INTEGRATION OF ELECTROCHEMICAL CELL FOR ENHANCEMENT OF BIOGAS PRODUCTION FROM CATTLE MANURE AND MOLECULAR CHARACTERIZATION OF ISOLATED MICROFLORA

M.Sc Thesis
2021 A.D

Submitted To
Central Department of Biotechnology
Institute of Science and Technology, Tribhuvan University
Kirtipur, Kathmandu, Nepal

Submitted By:
Bikram Prajapati
M.Sc. Biotechnology
Roll No.:BT 404/073
T.U. Regd. No.: 5-2-37-1655-2012

Supervisor:
Mrs. Jarina Joshi (PhD)
Assistant Professor
Central Department of Biotechnology
Tribhuvan University
Kirtipur, Kathmandu, Nepal

Central Department of Biotechnology

Tribhuvan University

Kirtipur, Kathmandu, Nepal

Date……

RECOMMENDATION

This is to certify that the research work entitled "INTEGRATION OF ELECTROCHEMICAL CELL FOR ENHANCEMENT OF BIOGAS PRODUCTION FROM CATTLE MANURE AND MOLECULAR CHARACTERIZATION OF ISOLATED MICROFLORA" has been carried out by Mr. Bikram Prajapati 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.

……

Asst.Prof. Jarina Joshi (PhD)

(Supervisor)

Central Department of Biotechnology

Tribhuvan University

Kirtipur, Kathmandu, Nepal

Central Department of Biotechnology
Tribhuvan University
Kirtipur, Kathmandu, Nepal
Tel. No.: +977 1 4336221

Date……

CERTIFICATE OF EVALUATION

This is to certify that this thesis entitled "INTEGRATION OF ELECTROCHEMICAL CELL FOR ENHANCEMENT OF BIOGAS PRODUCTION FROM CATTLE MANURE AND MOLECULAR CHARACTERIZATION OF ISOLATED MICROFLORA" presented to evaluation committee by Mr. Bikram Prajapati is found satisfactory for the partial fulfillment of Master of Science in Biotechnology.

…… ……

Head of Department **External Examiner**

…… ……

Internal Examiner **Supervisor**

ACKNOWLEDGEMENTS

First of all, I offer thanks to God for protection and ability to do work.

I would like to express my deepest gratitude to my research supervisor Mrs. Jarina Joshi (PhD), lecturer of Central Department of Biotechnology for giving me chance to work with her and also I would like to thank for her supervision, encouragement, criticism and patience to guide me during my research work.

I would like to thanks Central Department of Biotechnology and Head of the Department Prof. Dr. Krishna Das Manandhar for helping me conduct my research. My appreciation goes to all the teachers Prof. Dr Rajani Malla, Prof. Dr. Tribikram Bhattarai, Prof. Dr Pramod Aryal, Prof. Dr. Ganga Kharel, Prof. Dr. Gauri Shankar Manandhar, Mr. Mitesh Shrestha, Mr. Suresh Subedi, Dr. Smita Shrestha, Ms. Pragati Pradhan, Mrs. Preity Regmi, Mrs. Alina Sapkota.

I would also like to convey my sincere appreciation to Sailendra Koirala and Shyam Adhikari for providing me the cow dung during my thesis experiments. I would like to especially thank to Dr. Suman Bajracharya for instigating the knowledge about the research topic. I am thankful to Dr. Rabindra Prasad Dhakal, director of Renewable energy department at Nepal Academy of Science and Technology (NAST) and Prof. Dr. Rameshwar Adhikari Research Centre for Applied Science and Technology for allowing me to carry out some part of my research work under their guidance. I owe my gratitude to Mr. Din Bandu Parajuli for his assistance during my thesis work at NAST. I am grateful to Mrs. Elen Pradhan, Laboratory Assistant and all the supporting staff especially for their support in arranging a suitable environment in which the research work was carried out easily. I take this opportunity to record my sincere thanks to all the members of the Central Department of Biotechnology for their assistance.

I want to pay my special regards to Pradip Dhungana, and thanks to my senior Pranita Poudyal, Ranjeeta Odari Apshara Parajuli, and Pradip Chaudhary for their continuous support and tolerating my endless queries during my thesis period. I convey my tender thanks to Sawan Chaudhary, Sagar Dahal, Sunil Regmi, Padma Ratna Manadhar, Yujeen Chapagain and all my friends, seniors and juniors for their encouragement and moral support.

Finally, I wish to thank my family, for providing a loving and supportive environment for me.

Bikram Prajapati

LIST OF ABBREVIATION

AcoD- Anaerobic Co-Digestion

AD- Anaerobic Digestion

BES-Bioelectrochemical system

BSA- Bovine Serum Albumin

BSP- Biogas Support Programme

BSP-Nepal- Biogas Sector Programme

BTU -British thermal unit

CNG- Compressed natural gas

CMC- Carboxymethyl cellulose

DET- Direct Electron Transfer

DNS- 3, 5-dinitrosalicylic acid

GHS-Green House Gas

IEA-International energy Agency

IET -Indirect Electron Transfer

L- Liter

LPG- Liquid Petroleum Gas

MES - Microbial Electrochemical Systems

MET -Mediated Electron Transfer

MFC -Microbial Fuel Cell (

MTOE-Million Tons of Oil Equivalent

NAST- National Academy of Science and Technology

OECD-Organization for Economic for Cooperation and Development

OLR- Oxygen Loading Rate

RECAST - Research Center for Applied Science and Technology

RES-Renewable energy system

TSS- Total Suspended Solids

V- Voltage

VFA- Volatile Fatty Acids

VSS- Volatile Suspended Solids

K Pa -Kilo Pascal

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Nepal is a developing country where most of the population is still living in rural areas. A continuous supply of cooking gas as Liquid Petroleum Gas is difficult and expensive. So biogas is a good alternative for them. Biogas production is affected by several factors; temperature is an important factor to be considered during anaerobic digestion (AD) for effective degradation of organic waste. Though most of the rural areas' of Nepal have assembled biogas plant, due to the climatic variation, during winter season, production of biogas is less. Enhancement of biogas production can be done by various methods. Among them integration of microbial electrochemical cell (MEC) system in existing AD is a new and innovative technique where a small voltage of electricity supplied to reduce CO_2 produced in digester to methane with help of methanogens as a biocatalyst. AD using cow dung is cheap and clean method of production of biogas which help to reduce serious environmental and health problems. During this work, integration of microbial electrochemical cell (MEC) system in conventional anaerobic digester showed reduction percentage was 2.7% and 8% greater in 1 L and 5 L digester respectively in MEC compared to conventional control setup while reduction of soluble reducing sugar was 33% and 9% greater in 1 L and 5 L digester at 15°C compared to control setup. At room temperature (23-29°C), reduction percentage of COD was about 11% and 18% higher in comparison to controlled digester. Likewise, reduction percentage of soluble reducing sugar was 32% higher in 1L and 19% higher in 5 L digester compared to the control digester. Biogas production was enhanced by about 28% compared to control setup even at temperature of 15°C in both 1 L and 5 L digester. Similarly, enhancement of biogas in 1 L digester and 5 L digester was 35.18±0.52% and 41.17% respectively at room temperature. Despite of enhancement, the reaction of microbial electrochemical cell was done successfully for short period of time which is not enough for complete digestion of cow dung. Hence, further study on the MEC for elongated digestion of organic waste and assembly of fed batch system is necessary. We analyze the change in different parameters for already existing 6000 L biogas plant. There was very negligible change in COD, soluble reducing sugar as there was a provision of continuous feeding of substrate every day. For the identification of microorganism, among six isolates 14IN and 18IN showed significant level of cellulase production while doing hallo zone test with congo red. PCR product of 16s rRNA of while sequenceing showed the microorganisms 14IN and 18IN were *Bacillus licheniformis* and *Bacillus aerius* respectively.

