, the bacteria were mostly found to be gram –ve. Some were in a definite pattern while some were scattered.

4.11.3 gDNA extraction and PCR amplification of P1D and P2D isolates.

The genomic DNA of first two bacterial isolates were extracted and PCR was performed successfully. The size of the PCR product was found to be near 400bp. The sequence of isolate P1D and P2D was obtained from Xcelris Lab, India and further BLAST was done to find the most probable genus of bacteria.

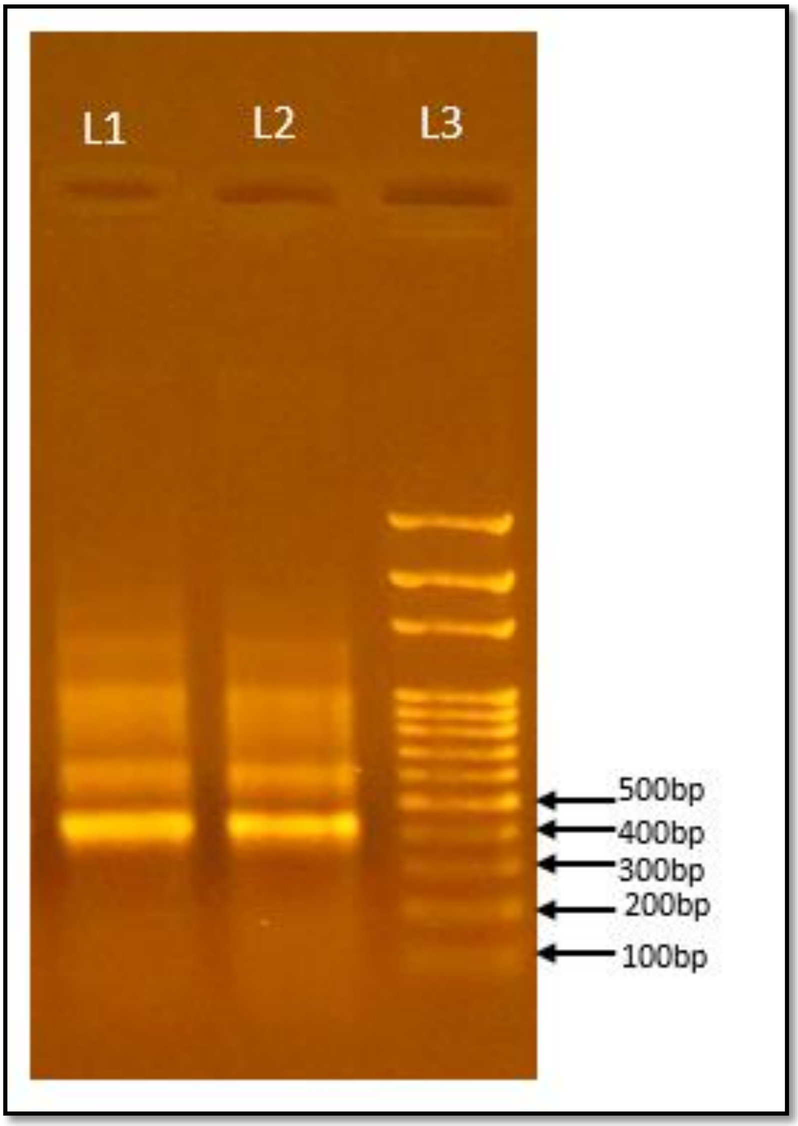

Figure 4.15 : Agarose Gel electrophoresis (1.5% agarose) of unpurified PCR product. L1 and L2 PCR product of P1D and P2D and L3 100bp Biodyne ladder.

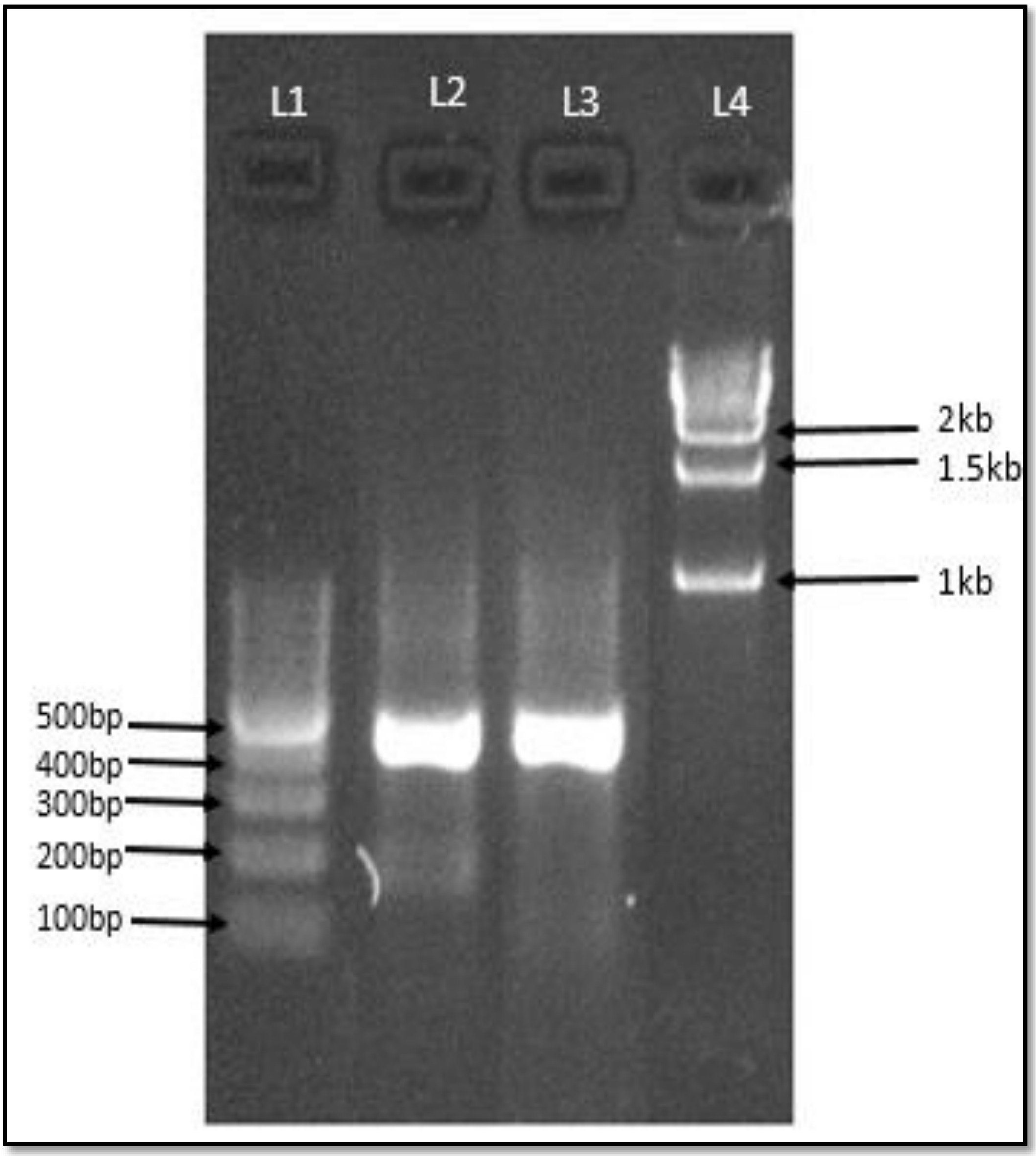

Figure 4.16 : Gel electrophoresis (1% agarose) of purified PCR product. L1 and L4 contained 100bp and 1kb ladder respectively .L2 and L3 is the PCR product of P1D and P2D respectively.