Keywords: Biogas, microbial electrochemical cell, anaerobic digestion, chemical oxygen demand.

1 INTRODUCTION

1.1 Background

Nepal is one of the developing country in the world where around 83% of the population are still living in rural areas of the country (CBS, 2012). Most of the people in Nepal depend on agricultural sector which contributes to 33% of the national GDP. Despite being an agricultural based economy, most of the people are farmers where the production of product is only limited to consumption at the household (MOF, 2012). Among this animal farming is the one of the sector which peasants of Nepal adopt for their living. The wastes produced by the animals are mostly neglected and dumped in the environment mostly due to the lack of knowledge and cost to reuse it. This inappropriate disposal of the manures can cause serious environmental and health problems like pathogen contamination, foul odor, air borne disease, water borne disease, greenhouse gas (GHG) etc. (Harikishan & Sung, 2003). To prevent pollution, most of the objectives of the Kyoto agreement were related to human and animal health safety which required sustainable solution for recycling of animal cattle manure and organic wastes. Biogas production using different technologies like anaerobic co-digestion, pre- or post-treatment play an important role to fulfill these objectives (Holm-Nielsen et al., 2009).

Nepal is an agricultural country, so tons of agricultural wastes and cattle manure are generated annually. Most of the agricultural wastes are being disposed in the bare land and cattle manure are used as guitha for cooking or used as a fertilizer directly due to lack of knowledge and technology. During winter season, production of gas from digester decrease so people are forced to select guitha as their energy source for cooking which can add to the environmental pollution negatively. So, new technology for production of biogas is much needed to overcome this problem and generate clean secondary energy even in the low temperature. In the term of a fully renewable energy system (RES), biogas is storable in the gas network which provides flexibility for buffering the fluctuant energy supply from secondary sources like wind and sun, as well as a fuel for transport (Hamelin et al., 2020). Anaerobic digestion process is interesting method for renewable energy production but production on industrial scale, spontaneous biological reactions needs good knowledge of the phenomena involved. Development of appropriate models to be used in control theory is now a high priority to optimize fermentation processes and solve important problems to develop renewable energy from biodegradable organic waste (Fedailaine et al., 2015)

Animal manure is complex organic molecule, which is compose of cellulose, hemicellulose, lignin and other contents such as pectin. Cellulose and hemicellulose can be degraded further to produce fermentable sugars, e.g., glucose, arabinose and xylose. These simple form of sugars can be further converted into various important products like biogas, bioethanol etc (Joshi et al., 2019). These sugars are converted into valuable products by mean of anaerobic digestion (AD) and anaerobic fermentation process.

Complex organic molecules from organic materials are degraded in simpler from in anaerobic condition by microbial metabolism leading to formation of methane, carbon dioxide, H_2S, H_2 and microbial biomass. This process of degradation of organic molecule into biogas form is known as anaerobic digestion. Anaerobic digestion is also known as waste to money technology in which the wastes like cattle manure, solid waste, kitchen waste, sewage slurry, food wastes etc can be converted to energy. The biogas produced from digestion generally composed of around 48–65% methane, 36–41% carbon dioxide, up to 17% nitrogen (Rasi et al., 2007). Various consortia of microorganisms are involve with different roles in the overall process scheme are needed for the AD process (Ferry, 1993). The potential for the leaching of nitrates into ground water, release of nitrates and pathogens into surface water and the emission of odors from storage logons is significantly reduces with the help of anaerobic digestion. The digested material can be used as valuable fertilizer due to the increased availability of nitrogen and it also reduced the survival of the pathogens (Weiland, 2010). Anaerobic digestion system has been used from decades to manage the municipal wastes and recently it has been used to process the industrial, household and agricultural wastes.

The biogas production is affected by several factors like condition of digester, microbial consortia presence in digester, pH, temperature, trace elements, nutrients, the ratio of C/N and ammonia. These parameters should be kept in equilibrium and dynamic condition in order to grow methanogenic and acetogenic bacteria for the production biogas at its peak level. The pH of the digester should be maintained in range of around 6.6 to 7.6 for the methanogenic bacterial growth. Nutrient available for the microbial consortia play vital role in order to enhance the biogas production. Nowadays, there are lots of researches going around the world on temperature effect in biogas production. Previously, mainly mesophilic condition of AD was studied but due to the different environmental condition around the world thermophilic and psychrophilic condition are being exploring by the researchers. According to (Bouallagui et al., 2004) anaerobic digestion can take place at psychrophilic

temperatures below 20° C. Inhibition factors presence in the substrate (cow dung) play another important role. Exceed amount of inhibitor can in halt the growth of methanogenic bacteria which may be the reason for loss in biogas production. Adequate amount of nutrient and trace element should be available for proper microbial growth in the digester. Bioelectrochemical system (BES) is one of the emerging technique which uses microorganisms as the catalyst on one or both electrode system (Hamelers et al., 2010). According to Logan et al., (2008) in presence of electrochemically active microorganisms, with apply of a small voltage in a specially designed microbial electrolysis cells (MECs), can result in a high yield of hydrogen gas at anodic side. During AD, CO_2 gas is also produced as a byproduct which can be captured and utilized by electromethanogens and convert into CH_4 (Cheng et al., 2009). Methane production is coupled to carbon dioxide capture with use of microbial electrochemical cell, this may offer perspectives for industry to reduce their greenhouse gas emissions. The MEC is a modified type of microbial fuel cell (MFC) that has been used to efficiently store electrical energy as a biofuel i.e hydrogen (Logan et al., 2008). The voltage produced on the anode by electrogenic bacteria using acetate as a substrate (E_{An} = -0.2 V) is insufficient to evolve hydrogen gas at the cathode (E_{cell} = -0.414 V, pH 7). Addition of small voltage can produce hydrogen using MECs at very high energy efficiencies evaluated in terms of electrical energy (200-400%) or both electrical energy and substrate heat of combustion energy (82%) (Cheng & Logan, 2007). The study of Villano et al., (2010) explained the performance of a microbial biocathode, which is capable of reducing carbon dioxide to methane, at rates of 0.055 ± 0.002 $mmol d^{-1} mgVSS^{-1}$.