4.11.4 Chromatogram of the sequenced microbes

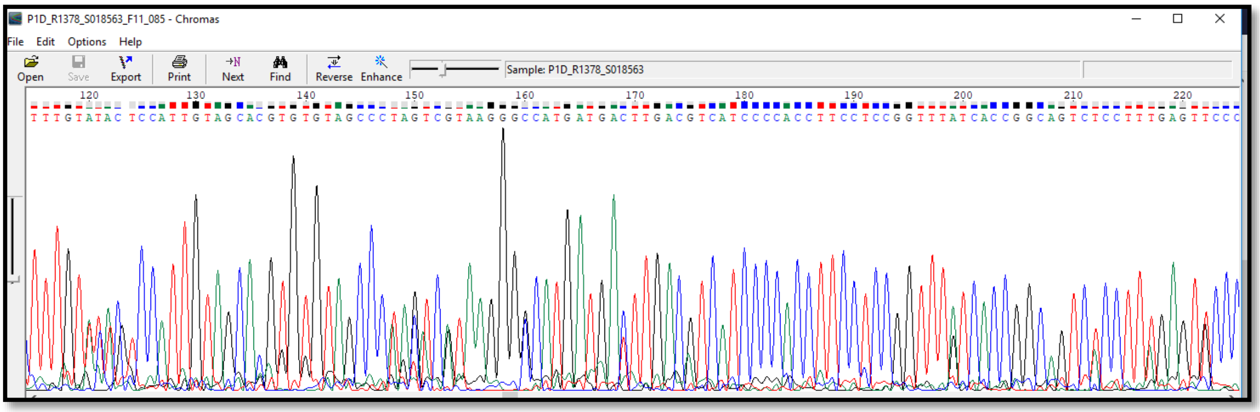

Figure 4.17 : Chromatogram of P1D isolate

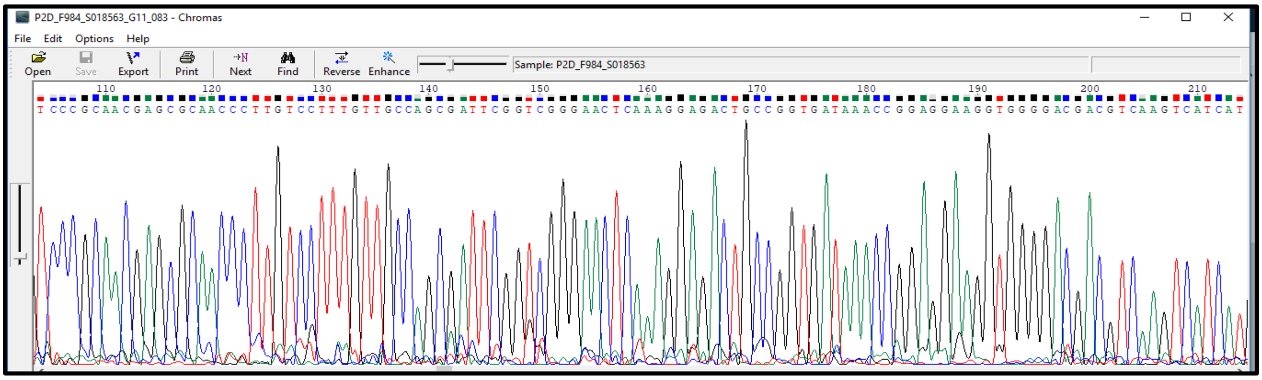

Figure 4.18 : Chromatogram of P2D isolate

The chromatogram of the microbes (P1D and P2D) was obtained after sequencing and upon blast it showed maximum similarity with *Serratia liquefaciens* and *Zobellella taiwanensis* respectively.

4.11.5 Alignment of the isolates with other bacterial species

Sequence similarity searches was performed for the isolated bacterial sequences against the nonredundant database maintained by the National Center for Biotechnology Information (NCBI) using the "Basic Local Alignment Search Tool‖ (BLAST) as the tool to infer functional and evolutionary relationships between sequences as well as help to identify members of gene families. The obtained sequence of P1D and P2D showed maximum similarity with *Serratia liquefaciens* and *Zoballella taiwanensis* respectively.

Table 4.5: Data of sequence alignment of the isolates.

	Max score	**Total score**	**Query cover**	**E value**	**Identity**	**Accession**
P1D						
Serratia liquefaciens strain Sneb2480	592	592	90%	5.00E-165	92%	MG132665.1
Serratia liquefaciens strain FC2971	592	592	90%	5.00E-165	92%	MH628238.1
Serratia liquefaciens strain CH83	592	592	90%	5.00E-165	92%	MH620755.1
Serratia liquefaciens strain TPD7002	592	592	90%	5.00E-165	92%	MH190215.1
Serratia liquefaciens strain 263.12.2	592	592	90%	5.00E-165	92%	MG871160.1
P2D						
Zobellella taiwanensis strain DN-7	721	721	98%	0	96%	KM361042.1
Zobellella taiwanensis strain AT 1-3	721	721	98%	0	96%	FJ999669.1
Zobellella taiwanensis strain ZT1	721	721	98%	0	96%	NR_043630.1
Zobellella taiwanensis strain F4	715	715	98%	0	96%	KJ197182.1
Zobellella taiwanensis strain F28	715	715	98%	0	96%	KJ398233.1

4.11.6 Construction of phylogenetic tree

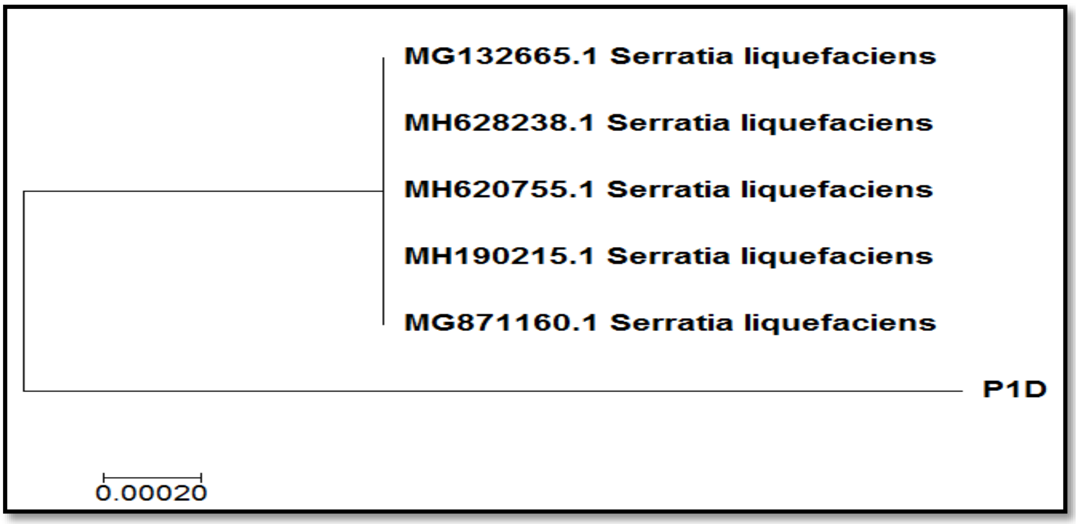

Figure 4.19 : Phylogenetic tree of P1D isolate

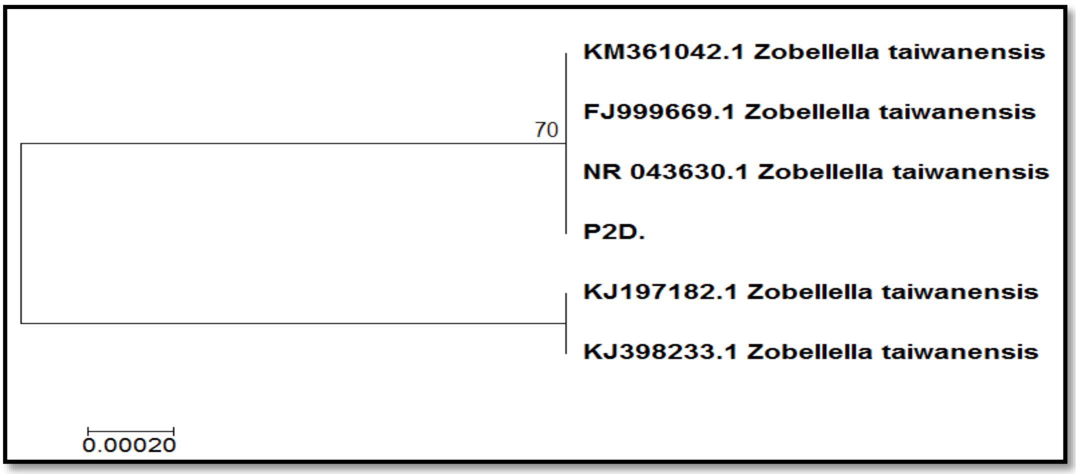

Figure 4.20 : Phylogenetic tree of P2D isolate

The construction of phylogenetic tree also provided the extent of biological diversity of the sequence of P1D isolate with *Serratia liquefaciens* and the sequence of P2D with *Zoballella taiwanensis* respectively.

CHAPTER 5. DISCUSSION

There are a lot of research done regarding environmental analysis of cow dung. On performing the determination of analytical parameters, the total soluble protein in the sample was found to be 116.25mg/L, phosphorus content was found to be 0.537 mg/L, the reducing sugar was found to be nearly 0.514mg/L, the potassium content was found to be 0.015mg/L. Similarly, the heavy metal arsenic was found to be 1.3 ×10-7 mg/L. The pH value of the sample was 7.35. In addition, the TSS, VSS and moisture content was found to be 19.7%, 10.7% and 66.78 % respectively. Our analysis showed similar result with the research conducted as the value of TSS and VSS is estimated to be within the range of 15-20% and 10-15% respectively (Tomlinson *et al.,* 1996). All these specific analyses were performed in triplicate to ensure reliable and reproducible results. The presence of P and K ensured the fertilizing capacity of the cow manure. TVS/TSS ratio was high, indicating high organic content. Five gram of sample was dissolved in 100ml of water for all the analysis to maintain the similar unit of comparison i.e. mg/L. However most of the values of protein and phosphorus in the sample would probably depend on the feed of the cattle too (Hoekstra *et al.,* 2002).TSS testing measured the total concentration of suspended (non-soluble) solids in the effluents. Several researches have proved that TSS data is critical in determining the operational behavior of a waste treatment system (Gujer and Zehnder, 1983). TSS values in effluent are often related to excessive solids generation due to an increase in BOD loading or can indicate problems with the bacteria like nutrient deficiency (Feng *et al.,* 2015).

Similarly, the COD value was found to be 438mg/L .COD values without digestion was very high. In addition, considerable decline in those values were observed after the anaerobic digestion. After the process of digestion the COD value was nearly 256 mg/L. Among the three stages of anaerobic fermentation of waste sludge, COD was high in the first two stages which mainly included soluble protein, soluble polysaccharide and VFA. Generally, COD in anaerobic fermentation kept increasing at the beginning of fermentation along with the hydrolysis of insoluble organic matters. Afterwards, COD decreased when the soluble organic matters were gradually mineralized to CH_4 and CO_2 (Feng *et al.,* 2015).