1.2 Current studies

1.2.1 Status of Biogas and its enhancement in Nepal

Traditionally people of Nepal use cattle manure cake (guitha), firewood, kerosene, agricultural residue and electricity as their main source energy. After the introduction of biogas in 1990 by Biogas Sector Partnership (BSP) Nepal, the user of biogas produced from cattle manure were increased rapidly. The present state of biogas production from cattle manure, human excreta and kitchen wastes has been implemented in anaerobic digester which helps to substitute the traditional method in some places. But due to various problems, methane gas production has been stopped in many digesters. This may be due to the lack of enough gas production from the digester to fulfill required energy. In the context of Nepal, fixed dome below ground biogas plants have been used which

was a modification of the Chinese and Indian fixed dome models (Henderson, 1997; Khoiyangbam et al., 2004). 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). Various researches has been done in Anaerobic co-digestion (AcoD) which was very helpful for people as they can use agricultural and kitchen wastes along with cattle manure. According to Subedi & Baral, (2021) biogas can even be produced at psychrophilic condition of Nepal but the enhancement of biogas should be necessary for the proper biogas production. The main problem regarding generation of biogas at cold temperature and during winter season should be addressed by providing fund for those topic researches in Nepal for further enhance in of biogas in Nepal.

1.2.2 Enhancement of biogas around the world

The biogas production is quite promising for the sustainable development of the country. The organic biomass contain lots of potential for the production of the clean energy, however to meet increasing demand of the energy conventional technique of biogas production is inadequate. Hence, new technique for the enhancement of the biogas is much needed in present day context. Analyzing these scenarios they tend to enhance the current energy source for the future energy demand. Apparently in many European countries, the production of biomass as a substrate for the biogas plants has been developed. Meanwhile the government of Germany has taken steps in 2011 to reduce even monoculture maize production for energy purposes (Graaf & Fendler, 2010).

Anaerobic co-digestion is the one of the most effective mode of enhancement and management of the organic wastes. The benefit of the co-digestion is mainly due to the optimization of the various nutrient balances in the substrates mixture when co-digesting nitrogen rich substrates with carbon rich substrates which result in production of high methane yield (Giordano, 2012). AcoD technology not only reduces the volume of wastes to be disposed and avoids soil and groundwater pollution, but also makes available a renewable and inexpensive energy, e.g. biogas that, unlike the fossil fuels, keeps stable in the atmosphere the balance of greenhouse gases, such as CO2. According to Labatut and co-workers (2011), studies reported that the optimal operational conditions in terms of percentages of co-substrates cannot be univocally defined but should be investigated for each specific case. So more research should be done to get high yield of methane from different substrate should be conducted. It is an innovative and effective strategy for reducing the ammonia inhibition during AD process since it aims at favoring synergisms, dilutes harmful compounds,

increases the substrate quality , and enhance the biogas production (Labatut et al., 2011). AcoD of animal manure with lignocellulosic residues offers a promising route of efficient biogas production which is also in line with climate friendly farming practices. 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 same plant will be more effective for reasonable production of energy and supply. This removes the total cost for energy generation by around 50% reducing total transportation cost (Stu etr al., 2011; Walla & Schneeberger, 2008).

Pretreatments are the initial process of the hydrolysis of complex polymers for the easy fermentation and digestion process. These process can be carried out by various method like physical pretreatment, rapid decompression, auto hydrolysis, acid- or alkali pretreatments, solvents (e.g. for lignin or cellulose) pretreatments or leaching, supercritical, oxidative or biological pretreatments, as well as combined gasification and fermentation, integrated biogas production and pretreatment, innovative biogas digester design, co-digestion, and bio-augmentation (Horváth & Taherzadeh, 2016). However in a mean while the process of pretreatment is quiet expensive in term of total energy production in context of house hold biogas production. This process is mainly applicable for the industrial purpose of biogas production.

Traditionally the design of digester is done for simple process of digestion. As followed by various researches of chemical and biological processes till now there was need of up gradation of digester design for optimum digestion and biogas production. The design usually aims for the digester to provide a good environment that enables efficient contact between the microorganisms and the substrate. However, in case of use of certain chemicals, the AD process can be inhibited, even though these methods can successfully release dissolved intermediates for the AD process (Rodríguez & Encina, 2016). Another innovative design is the use of two- or multi-stage digesters. Due to the need of different environment and need of nutrition separating the methanogenesis step from the acidogenesis step during the AD process has been found to give optimal conditions to the different microorganisms involved in the AD processes. Which results in achieving a better process control and ability to handle higher oxygen loading rates (OLRs) than that in the single digesters (Demirel & Yenigün, 2002).

Microbial electrochemical cells technology derived from microbial fuel cell (MFC) in which small amount of voltage in supply is added externally. MECs are a new technology, and thus many researchers may be unfamiliar with the construction

of these reactors and factors that can affect performance. MECs share many attributes with MFCs because the design of the anodes and the electrogenic reactions occurring there are similar. MECs work under oxygen free conditions and promote the growth of obligate anaerobic bacteria such as exoelectrogenic *Geobacter* spp, *Pseudomonas* spp. and *Shewanella* spp as well as nonexoelectrogenic fermentative or methanogenic microorganisms(Liu et al., 2008). Sodium acetate, sodium propionate, sodium butyrate, glucose and starch served as different substrates for MEC anodic culture experiments under the same condition. MEC hydrogen production is environment friendly with the advantages of wide availability of substrate sources with high hydrogen conversion rate. Different researches show that all types of organic wastes (e.g. livestock manure, domestic sewage, activated sludge, and industrial wastewater) can be used as ideal substrate sources for MEC hydrogen production (Shao et al., 2019).

1.3 Rationale

For the sustainable development of the country, development in the field of renewable energy source is one of the most important parts which helps people to reduce their living cost as well as make environment clean. In order to fulfill present energy need, instead of fossil fuels, biogas production from lignocellulosic biomass and manures of different animals has attracted promisingly to the people of the world. The biogas which contain methane as the flammable gas proved to be lesser harmful to environment than other commercial and fossil fuels. Due to lower production cost and lesser environmental impact of the biogas, it is one of the promising alternative energy sources. Nepal is a sub-tropical country which has low land, high land and mid land, showing different climatic conditions throughout the year. The temperature of these area changes each season which affect the production of biogas. During winter season, the temperatures of the hilly and mountain area drop down below 10°C which is not suitable for the production of biogas. Lower temperatures decrease the production of biogas. Due to this peasant are forced to choose commercial or fossils fuels which are a burden both economically as well as environmentally. To overcome this problem of temperature barrier new technology should be developed to enhance the production of biogas production.

1.4 Research hypothesis

Renewable energy sources are most reliable energy option for the sustainable development by using cattle manure. Biogas production helps in maintaining the environmental resources intact for many year even after continuous us of it. Enhancement of biogas can help us in adverse environmental conditions even during the shortage of other fuels.

1.4.1 Null hypothesis

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

1.4.2 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

- Production of biogas in lab and pilot scale plant and its enhancement using electrochemical cell.

1.5.2 Specific objectives

- Analysis of chemical parameters of cow dung.
- Observation of biogas production in lab and pilot scale anaerobic digester using cow dung.
- Develop the biological process to utilize voltage supply for methane production using naturally occurring microorganism.
- Analysis and comparison of COD and soluble reducing sugar before and after biogas production.
- Identification of prevalent microorganism in anaerobic digester.

1.6 Research Scope

The world is heading toward sustainable development technology to maintain clean environment for the sake of future generation. In order to meet the goal most of the European countries are adopting renewable energy sources which are more reliable than other fossil fuels. These new technologies are facing various problems involving technical and different environmental parameters. Among these biogas production from waste biomass is cheap and reliable means of energy sources which is even affordable for rural population. Varying climatic condition has remained the main problem for the continuous biogas production all year around in context of Nepal. According to new hypothesis, use of microbial electrochemical cell (MEC) in anaerobic digester helps in enhancement of biogas even at the low temperature. The prevailing disruption of the use of bovine manure during winter season and cold environmental condition can be overcome. Moreover, biogas can become cheap and reliable means of energy source and will be adequate to use as household fuel. Even in the adverse condition, there will be possibility of enough supply of household cooking fuels. So country like Nepal should keep use of renewable product with high priority for the sustainable development. Like other renewable energy, biogas can be used in various sectors for example can be used for production of electricity, can be used in the vehicles as clean fuels if it is produced in commercial scale which can replace the petroleum products. Nepal being an agricultural country, most of the people relies on farming or animal rearing occupation. Due to lack of knowledge, people throw away valuable byproduct like manure and agricultural wastes which can be used for production of biogas. Still many part of country use forest wood for cooking leading to deforestation and adverse climatic conditions like flood and landslide. Biogas can help in reuse of these waste materials, reduction in dependency on forests and petroleum product in rural area. The new method of enhancement of biogas can help nation economically as well as environmentally throughout the year irrespective of the climatic fluctuation.

2 LITERATURE REVIEW

2.1 World energy demand

The world energy consumption will be more than 1400 MTOE by 2018 as predicted by International Energy Agency (IEA). The total energy production was 14,421 MTOE in 2018 and approximately 81% of come from fossil fuels and remaining was covered by natural gas, coal, oil and renewable energy. At this time the amount of energy demand has fulfilled but the energy demand for the later years it will be increased and the over dependence on fossil fuels which will result in increased amount of atmospheric CO_2 (IEA, 2020).

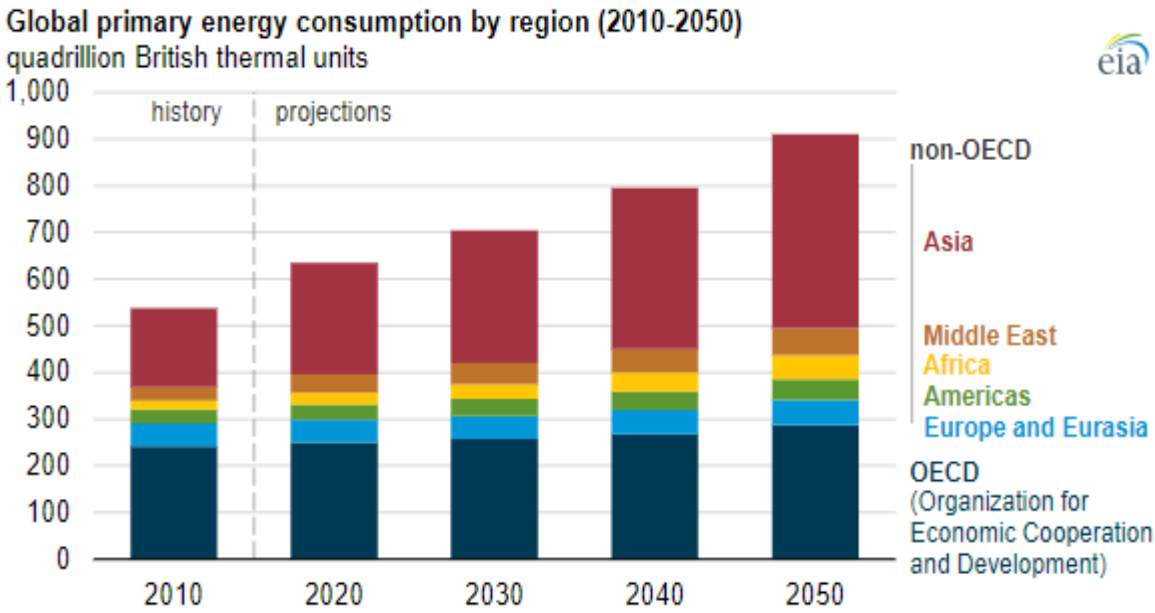


Figure 2. 1: Global primary energy consumption by region (2010-2050)(IEA, 2020)

Worldwide energy utilization was approx. 520 quadrillion BTU (British thermal unit) in 2010 and is relied upon to increment by 56% (820 quadrillion BTU) by 2040. According to the U.S Energy information Administration (EIA) estimated that world energy consumption will increase by nearly 50% between 2018-2050 in non-organization for economic cooperation and development (OECD) in Asian region.

2.2 Energy demand of Nepal

Nepal is the least developed country in the world so the consumption of energy is also low which is about 2.8 million btu in 2009 (US EIA, 2013). In Nepal, the energy demand was dominantly fulfilled by the traditional type of energy source like fuel wood, agricultural residue, animal dung etc. It was estimated that about

70% of the energy supply was fulfilled from the fuel wood, 13% from petroleum products, 3% from electricity, 1% from biogas, 4% from dung and 6% from coal .It is estimated that reduction of the fuel wood will be occur to 43% by the 2030 at the 6% of GDP growth rate which will be replace by the electricity, petroleum product and renewable energy (NEC, 2015). As predicted the supply of energy from electricity increase due to high implementation of hydropower but still dependency on the fuel wood is high in Nepal will result deforestation which can lead to serious damage to environment. To manage this problem electricity source is not enough so the renewable energy source must be enhanced to fulfill the future demand of energy to save the forest and the sustainable development of Nepal (MOF, 2012).