The major duration of the research was invested in optimizing the apparatus and choosing the right one. It was done particularly for facilitating the maintenance of the anaerobic condition as well as conducting daily sampling for analysis of the chemical changes in the digester. Out of the several type of vessels (i.e. Reagent bottle, Saline bottle, H shaped two chambered reactor and aspirator bottle), aspirator bottle was found to be appropriate because of its flexibility to add a cork in its opening so electrodes could be inserted along with the sampling tube. It helped to conduct sampling without disturbing the anaerobic state of the digester.

The reducing sugar initially was found to be 0.515mg/L but after digestion in the control condition the values indicated minimal decline and was found to be 0.411 mg/L while in the MEC the values resulted to 0.013mg/ L. The reducing sugar concentration decreased by 97.85% with the use of MEC whereas decrease of only 28.30% was observed in control. The result signifies that the conversion into the soluble sugars is higher and faster in MEC than in the simple anaerobic digestion. The digestion process begins with bacterial hydrolysis of the input materials. Insoluble organic polymers, such as carbohydrates, which were considered to have broken down to soluble derivatives that become available for other bacteria. Later, due to rapid conversion the acidogenic bacteria converted the sugars and aminoacid into carbondioxide, hydrogen, ammonia, and organic acids. These resulting organic acids later converted into acetic acid with the same group of bacteria. Finally, methanogens converted these products to methane and carbon dioxide. The methanogenic archaea populations play an important role in anaerobic waste treatments (Tabatabaei *et al.,* 2010).

The pH variation of both electrochemical cell and control during the digestion was noted on daily basis as the digestion began. H^+ was consumed for the cathodic methanogenesis, which led to the increase of pH. Accordingly, the pH in the MEC-anaerobic reactors was higher than that in the control. The pH of the digesters all increased and was finally up to alkaline pH ranges. At the end of the digestion, the pH reached about 7 and 9 in the MEC and control respectively. It suggested that the pH of the digesters increased with the application of voltage. It is due to the excessive utilization of H^+ by the cathodic reduction of CO_2 or H^+ for producing CH_4 or hydrogen, respectively (Wagner *et al.,* 2009). Methanogens grew at a neutral pH range (6–8) and then the alkali pH (9) might have inactivated methanogens to decrease the methane production at 2 V. Due to which production of the gas was limited only for 5 days.

In this research, the main objective was to enhance the production of biogas at low temperature. Therefore, the production of gas was evaluated at different temperature ranges (18˚C, 28˚C and 37˚C). The optimum temperature for the highest production was 37 ˚C without the supply of electric potential and the production was negligible at 18˚C. However, even at 18˚C with the supply of certain voltage a significant increase in the production was observed. The effect of electric potential in the production of gas was determined. The voltage was supplied varying from 1—4 V. At 2V the yield of biogas was dramatically high. Additionally, the gas produced at 18˚C with the supply of 2V electric potential was about 3 times higher than the biogas produced at 37˚C without the implementation of electric potential.

KOH pellet was kept within the barrel of the syringe where the gas was collected. It was performed to exclude the CO_2 content as KOH is found to absorb CO_2 rapidly which gives K_2CO_3 and water and potassium carbonate is thermodynamically stable compound. KOH is known as a strong base (alkali) and CO_2 as an acidic oxide. So, the two readily reacted to form salt and water. CO_2 content in the total biogas was found to be 20%. It was performed due to the unavailability of specific instrumentation to detect the amount of the components of the biogas.

Graphite felt was used as electrode in the MEC because it is inert and has high surface area (Bajracharya *et al.,* 2015). Furthermore, MWCNT-modified graphite felt was used to distinguish the change. The production of the biogas increased by nearly 13% with the use of MWCNT coating in the graphite felt. Carbon nanotubes (CNTs) combine in a unique way and is considered to provide high electrical conductivity, high chemical stability and extremely high mechanical strength. (Merkoçi *et al.,* 2005) Electrochemical reduction of carbon dioxide to useful chemical materials is of great significance to the virtuous cycle of CO_2. However, some problems such as high over potential, high applied voltage, and high energy consumption exist in the course of the conventional electrochemical reduction process. The new electrodes (MWCNT coated) may significantly decrease the over potential of CO_2 reduction (Zhao *et al.,* 2012).

The structural changes in the graphite felt electrode after MWCNT coating and bacterial adhesion on the membrane was analyzed by using a SEM. The surface of the UGE and CGE was smooth and showed flaky structure before inoculation of microorganisms. While, obvious changes occurred and carbon microspheres was formed and coated on the surface of electrode for the CGE. SEM images of the CGE after attachment of microorganisms showed some drastic changes. The microorganisms groups and electrode substrate were significantly observed to be attached to the CGE. It is generally known that the interesting groups of microorganisms play a key role in the electron transfer from bacteria to the electrode (Kalathil *et al.,* 2016; Zhang *et al.,* 2011), which means more electrons were produced by microorganisms due to large number of microorganisms attaching on electrode. The results indicate that the CGE with larger amounts of microorganisms is beneficial to the electrochemical activity of MECs.

Cyclic voltammetry was employed to investigate the bioelectrochemical behaviors of microorganisms in the anaerobic reactor with the MEC. The cyclic voltammogram consisted of 2 anodic peak. It presented high current, suggesting that the bacteria attached to the anode actively participated in for anodic electron transfer. The two peaks were observed between 0.02 A-0.03 A by applying 0.01- 0.2 V electric potential. There was a previous report that observed a similar peak at 0.15 V representing the electrochemical activity of methanogens (Bo *et al.,* 2014). These peaks probably indicate that the microorganism in the consortium possesses electrochemical activity to accept electrons from the anode or extracellular electron transfer components. CV was performed by cycling the potential of a working electrode, and measuring the resulting current (Nicholson, 1965).

Microbes were isolated from the acclimatized cow dung by using specific media so that anaerobic organisms would grow. Isolation of methanogens required strictly anoxic environment which was difficult to maintain. Growth of anaerobic bacteria was facilitated by DMSZ 825 media (Appendix –I). Five isolates were selected and subjected for microscopic study and gDNA isolation. Only two isolates (P1D and P2D) were chosen to perform 16s rRNA PCR amplification because of their distinct gDNA bands. The amplicons (P1D and P2D) that were sent for sequencing resulted to have close identity with *Serratia liquefaciens* of family Enterbacteriaceae and *Zobellella taiwanensis* of family Aeromonadaceae respectively on performing blast. The efficiency of tree resolution while constructing phylogenetic tree was considered successful as the clades had at bootstrap value higher than 50%.The values ≥50% bootstrap value are considered reliable(Whitford *et al.,*1998).

The isolates were Gram-negative and facultative anaerobes. *Serratia liquefaciens*, a species of gram-negative bacteria in the family Enterbacteriaceae and genus Serratia. It is found in plants and the digestive tract of rodents. It is straight rod shaped bacterium. It is the most prevalent Serratia species in the natural environment. *Serratia liquefaciens* identified as the dominant psychrotrophic microorganisms with high spoilage potential of milk. The storage of fresh raw milk at low temperature does not prevent proliferation of psychrotrophic bacteria that can produce heat-resistant proteolytic enzymes contributing to the reduced shelf life of dairy products (Malchado *et al.,* 2015). *Zobellella taiwanensis* is a gram- negative bacteria in the family Aeromonadaceae and genus Zobellella. It is denitrifying bacteria. Cells grown in broth cultures were straight rods that were motile by means of a single polar flagellum. Other studies showed that these isolates grew between 10 and 45 degrees ˚C. They were capable of anaerobic growth by denitrifying metabolism using nitrate or nitrous oxide as electron acceptors or, alternatively, by fermenting glucose, sucrose or mannitol as substrates. This denitrifying isolates could be classified as representatives of novel species in a new genus, Zobellella gen. nov (Lei *et al.,* 2016).

CHAPTER 6. SUMMARY

The present work was done to study the possibility of enhancement in the volume of biogas even at cold temperature conditions. Significant results were obtained with the prospect of overcoming the problem of the nation. Even at a very low voltage i.e. 2 V the production of gas was observed at 18˚ C using MEC. Using the same system, the COD level was decreased by 41.55% and reducing sugar was curtailed by 97.85%.Though the prevalent methanogens couldn't be isolated due to the deprivation in maintaining the anaerobic condition, two strains that were facultative anaerobes were obtained. They played a major role in maintaining the motility of the electrons. Several molecular properties aided for determination of size of the PCR product and respective genus identification. In addition, a complete characterization of manure had been carried out in terms of organic matter, its biodegradability, nutrients (P, K), heavy metals (As), protein content and several other physical-chemical parameters were analyzed.

It is also seen that animal waste have substantial nutritional value. This allows the production of biogas at any place in the world. In a near future, every single anaerobic digestion plant, landfilling site, wastewater treatment plant will be a potential spot for a highly efficient interface between the electricity and the methane grids while treating waste organic matter ʻʻfor freeʼʼ. This field of research is still in its infancy. However, the results obtained by several studies, even at a real-scale, promise near-future application of this concept. The main issues related to the process scale-up are given by the choice of the electrode materials and the reactor design.

Biogas production in the agricultural sector is a very fast growing market in the developing nations and finds increased interest in many parts of the world. In the next few decades, bioenergy will be the most significant renewable energy source, because it offers an economical attractive alternative to fossil fuels. The success of biogas production will come from the availability at low costs and the broad variety of usable forms of biogas for the production of heat, steam, electricity, and hydrogen and for the utilization as a vehicle fuel. Many sources, such as crops, grasses, leaves, manure, fruit, and vegetable wastes or algae can be use, and the process can be applied in small and large scales.

CHAPTER 7. CONCLUSION

A laboratory scale electrochemical cell enhanced biogas production system which was feasible even at low temperature condition was developed in this research work. Among five only two isolates were characterized which were found to be identical with *Serratia liquefaciens and Zoballela taiwanensis.* Higher volumetric production rates were achieved with use of microbial electrochemical system resulting in 65 times more production than that of the control by supplying 2V of electric potential at 18°C. Nearly 13% increment in the biogas production was observed with the use of MWCNT coating in the graphite felt. The MEC showed the COD and reducing sugar removal efficiency of 41.55% and 97.85% indicating it to be suitable for waste/manure treatment. Recent studies show that the electromethanogenesis play significant role in the production of value added products .This work had been initiated by observing the global improvement in management of waste and converting waste to energy. However, the feasibility of this technology in our country is still unknown. Nepal has taken an initiative in using the litter for production of biogas but no work has been done with the use to MEC in production scale. So, lab scale finding can serve as the basis to attempt for the use of MEC in pilot scale. Moreover, this work is futuristic and more substantiate research needs to be done to prove any of the statement. Since the molecular mechanism hasn't been studied, so repetition of this work is required before drawing any conclusion.

Despite some problems, it is sure that methanogenesis rates increased gradually with time using MEC which signifies methane production with good substrate conversion efficiency.

7.1 Recommendation

- Extending the derived result in pilot scale is essential for using the enhancement technique in practical use.
- Better electron transporting media can be chosen for further enhancement in production.
- Another point to consider is the isolation and characterization of more methanogens.

CHAPTER 8. REFERENCES

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APPENDIX-I

A. Reagents

i. DMSZ 825 media

Table A.8.1 : Components of DMSZ 825 media

S.N	Components	Amount
1.	$CaCl_2$ x 2 H_2O	0.10 g
2.	K_2HPO_4	0.30 g
3.	KH_2PO_4	0.30 g
4.	$MgCl_2$ x 6 H2O	0.20 g
5.	KCl 0.10 g NaCl	0.60 g
6.	NH_4Cl	1.00 g
7.	Trace element solution (see medium 141)	10.00 ml
8.	Na-acetate	0.50 g
9.	Na-resazurin solution (0.1% w/v)	0.50 ml
10.	Vitamin solution (see medium 141)	10.00 ml
11.	Yeast extract	1g
12.	Na_2S x 9 H2O	0.50 g
13.	L-Cysteine-HCl x H_2O	0.50 g
14.	$NaHCO_3$	4.00 g
15.	Distilled water	1000.00 ml

Dissolve ingredients (except bicarbonate, vitamins, cysteine and sulfide), sparge medium with 80% H2 and 20% CO2 gas mixture for 30 – 45 min to make it anoxic. Add and dissolve bicarbonate and adjust pH to 7.0, then dispense medium under 80% H2 and 20% CO2 gas atmosphere into anoxic Hungate-type tubes or serum vials to 30% of their volume and autoclave. After sterilization add cysteine and sulfide from sterile anoxic stock solutions autoclaved under 100% N2 gas. Vitamins are prepared under 100% N2 gas atmosphere and sterilized by filtration. Adjust pH of complete medium to 6.8 – 7.0, if necessary. For incubation use sterile 80% H2 and 20% CO2 gas mixture at two atmospheres of pressure. Note: If the medium is being used without overpressure then adjust pH with a small amount of sterile anoxic 1 N HCl, if necessary.

ii. Trace element solution

Table A.8.2 : Components of Trace Element solution.

S.N	Component	Amount
1.	Nitrilotriacetic acid	1.50 g
2.	$MgSO_4$ x 7 H_2O	3.00 g
3.	$MnSO_4$ x H_2O	0.50 g
4.	NaCl	1.00 g
5.	$FeSO_4$ x 7 H_2O	0.10 g
6.	$CoSO_4$ x 7 H_2O	0.18 g
7.	$CaCl_2$ x 2 H_2O	0.10 g
8.	$CuSO_4$ x 5 H_2O	0.01 g
9.	$KAl(SO_4)_2$ x 12 H_2O	0.02 g
10.	H_3BO_3	0.01 g
11.	Na_2MoO_4 x 2 H_2O	0.01 g
12.	$NiCl_2$ x 6 H_2O	0.03 g
13.	Na_2SeO_3 x 5 H_2O	0.30 mg
14.	Na_2WO_4 x 2 H_2O	0.40 mg

Distilled water 1000.00 ml First dissolve nitrilotriacetic acid and adjust pH to 6.5 with KOH, then add minerals. Adjust final to pH 7.0 with KOH.

iii. Vitamin solution:

Table A.8.3 : Components of Vitamin Solution.

S.N	Components	Amount
1.	Biotin	2.00 mg
2.	Folic acid	2.00 mg
3.	Pyridoxine-HCl	10.00 mg
4.	Thiamine-HCl x 2 H_2O	5.00 mg
5.	Riboflavin	5.00 mg
6.	Nicotinic acid	5.00 mg
7.	D-Ca-pantothenate	5.00 mg
8.	Vitamin	B12 0.10 mg
9.	p-Aminobenzoic acid	5.00 mg
10.	Lipoic acid	5.00 mg
11.	Distilled water	1000.00 ml

iv. PCR components

The PCR mixture was prepared in PCR tubes with the following components.

Table A.8.4 : PCR components

S.N	Components	Volume(µl)
1.	Master mix (2X) (Polymerase, 2X Reaction Buffer, and dNTP Mix)	12.5
2.	Forward Primer	2.5
3.	Reverse Primer	2.5
4.	Nuclease Free water	5
5.	Template	2.5

B. Determination of As and K

1. Digestion in flask with H_2SO_4- Salicilic acid – H_2O_2
2. Sulphuric Acid, 96 % (w/w), 18 mol/L (U = 1.84 g/cm3).

3. Hydrogen Peroxide, 30 % (w/w).
4. Salicylic Acid, Powder.
5. Digestion Mixture - Put 18 mL water in a 250-mL erlenmeyer flask. While cooling, add in small portions 100 mL of sulphuric acid (4.1) (CAUTION). Then dissolve 6 g of salicylic acid (4.3) with the aid of a magnetic stirrer.
6. Carborundum Beads

C. Determination of COD

1. Phthalate standard solution, 1.0 mL = 1.0 mg COD: Dilute 100 mL potassium acid phthalate standard solution I to 1,000 mL with demineralized water. This solution is used to prepare working standards at time of analysis.
2. Digestion solution: Potassium dichromate-mercuric sulfate: To approx 700 mL demineralized water, add 10.216 g $K_2Cr_20_7$ and 33.0 g $HgS0_4$. CAUTION: Hazardous. Slowly, and with constant stirring, add 167 mL concentrated H_2S0_4 (sp gr 1.84). Mix until dissolved. After the solution cools, dilute to 1 L with demineralized water.
3. Catalyst Solution: Dissolve 22 g Ag_2SO_4 in a 9-pound bottle of concentrated H_2S0_4 (sp gr 1.84).

D. Determination of Phosphorus by colorimetric method

1. Stock Solution, PO_4 concentration 1000 mg/L Merck nr 1.19898.
2. Stock Solution, PO_4 concentration 1000 mg/L - Dissolve 1.432 g potassium dihydrogen phosphate, KH_2PO_4 (see remark 2), in about 900 mL water in a volumetric flask of 1000 mL. Make up to 1000 mL with water.
3. Ascorbic Acid Solution - Dissolve 1.76 g ascorbic acid, $C_6H_8O_6$, in 100 mL ultra pure water and mix. Prepare fresh daily.
4. Ammonium Molybdate Solution - Dissolve 40 g ammonium molybdate tetrahydrate, $(NH_4)_6Mo_7O_{24}.4H_2O$, in ultra pure water and make up to 1000 mL. This solution should be stored in a bottle made of hard glass.
5. Potassium Antimonyl Tartrate Solution - Dissolve 0.274 g potassium antimonyl tartrate, $KSbOC_4H_4O_6.5H_2O$, in ultra pure water and make up to 100 mL with ultra pure water.
6. Sulphuric Acid Solution 2.5 mol/L - Dilute carefully, in portions, 140 mL concentrated sulphuric acid (96 %) in about 500 mL ultra pure water in a 1000-mL volumetric flask. Allow the mixture to cool off and make up to volume with ultra pure water.
7. Anti-coagulation Agent - Wetting agent Aerosol 22, Merck nr 13908.