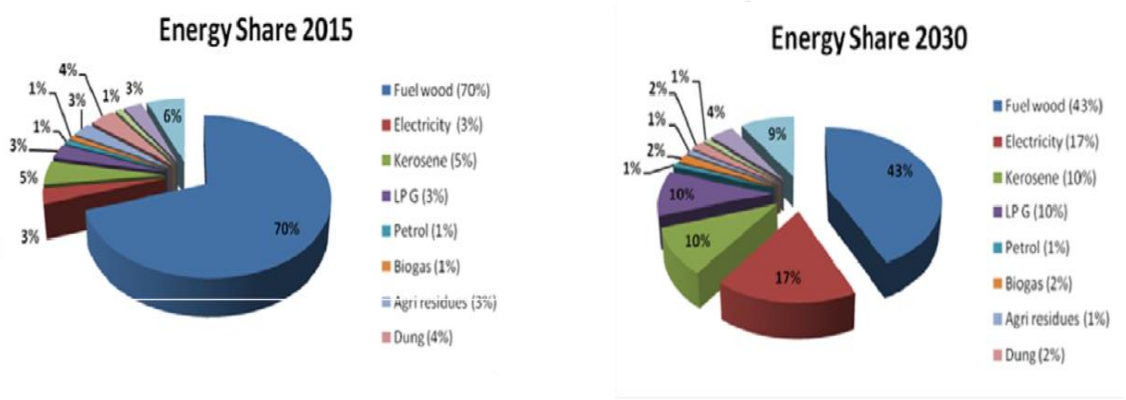


Figure 2. 2: Projected energy use and energy distribution in 2015 and 2030 at 6% of GDP growth

Nepal is predominately an agricultural country where this sector contributes to 33% of the national (MOF, 2012). The livestock farming occupies an important role in the economics. It contributes to the economics in different ways like energy, food, raw materials for pharmaceutical and industrial products and manure for various purposes (Saadullah, 2001).The number of households with cattle or buffaloes in Nepal is estimated to be 2,784,583 with potential biogas households of 1,937,006. Based on the reduction in firewood consumption, we estimate that a household with biogas saves about 250 kg of firewood per month. Thus saving of firewood from each household per year is about 3 tons. This reduction in demand for firewood helps to conserve forests. Similarly the saving of cow dung being directly burnt is 48 kg per month. According to Mendis and van Nes, emission coefficients for non-sustainable fuel wood and kerosene are 1.5 tons CO2 per ton and 2.5 tons CO2 per 1000 l of kerosene. Based on these emission factors, a rural household with biogas reduces about 4.5 tons CO2 being released in to the atmosphere each year. In other words, every biogas

system in Nepal avoids nearly 4.5 tons of carbon emissions per year by reducing the use of firewood in the kitchen. Results show that households whose main cooking fuel is biogas collect 1200 to 1400 fewer kg of firewood annually than households that use firewood as their main fuel (Bluffstone & Toman, 2014).

2.3 Biomass as renewable energy source

Biomass is the general term for variety of different materials: wood, sawdust, straw, seed, waste manure, household waste, wastewater etc which are generally derived from growing plants or from animal manure. Basically it can be said that the solar energy which are stored in plants and animals or in wastes is called biomass energy. These energy are use directly by burning as heat energy or by converting it to another form like electricity, biogas (Demirbaş, 2006; Moren et al., 2019). Biomass is a resource that is present in a variety of different materials: wood, sawdust, straw, seed waste, manure, paper waste, household waste, wastewater, etc. Biomass resources have traditionally been used, and their use is becoming increasingly important due to their economic potential, as there are significant annual volumes of agricultural production, whose by-products can be used as a source of energy and are even being promoted as so-called energy crops, specifically for this purpose (Bluffstone & Toman, 2014).

2.3.1 Biogas

Biodegradation of organic matter such as food, plant debris, animal manure, sewage sludge, biodegradable portions of municipal solid waste, etc produces a gas which contain about 40-70% methane as well as carbon dioxide and other gases. This mixture of gas is commonly known as biogas. Biogas burns cleanly without foul smell similar to LPG or CNG when ignited (Abbasi et al., 2012). The formation of methane gas is a natural process when biomass is decompose in absence of oxygen but in presence of group of natural organism like methane bacteria which are metabolically active.(book). The biogas production is determined by the biodegradable organic matter content of the raw material subjected to the microorganism's action, by the C/N ratio, temperature, sub layer, pH etc (Letters, 2014).

Cellulose is a polysaccharide built with a linear chain counting from several hundred to several thousand repeating D-glucose units that are bound together covalently by (1–4) β glycosidic linkages the most common natural polymer in nature which is characterized by biocompatibility, biodegradability and high chemical reactivity (Pitol-filho, 2012). The cellulose disintegration products such as cellobiose and soluble cellulodextrin of higher order can be transformed into

methane and carbon dioxide after a series of transformations (Bhadra et al., 1986)

Methane can be produced naturally as well as artificially. In nature, methane is produced by various microorganism involvements at different phases of anaerobic digestion while in other method CO_2 is captured at a stationary point of fuel use which is facilitated by using pure oxygen combustion; the captured CO_2 is then transported to a location with abundant renewable energy. The renewable energy is used to generate electrolytic hydrogen, which is reacted over a catalyst with the captured CO_2 to re-synthesize methane (Hashimoto et al., 2001). Combination of biological and electrochemical technique for methane production results great promise as it does not require precious metals to produce final product however use of some cheap metal can further enhance the production of product (Spinner et al., 2012). Electromethanogenesis is the new approach to produce methane by direct electron transfer from a carbon electrode using microorganisms of Archea domain which can fix CO_2 to methane (Siegert et al., 2014). Carbon felts are eco-friendly typically used porous carbon electrode for the development of electro active bio-films in bioelectrochemical system (Bajracharya et al., 2015).

2.3.2 Sources of biogas

Biodegradable waste can be used as a feedstock to produce useful energy which ultimately leads towards waste minimization at the same time. Therefore, waste treatment plants using various organic substrate to produce biofuel and electricity are common in many countries (Mustafa, et al., 2016). Different substrate and their methane content are shown in Table (2.1).

Table 2. 1: Different substrate for methane production and their methane content

Substrate	Methane content (%)	References
Maize	53.47	(Mursec, 2009)
Sorghum	51.81	(Mursec, 2009)
Bovine manure	46.5	(Fantozzi & Buratti, 2009)
Chicken manure	66.6	(Fantozzi & Buratti, 2009)

Pig manure	65	(Ahn et al;, 2010)
Food waste	73	(Zhang et al., 2007)
Wheat straw	78	(Letters, 2014)
Sugar beet	55.82	(Mursec, 2009)
Microalgae (Chlamydomonas reinhardti)	66	(Mussgnug et al., 2010)
Microalgae (Dunaliella salina)	64	(Mussgnug et al., 2010)

2.3.3 Biogas program in Nepal

Cattle manure, human excreta, agriculture residues and organic waste are used in anaerobic bioreactors to produce methane gas. These kind of organic waste are easily available in farms, rural people of many developing countries have been benefited from this technology. Nepal being a least developed countries with the majority of people involved in subsistence agricultural field, biogas technology in Nepal has been benefitting the country in improving health, environment, economy and energy conservation (Gautam et al., 2009).

The first biogas plant in Nepal was built in 1955 in the Kathmandu as a demonstration how we can use organic waste as energy. 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)

More than 2, 60,899 biogas plants have been installed in Nepal till 2003. The progress of the biogas sector in Nepal has been possible due to the joint effort of different stakeholders in which pivotal role has been played by Biogas Support Program (BSP) an independent non-profit organization, through the financial assistance provided by the Netherlands (Nakarmi & Dhital, 2016; Bajgain & Mendis, 2005).