8. Mixed Reagent - Add successively with a graduated cylinder and mix after each addition: 50 mL sulphuric acid , 15 mL ammonium molybdate solution , 30 mL ascorbic acid solution and 5 mL potassium antimonyl tartrate solution. Prepare fresh daily.
9. Diluted Mixed Reagent - Mix 80 mL of the mixed reagent with 300 mL ultra pure water; if samples of digestion (digestion with H_2SO_4 - salicylic acid - H_2O_2 - Se) are to be measured, add 0.5 mL of anti-coagulating agent

E. Estimation of Protein Lowry method

1. Phosphate buffer-(0.1M, pH 7.6)
2. Alkaline Na_2CO_3 reagent-Dissolve 2g Na_2CO_3 in 0.1N NaOH and make up the volume to 100ml with 0.1 N NaOH.
3. Copper Sulphate Reagent-Prepare 0.5% $CuSO_4.5H_2O$ in 1% sodium potassium tartarate solution
4. Alkaline copper sulphate solution- Add 1ml of Reagent 3 to 50 ml of Reagent 2. This mixture is unstable and should be prepared fresh.
5. Folin’s Reagent-Dilute the reagent appropriately so that it is 1N in respect of its acid content.
6. BSA-100µg/mL solution in distilled water

F. Apparatus required in the research

The various materials required in this study are

Anaerobic jar
Aspirator Bottle
Autoclave
Burner
Centrifuge
Electric heating plate
Electric Weighing Balance
Gel electrophoresis tank, doc
Glass wares
Hot air oven
Hot water bath
Hot water bath
Laminar air flow
Magnetic Stirrer
Microfuge tubes
Micropipette tips
PCR machine
pH meter
Pipette
Refrigerator
Syringe
UV-illuminator
UV-spectrophotometer
Voltmeter

G. Formula for calculation

$$\text{TSS- } \frac{\text{weight of dry solid}}{\text{Volume of Sample}} \times 1000000 \text{ mg/L}$$

$$\text{VSS- } \frac{\text{weight of volatile suspended solid}}{\text{Volume of Sample}} \times 1000000 \text{ mg/L}$$

APPENDIX II

DETERMINATION OF STANDARD GRAPH

PROTEIN

Table A.8.5 : Data for estimation of Protein

Test Tube Numbers	Volume Of BSA (ml)	Amount of BSA (µg)	Volume of d/w (ml)	Volume of alkaline copper sulphate(ml)	Volume of Folin’s Reagent (ml)	Absorbance at 660 nm
Blank	-	-	1	5	0.5	0
1	0.2	20	0.8	5	0.5	0.197
2	0.4	40	0.6	5	0.5	0.335
3	0.6	60	0.4	5	0.5	0.409
4	0.8	80	0.2	5	0.5	0.500
5	1.0	100	0	5	0.5	0.636

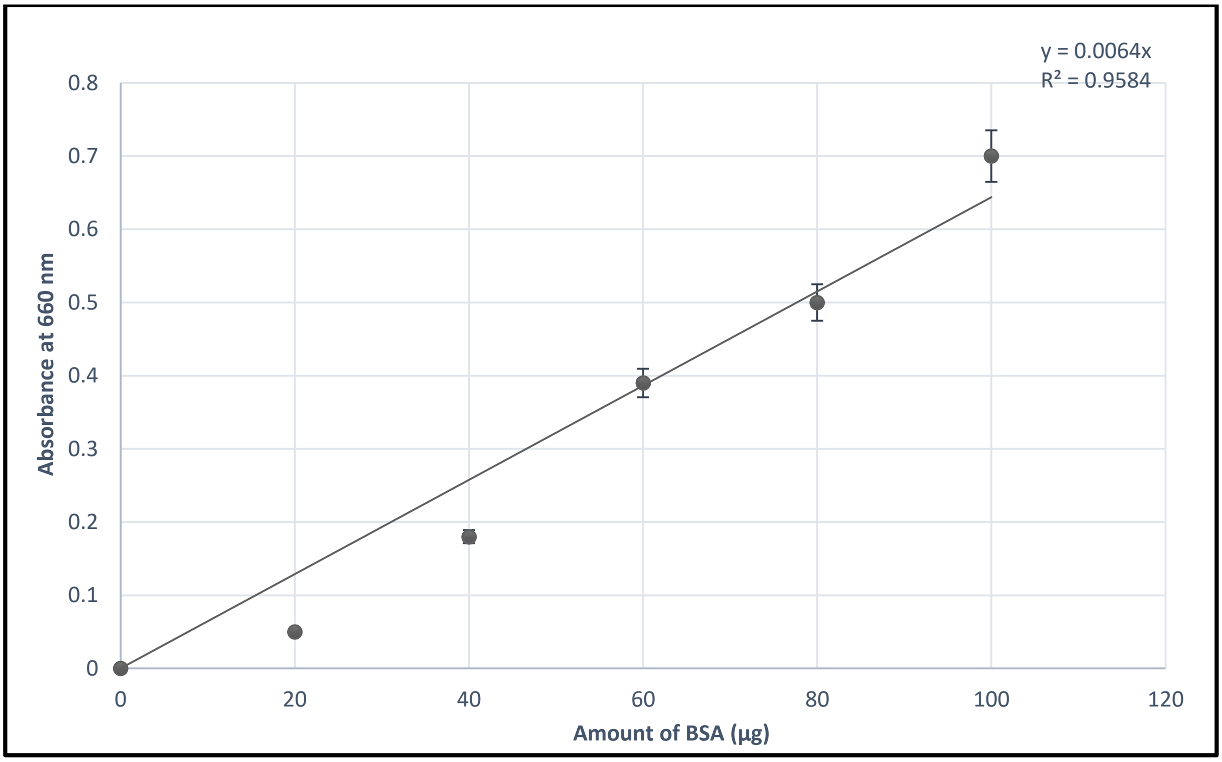


Figure A.8.1 : Standard graph for estimation of Protein

REDUCING SUGAR

Table A.8.6 : Data for estimation of reducing sugar.

Test Tube Numbers	Concentration Of glucose (µg/ml)	Volume of DNS(ml)	Volume of water (ml)	Absorbance at 540 nm
Blank	0	0.2	2	0
1	10	0.2	2	0.067
2	25	0.2	2	0.076
3	50	0.2	2	0.205
4	100	0.2	2	0.430
5	150	0.2	2	0.579
6	200	0.2	2	0.876
7	250	0.2	2	1.237
8	300	0.2	2	1.287
9	400	0.2	2	1.911
10	500	0.2	2	2.277

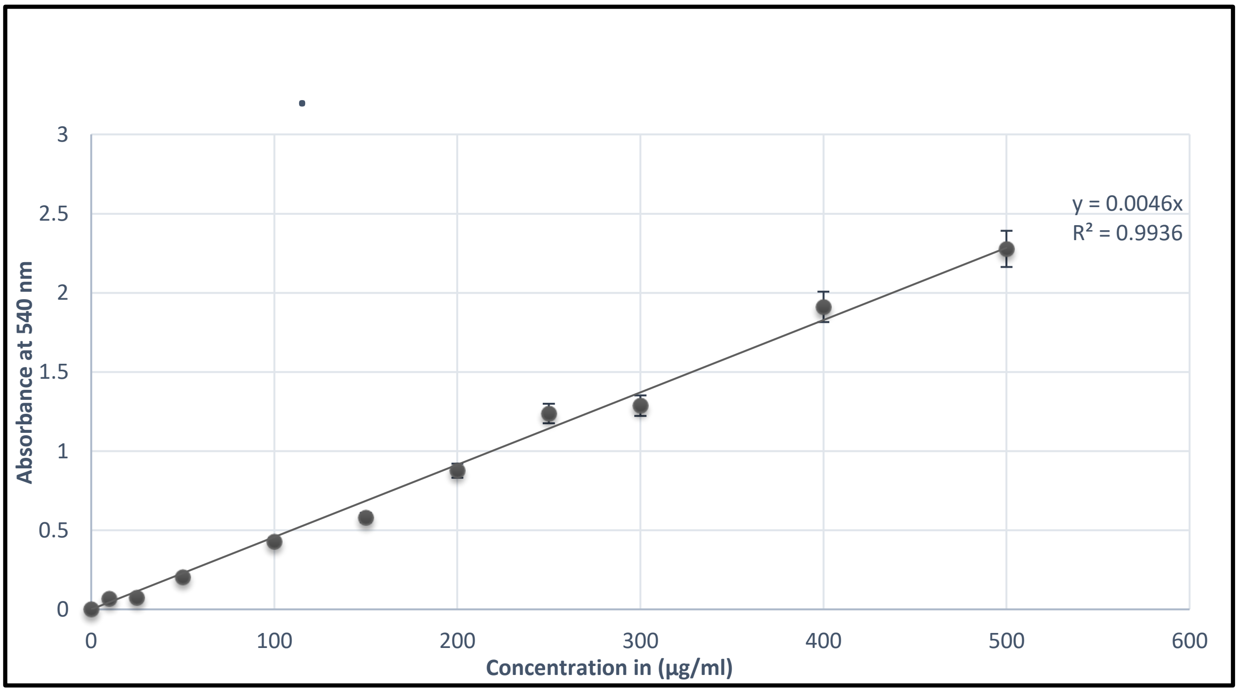


Figure A.8.2 : Standard for reducing sugar test.