2.4 Anaerobic digestion

Anaerobic digestion(AD) is the biological methanogenesis process in which several different consortium of microorganism is involve in degradation of much of the carbonaceous matter, protein and lipids occur which results in production of biogas (Chynoweth et al., 2001). The biological gasification is a process where breakdown of the biomass is occur result in production of methane. This decomposition process involves the biological breakdown or conversion of organic material to mainly methane (CH_2), carbon dioxide (CO_2) and H_2O in the absence of oxygen. It is a complex process including intermediate molecules such as sugars, hydrogen and acetic acid, before biogas is produced (Chynoweth et al., 2001).Raw biogas is composed of 40-75% CH_4 and 25- 65% CO_2 but for effective fuel source the percentage ratio of methane should be increased.

Anaerobic digestion process is mainly occurs in many anoxic environments including watercourses, sediments, waterlogged soils and the mammalian gut naturally and AD can also be applied to a wide range of feedstock's including industrial and municipal waste waters, agricultural, municipal, food industry wastes, and plant residues (Ward et al., 2008). AD of has been used to convert organic matter residue into different valuable products like biogas, which can be further converted into other renewable energy source green electricity, vehicle fuel. On other hand the digested substrate can be used as fertilizer in agricultural field which help reduction in production of greenhouse gas (GHG) in environment by recycling the carbon into environment. Production of biogas from AD requires man power for production, collection and transport of AD feedstock, manufacture of technical equipment, construction, operation and maintenance of biogas plants. This means that the developmen and establishment of new enterprises in biogas production in nation can be done. These plant with significant economic potential, increases the income in rural areas and creates new oppertunities (Comino et al., 2009)

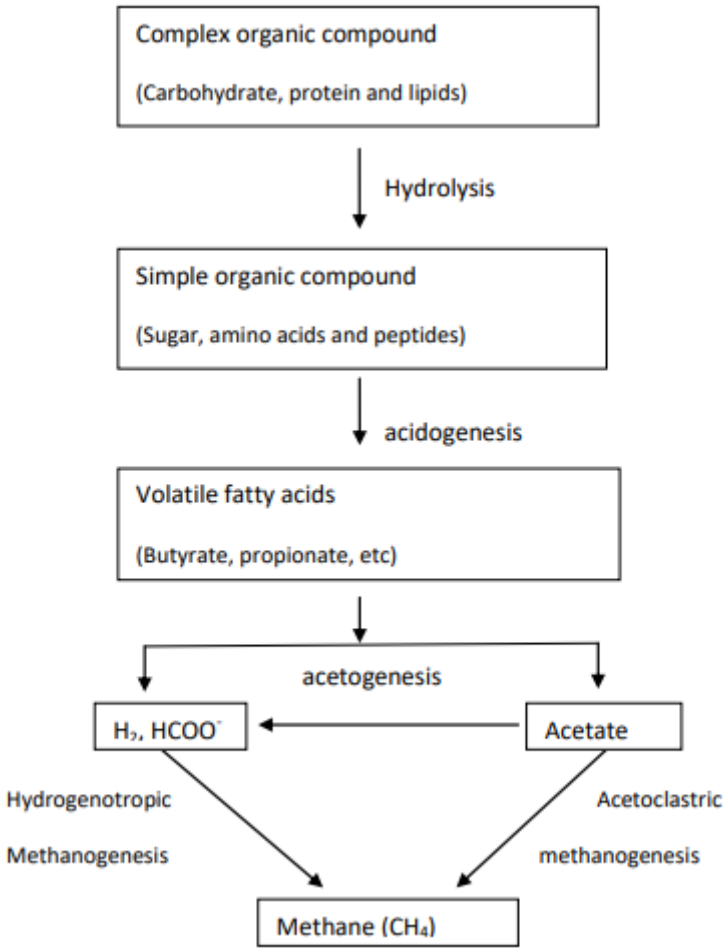


Figure 2. 3: Methane formation pathway (Zehnder & Gujer, 1983)

According to Zehnder & Gujer (1983), there are six process in which biogas is produce from complex polymers which are: hydrolysis of biopolymers, fermentation of amino acids and sugars, anaerobic oxidation of long chain fatty acids and alcohols, anaerobic oxidation of intermediary products such as volatile acids except acetic acid, conversion of acetate to methane and conversion of hydrogen to methane. Methane generation is mainly contributed by conversion of acetate (70%) whereas conversion of hydrogen only contributes 30%. This may be due to insufficient availability of hydrogen or other reducing equivalents, CO_2 reduction is limited.

2.4.1 Different phases of anaerobic digestion

Methane formation is complex process in which polymer can be converted to methane. As mentioned above the six processes are carried out in four different phases of AD which are hydrolysis, acidogenesis, acetogenesis/dehydogenesis and methanogenesis.

2.4.1.1 Hydrolysis

Biomass contains large organic polymers which are hard to break during the AD. To access the energy potential in the AD microorganism need to break down these chains. The process of the breakdown of the complex polymer into simple monomer is known as hydrolysis. These constituent parts such as sugar amino acid and peptides are readily available for bacteria. Thus first phase of AD is the essential and rate limiting step for continuation of the anaerobic digestion into further next step (Henze et al., 2002). Hydrolysis of biomass produce acetate, hydrogen and volatile fatty acids (VFAs) are produce. Acetate and hydrogen can be directly used for methane production and VFAs is further process in acidogenosis process.

2.4.1.2 Acidogenesis

After hydrolysis, the biological processes which convert monomer or organic aids into VFAs like butyrate, propionate etc in the presence of acidogenic (fermentative) bacteria. Along with VFAs ammonia, CO_2, H_2S were also produce as a byproduct which give an intense unpleasant smell to this phase of the process (EPA, 2006). Acidogenesis process work on two method hydrogenation and dehydrogenation. The basic pathway of transformations passes through acetates, CO2 and H2, whereas other acidogenesis products play an insignificant role. As a result methanogenes can directly use the new products as substrates and energy source for methane production. Accumulation of electrons by compounds such as lactate, ethanol, propionate, butyrate, higher volatile fatty acids is the bacteria's response to an increase in hydrogen concentration in the solution (Ziemiński & Frąc, 2012).

2.4.1.3 Acetogenesis

In third step of AD acetogenesis VFAs are simply converted to acetate and $H_2/HCOO^-$ form which can be easily converted methane. The acetogens are responsible for the digestion of VFAs to further simpler form (Mac & Llabr, 2000). During this phase production of hydrogen can negative affect so symbiosis is necessary for acetogenic bacteria with autotrophic methane bacteria using hydrogen, here in after referred to as syntrophy (Bok et al., 2005). Acetogenesis is a phase which signifies the efficiency of biogas production, because around 70% of methane arises in the process of acetates reduction.