COD

Table A.8.7 : Data for calculation of standard graph for COD

Test Tube Numbers	Amount Of phthalate (mg/L)	Volume of ref.Solution (ml)	Volume of digestion solution (ml)	Volume of Catalyst solution (ml)	Absorbance at 600 nm
Blank	-	10	6	14	0
1	20	10	6	14	0.032
2	50	10	6	14	0.07
3	100	10	6	14	0.11
4	200	10	6	14	0.18
5	400	10	6	14	0.33
6	600	10	6	14	0.48

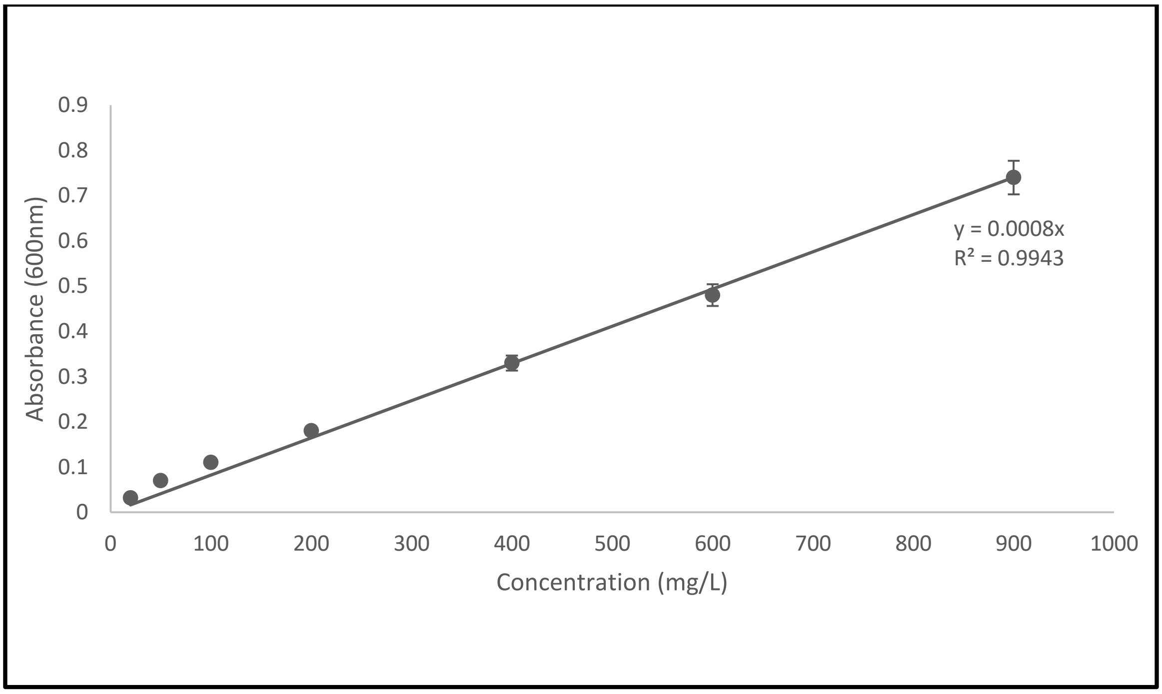


Figure A.8.3 : Standard curve of Chemical Oxygen Demand

PHOSPHORUS

Table A.8.8 : Data for estimation of standard of Phosphorus

Test Tube Numbers	Amount Of phosphorus (mg/L)	Volume of ref.Solution(ml)	Volume of ultra pure water(ml)	Volume of Diluted Mixed reagent (ml)	Absorbance at 600 nm
Blank	-	0	10	3.8	0
1	0.01	1	9	3.8	0.02
2	0.03	1	9	3.8	0.043
3	0.05	1	9	3.8	0.055
4	0.1	1	9	3.8	0.212
5	0.2	1	9	3.8	0.36
6	0.3	1	9	3.8	0.412
7	0.4	1	9	3.8	0.597
8	0.5	1	9	3.8	0.768

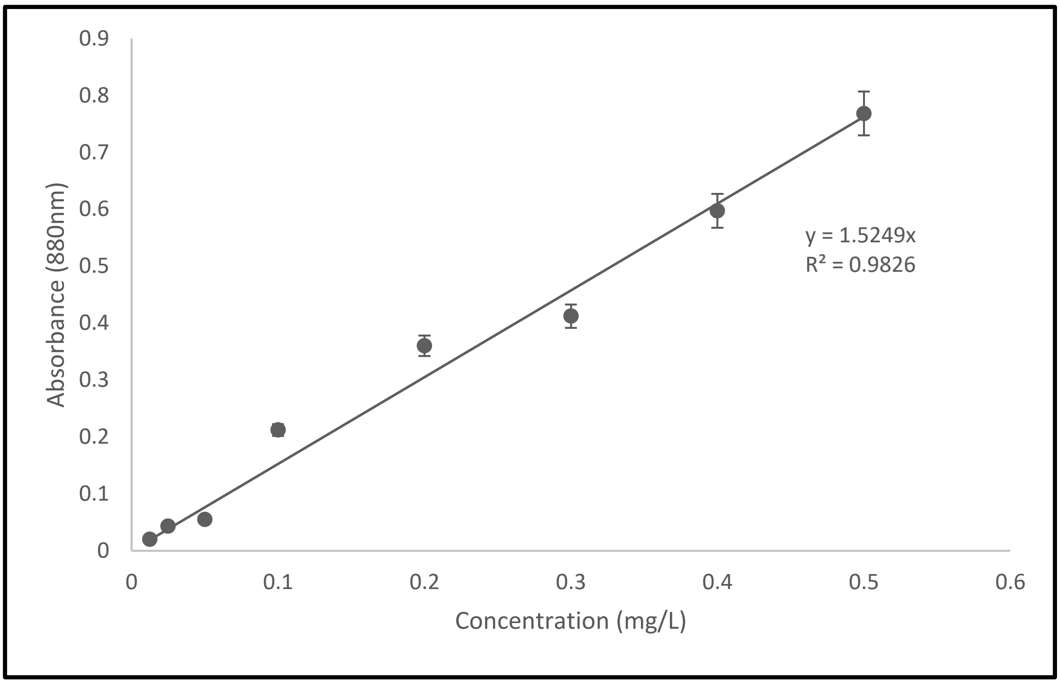


Figure A.8.4 : Standard curve of phosphorus estimation

APPENDIX III

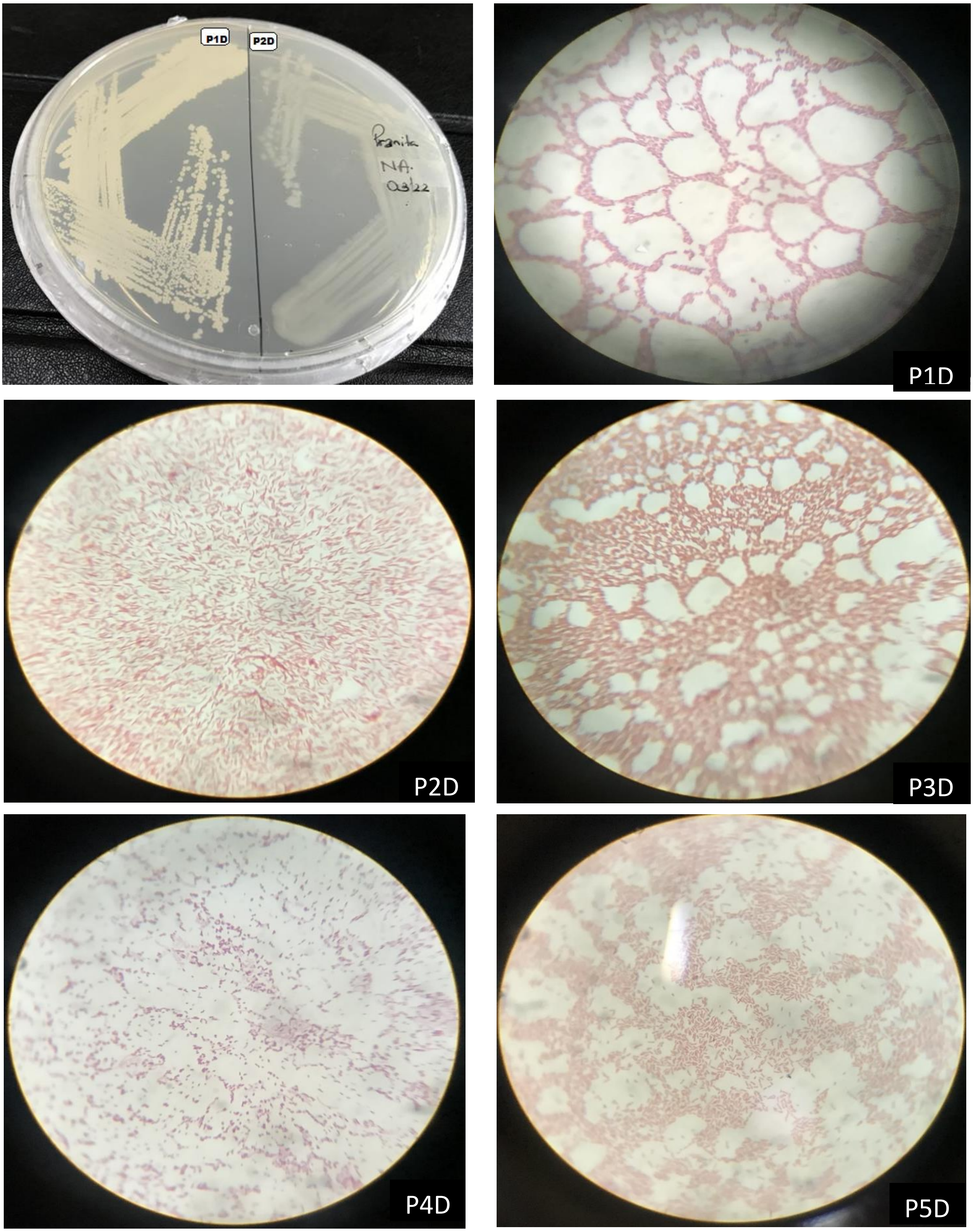


Figure A.8.5 : Microscopic examination of the isolated microbes and its culture in MMI media

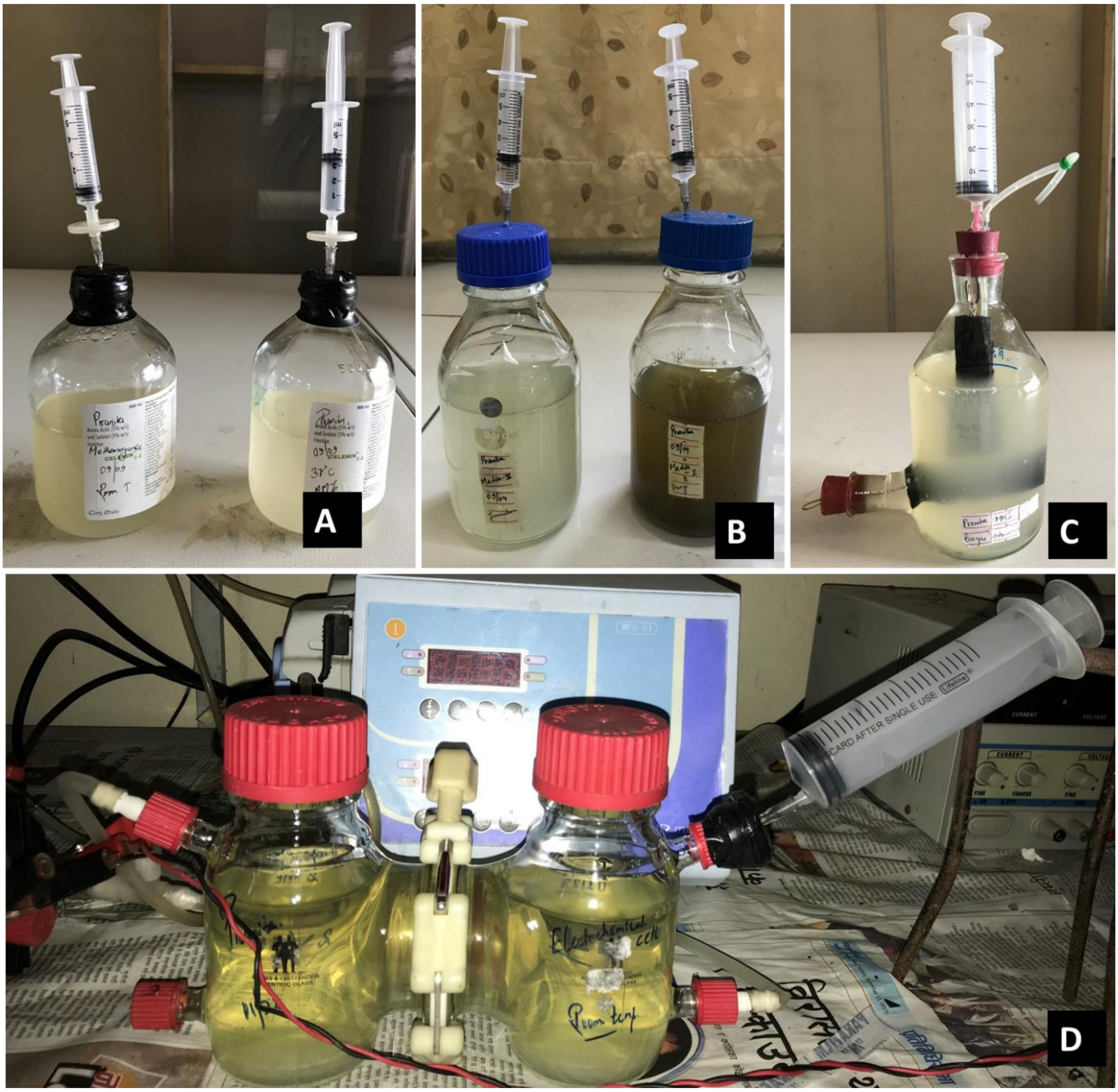


Figure A.8.6 : Optimization of various models for choosing the right apparatus.

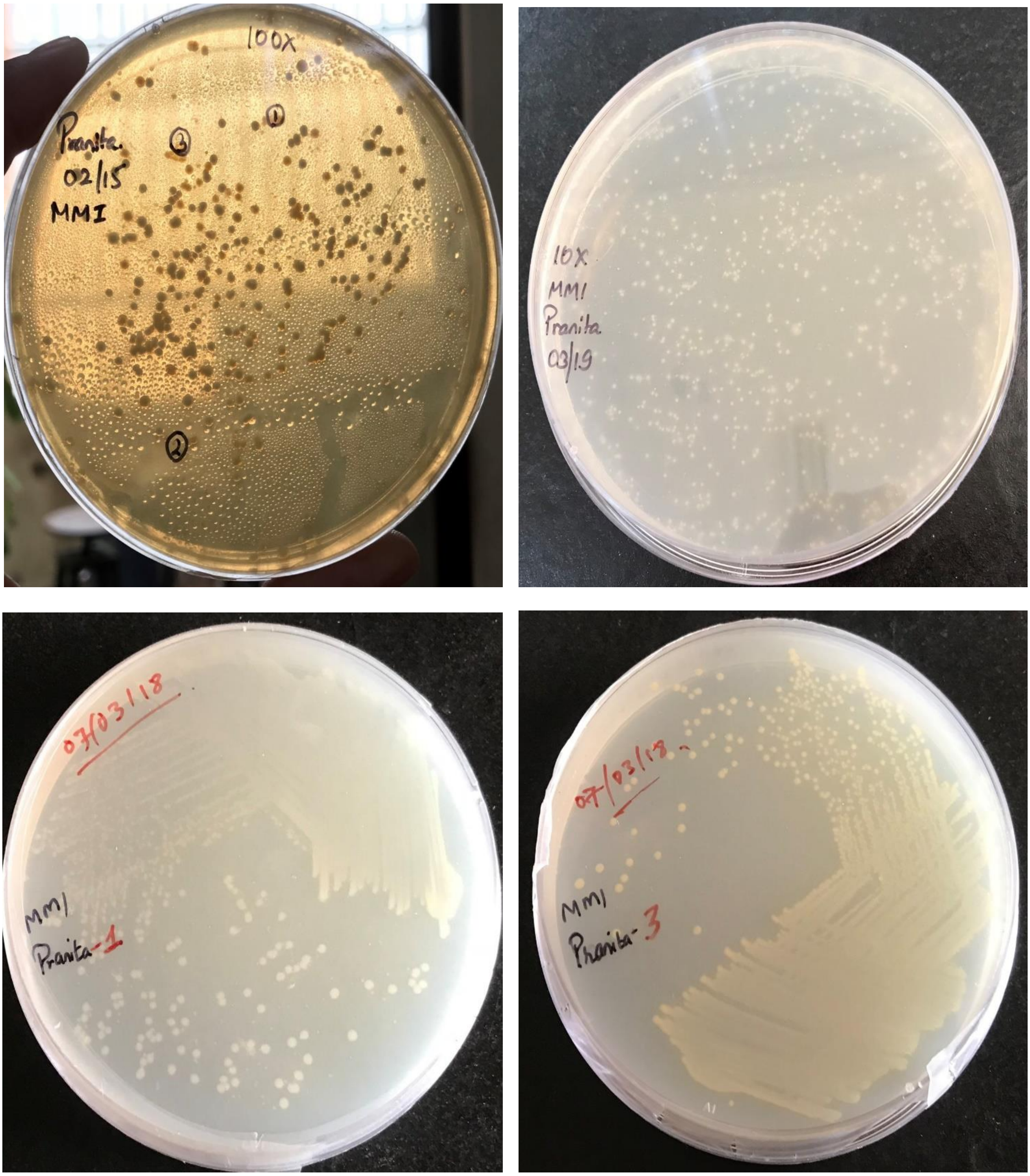
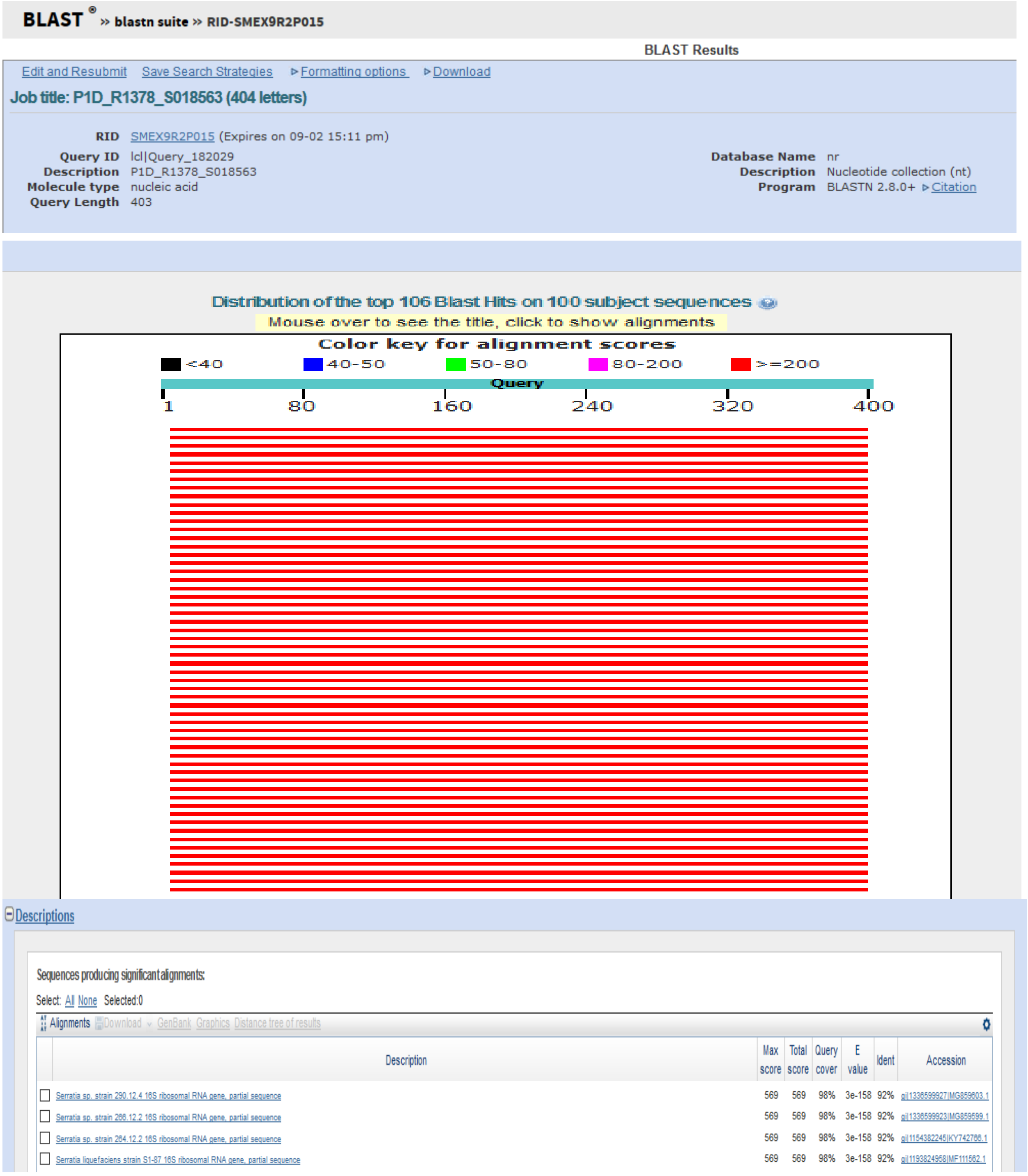