2.4.1.4 Methanogenesis

In terminal phase of the AD acetic acid, hydrogen and carbon dioxide like intermediate products are converted into a mixture of methane, water and carbon dioxide by the methanogenic bacteria. Due to end product methane production methanogenesis is the key pathway for production of biogas and is commonly considered to be the rate-limiting step of the whole (Chen et al., 2008). Only 30% of methane is produced by autotropic methane producing bacteria rest are produced by heterotropic methane producing bacteria with use of acetate (Demirel & Scherer, 2008).

2.5 Involvement of microorganism in different phase of AD

In the process of anaerobic digestion, the acid forming and the methane forming microorganism consortiums differ widely in terms of physiology, nutritional needs, growth kinetics, and sensitivity to environmental conditions (Pohland & Ghosh, 1971). Under symbiotic effects of different anaerobic and relatively anaerobic bacteria, complex organic substances are decomposed into simple, chemically stabilized compounds – mainly of methane and carbon dioxide (Naik et al., 2010). The lignocellulosic biomass which contains cellulose, hemicellulose, and lignin, the composition interact to create a highly resistant and recalcitrant biomass structure. In other to break down complex molecule like lignin, cellulose and hemicelluloses either pretreatment is required or hydrolytic enzymes like ligase, cellulase, cellobiase, amylase and protease producing microorganism is required for proper hydrolysis. Hydrolysis is mainly carried out by strict anaerobes such as Bactericides, Clostridia and facultative bacteria such as Streptococci, etc (Bryant, 1979). In acidogenesis phase primarily bacteria belonging to facultative anaerobes which utilize remaining oxygen accidentally, creating favourable conditions for the development of obligatory anaerobes such as *Pseudomonas, Bacillus, Clostridium, Micrococcus or Flavobacterium*. Cellulolytic strains of bacteria like actinomycetes and mixed consortia have been found to improve biogas production in the range of 8.4–44% from cattle dung (Attar et al., 1998).

The hydrogen and carbondioxide is converted into mixture of methane and carbon dioxide in acetogenesis phase. These process generally governed by methanogenic bacteria which utilize acetate like *Methanosarcina* spp. and *Methanothrix spp*. and hydrogen and formate utilizing species like *Methanobacterium*, *Methanococcus*, etc. In this phase the acetate bacteria

including those of the genera of *Syntrophomonas* and *Syntrophobacter* convert the acid phase products into acetates and hydrogen which may be used by methanogenic bacteria (Schink, 1997). Methanogenic bacteria binding hydrogen were found to belong to family *Methanobacteriaceae* (Mab et al., 1993). Methanogenes are largely differentiated morphologically. Methanogenes exhibit almost all shapes occurring in bacteria: Cocci (*Methanococcus*), rods *(Methanobacterium*), short rods (*Methanobrevibacter*), Spirillaceae (*Methanospirillum*), sarcina (*Methanosarcina*), filiforms (*Methanothrix*).

2.6 Role of substrates and compounds in phases of AD

The rheological behavior of sludge has a key role to play in heat and mass transfer during conversion of biomass to methane through anaerobic digestion (Miryahyaei et al., 2019). The chemical composition and structure of lignocellulosic biomass hinders the rate of biodegradation of solid organic waste by the microorganism. It has been recognized that hydrolysis of the complex organic matter to soluble compounds is the rate-limiting step of anaerobic processes for wastes with a high solid content. The composition of substrate i.e., protein, fat, fiber, cellulose, hemicellulose, starch and sugar content which are significant factors that impact the methane yield (Comino et al., 2009). Various research report that the rate-limiting for complex organic substrate is the hydrolysis step due to the formation of toxic byproducts like complex heterocyclic compounds and non-desirable volatile fatty acids (VFA) formed during hydrolysis step whereas methanogenesis is the rate limiting step for easy biodegradable substrates (Fernandes et al., 2009; Lu et al., 2008). Hence, this process can be divided into two phase acid formation and methane formation.

The product produced at the different phase are separate so the microorganism involve in these phase are also different. At the end of the AD acetate, methane, CO_2 and H_2 gas is produced. These products are harmful for the growth of initial digesting bacteria so for the compound like lignocellulogic biomass slow digestion is the most otherwise there will be rise in acidic concentration. High acidic condition inhibits the growth of methaogenic bacteria. Thus rate- limiting step depend on the substrate which is used for biogas. The fragile balance between acid forming and the methane forming microorganisms is very hard, which can result to reactor instability and consequently low methane yield (Demirel & Yenigün, 2002). The two main groups of microorganisms could be physically separated with the intention of making use of the difference in their growth kinetics to get maximum yield of methane (Pohland & Ghosh, 1971). In

order to achieve phase separation, several techniques have been employed such as membrane separation, kinetic control, and pH control (Fernandes, 1986,)

2.7 Role of various parameters in anaerobic digestion

The various parameters that control AD performance, such as temperature, pH, organic loading rate (OLR), C/N ratio, nutrients & trace element, and hydraulic retention time (HRT).

2.7.1 Role of temperature

Temperature plays a vital role in anaerobic digestion, since it determines the shapes of microbial ecosystems, and consequently regulates the constancy performance of anaerobic digestion process. Change in temperature can drastically decrease the methane production by interfering microbial growth moreover the imbalance in the bioavailability of acidogenic and methanogenic microorganisms can lead to instability in the AD system, has also been noted to be affected by operating temperature (Hupfauf et al., 2018). The digestion system can be performed in phycrophilic (<20°C), mesophilic (30-43°C) and thermophlic (50-60°C) temperature condition. But the methane yield of the various temperature conditions is different due to the effect of microbial growth. Mesophilic and thermophilic conditions are extensively used in most commercial-scale AD systems to maximum methane production rate (Nie et al., 2021). According to Dev et al.,(2019) structural transformation of membranes with high levels of unsaturated lipids is a common strategy of cold-adapted psychrophiles to maintain normal membrane fluidity and function during the digestion process while thermophilic archaea stabilize cellular components by synthesizing heat-shock proteins or concomitant proteins at high temperature (Lloyd et al., 2005).

The growth rate of methanogen is higher at thermophilic condition making the process faster and more efficient however thermophilic treatment suffers from some drawbacks such as lower stability compared to mesophilic treatment (Buhr, 1976). According to Ahring et al., (2001), increasing the operational temperature from 55 to 65°C results in an unbalance between the fermenting, acids-producing micro-organisms and acids-consuming micro-organisms. At high temperature the accumulation of the ammonia increase and lead to acidic condition and accumulation of VFAs increased, this acidic condition in halt the growth of methonogenic organism and hence the methane production is decreased (Angelidaki & Ahring, 1993).