Figure A.8.7 : Isolation of the anaerobic bacteria using spread plate technique and isolation of pure cultures.



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BLAST Results

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Job title: P1D_R1378_S018563 (404 letters)

RID	SMEX9R2P015 (Expires on 09-02 15:11 pm)		
Query ID	lcl|Query_182029	**Database Name**	nr
Description	P1D_R1378_S018563	**Description**	Nucleotide collection (nt)
Molecule type	nucleic acid	**Program**	BLASTN 2.8.0+ ▷Citation
Query Length	403		

Distribution of the top 106 Blast Hits on 100 subject sequences

Mouse over to see the title, click to show alignments

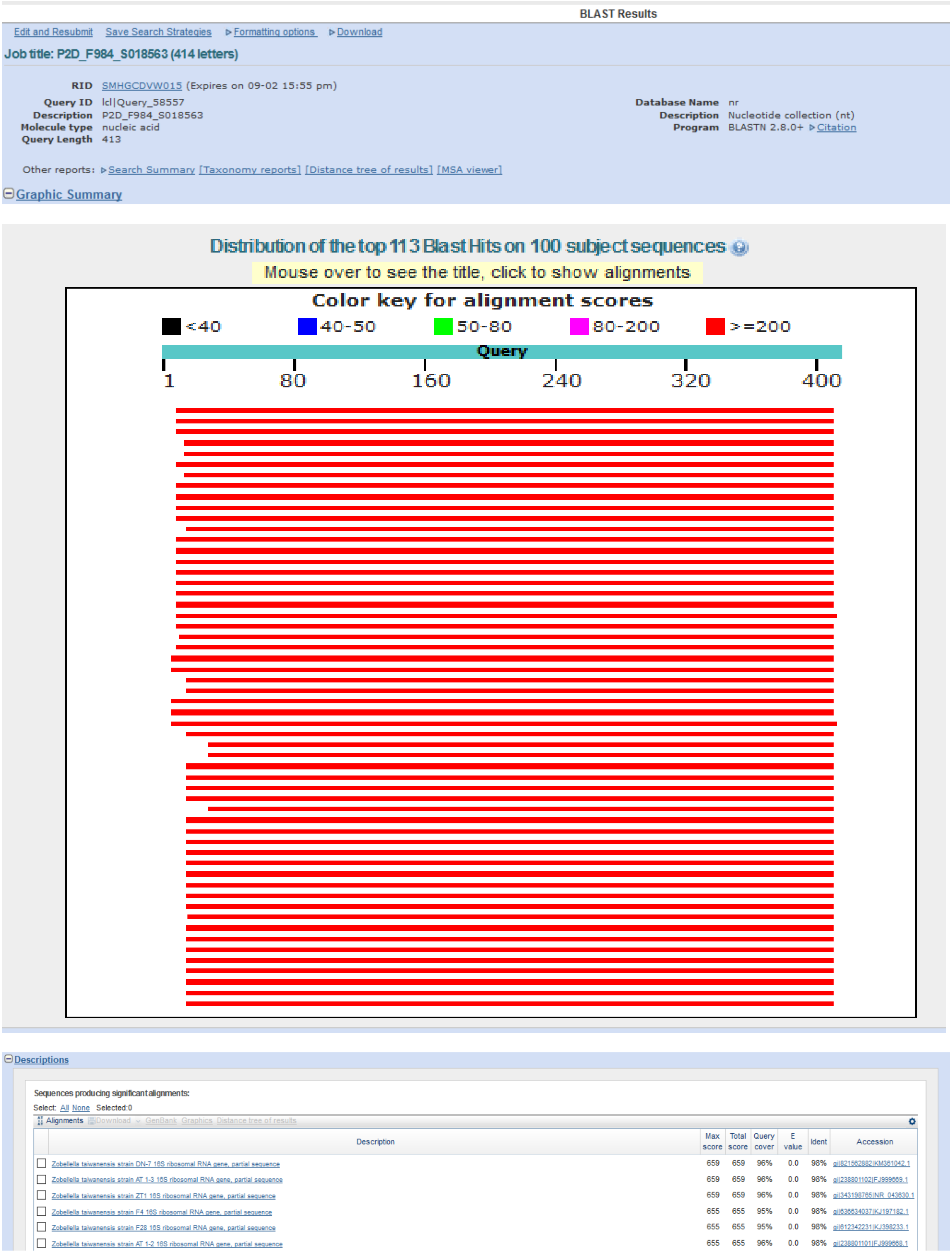
Descriptions

Sequences producing significant alignments:

Select: All None Selected:0

	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	Serratia sp. strain 290.12.4 16S ribosomal RNA gene, partial sequence	569	569	98%	3e-158	92%	gi|1336599927|MG859603.1
☐	Serratia sp. strain 266.12.2 16S ribosomal RNA gene, partial sequence	569	569	98%	3e-158	92%	gi|1336599923|MG859599.1
☐	Serratia sp. strain 264.12.2 16S ribosomal RNA gene, partial sequence	569	569	98%	3e-158	92%	gi|1154382245|KY742766.1
☐	Serratia liquefaciens strain S1-87 16S ribosomal RNA gene, partial sequence	569	569	98%	3e-158	92%	gi|1193824958|MF111562.1

Figure A.8.8 : Blast result showing percentage identity of isolate P1D with other bacterial species



BLAST Results

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Description P2D_F984_S018563
Molecule type nucleic acid
Query Length 413

Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.8.0+ ▷Citation

Sequences producing significant alignments:

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☐	Zobellella taiwanensis strain AT 1-3 16S ribosomal RNA gene, partial sequence	659	659	96%	0.0	98%	gi|238801102|FJ999669.1
☐	Zobellella taiwanensis strain ZT1 16S ribosomal RNA gene, partial sequence	659	659	96%	0.0	98%	gi|343198765|NR_043630.1
☐	Zobellella taiwanensis strain F4 16S ribosomal RNA gene, partial sequence	655	655	95%	0.0	98%	gi|636634037|KJ197182.1
☐	Zobellella taiwanensis strain F28 16S ribosomal RNA gene, partial sequence	655	655	95%	0.0	98%	gi|612342231|KJ398233.1
☐	Zobellella taiwanensis strain AT 1-2 16S ribosomal RNA gene, partial sequence	655	655	96%	0.0	98%	gi|238801101|FJ999668.1

Figure A.8.9 : Blast result showing percentage identity of isolate P2D with other bacterial species

Figure A.8.10 Some pictures during the research work

Figure A.8.11 : Participation in National and International Poster presentation and achievement of first prize in the essay competition on World DNA Day ’18 at NAST

APPENDIX IV

SEQUENCE OF 16S rRNA OF ISOLATE P1D and P2D

Isolate P1D

AGAATTCGCTAGAGATAGCTTAGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGT
CAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCA
GCACGTAATGGTGGGAACTCAAAGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGA
CGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGTATACAAAGAG
AAGCGAACTCGCGAGAGCAAGCGGACCTCATAAATTACGTCGTAGTCCGGATCGGAGTCTGCA
ACTCGACTCCGTGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATA

Isolate P2D

CTACCCTTGACATAGCAAGAAGTTTTCAGAGATGAATTCGTGCCTTCGGGAACTTGCATACAGG
TGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAAC
CCTTGTCCTTTGTTGCCAGCGATTCGGTCGGGAACTCAAAGGAGACTGCCGGTGATAAACCGGA
GGAAGGTGGGGACGACGTCAAGTCATCATGGCCCTTACGGGTAGGGCTACACACGTGCTACAA
TGGCGCGTACAGAGGGCGGCGAACCTGCGAAGGTAAGCGAATCCCAAAAAGCGCGTCGTAGT
CCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATG
CCACGGTGAATA