2.7.2 Role of pH

The pH of the solution is known to affect enzymatic activity due to the fact that only a specific and narrow pH range is suitable for the activation of each enzyme in the given solution thus an optimum pH is required for the maximum activity to be displayed. A pH range from 5.5 to 8.5 is reported for most methanogenic bacteria to function and the rate of methanogen bacterial activity may decrease if the pH is lower or higher than this range. The optimum temperature for the methanogenoic bacteria is 6.5 to 8 (Boe, 2006). Acid accumulation is the greatest risk for digester failure if the amount of VFAs into the digester increased sharply during low pH. The acidogenic bacteria would then flourish, producing high volumes of organic acids and further lowering the pH to below 5.0 which is lethal to methanogens and above pH 8 are toxic which results in the inhibition of biological functions of anaerobic bacteria. High pH could be due to prolific methanogenesis, resulting in a higher concentration of ammonia that would impede acidogenesis. This can now be opposed by adding a greater amount of fresh feedstock (Lusk, 1999; Ostrem et al., 2004).

2.7.3 Role of oxygen loading rate (OLR)

The degree of starvation of the microorganism depends on OLR. It determines whether microorganisms grow slow or fast. High OLR refer to fast growing of microorganism in anaerobic digestion whereas low OLR inhibit the growth of microorganism in anaerobic digestion due to the starvation. However, too high load of OLR is still toxic to microbiological environment of digester because all OLR is not utilized and produced organic acids which lead to the acidic condition. OLR rate is determined by rate of feeding of substrate and reactor temperature (Liu & Tay, 2004).

2.7.4 Role of C/N ratio

All the microorganism need nutrients and trace elements for grow. The ratio of C/N/P is essential for growth because these are the key elements synthesis of protein, amino acids, ammonia etc for microbial growth. Ammonia helps in buffering activity to neutralize acidification in anaerobic digestion son feedstock must contain proper ratio of C/N ratio. According to Rajeshwari et al.,(2000) the ratio of C/N/P should be around 100:3:1 for growth of methanogenic bacteria. Low C/N ratio generates ammonia production and increases in pH value more than 8.5 and considered harmful to methanogenic bacteria where as high C/N ratio will decrease methane production due to shortage in nitrogen element which is digested by rnethanogen (Hanafiah, 2016).

2.7.5 Role of nutrients and trace element

The problem of trace element deficiencies in anaerobic digestion (AD) for growth of microorganisms has been well-known for more than 30 years. The substrate used for AD determined whether to add or not add nutrients and trace element in given substrate. The organic waste and manure contain appropriate amount of micro and macro nutrient so the deficiencies will be irrelevant. But some research shows that addition of trace element increase in methane production (Pobeheim et al., 2010). According to Lindorfer et al.,(2012) Phosphorus and sodium showed the highest deviation in the macronutrients whereas molybdenum, nickel and cobalt of the micronutrients. However, the variation in zinc, selenium, tungsten, boron, iron and manganese was at a high level as well. In digesters the elements with the highest deficiencies were found to be cobalt, nickel and selenium.

2.8 Biochemistry and microbial background

2.8.1 Microbiology of methanogens

The microbiology in AD plants is normally regarded as a big black box and very few attempts have been made to characterize the actual micro flora in bioreactors. Recent research has demonstrated that AD plants within close distance of each other can possess different micro floras with different characteristics. Some microbial strains will add superior characteristics to the reactor system and this has major implications for the future of AD plants. Among them methanogens are responsible for the methane production. Methanogens contain neither catalase nor superoxide dismutase. Due to extraordinary sensitivity of these microorganisms to oxygen, their biochemistry, physiology and ecology are less known.

Methanogens are a morphologically diverse group of organisms found in anaerobic conditions; on the other, they are a physiologically coherent group of strict anaerobes which share the common metabolic capacity to produce methane (Balch et al., 1979). Methanogens are terminal organisms in microbial food chains which contain at least three interacting metabolic groups of strictly anaerobic bacteria that together convert complex organic biomass to CO_2 and CH_4. The complex organics are first converted to a mixture of volatile fatty acids, alcohols, carbon dioxide, and H_2 (Abbanat et al., 1989). The major part of the carbon flow in anaerobic reactor occurs between the fermentative microorganisms and the methanogens to produce methane & CO_2 and only

between 20 and 30 % of the carbon is converted into intermediary products (Marvin & Mackie, 1981).

Methanogenic bacteria can be differentiated into two groups: a) those that utilize acetate and produce methane and carbon dioxide, called acetoclasticic methanogen b) those which utilize carbonic anhydride from hydrogen and produce methane, called hydrogenotropic methanogen.

Typical reactions carried out by methanogen during anaerobic process

Reaction carried out by methanogen		
Hydrogen:	$4H_2 + CO_2$	$CH_4 + 2\ H_2O$
Acetate:	CH_3COOH	$CH_4 + CO_2$
Formate:	$4HCOOH$	$CH_4 + 3CO_2 + 2H_2O$
Methanol	$4CH_3OH$	$3CH_4 + CO_2 + 2H_2O$
Carbon monoxide:	$4CO + 2H_2O$	$CH_4 + 3H_2CO_3$

Source: (Demirel & Scherer, 2008)

2.8.2 Phylogenetic and habitat of methanogens

Methanogens belong to the kingdom of euryarchaeota in the domain of archaea. The archaea differ from bacteria in many aspects important to molecular work. Among these are cell wall composition, their sensitivity to antibiotics, their translation and transcription machinery, and their very strict demands to anaerobic culture conditions (Lange & Ahring, 2001). Despite of enormous phylogenetic diversity, methanogens can use limited amount of sugar as substrate (Woese, 1987).

Methanobacterium and *Methanobrevibacte*r belong to the family Methanobacteriales while *Methanosarcina* belongs to the family, Methanosarcinaceae. Different methanogens belongs to the different family according to their morphological and physiological characteristic nature. Some methanogens can tolerate low temperature while other can tolerate high salinity. Hence, the habit of the methanogen differs according to their nature of substrate utilization. *Methanolobus*, *Methanococcoides and Methanosarcina acetivorans* are the marine methylotrophic genera (Jones et al., 1987). Additional isolates have also been obtained from extreme environments such as geothermal springs. *Methanobacterium thermoautotrophicum* and *Methanothermus fervidus*

have been isolated from terrestrial hot spring waters (Zeikus et al., 1980) while Acetotrophic methanogens are less frequently found in hot springs.

2.9 Electromethanogenesis

The process of methane generation from bioelectrochemical carbon dioxide reduction with biocathode containing methanogens microbial electrolysis cells is known as electromethanogenesis. Methane generation was found to proceed on plain carbon cathodes, polarized at potentials more negative than -650 mV, in the presence of a hydrogenophilic methanogenic culture (Villano et al., 2010).

Methanogenesis is the terminal step in carbon flow in many anaerobic habitats, including marine and freshwater sediments, marshes and swamps, flooded soils, bogs, geothermal habitats, and animal gastrointestinal tracts. It has been reported that the generation of methane in the cathode of microbial electrochemical cells often coincides with the production of hydrogen (Cusick et al., 2011). Based on thermodynamic calculations, methane could also be produced electrochemically through carbon dioxide reduction with supply of 0.169 V under standard conditions, or -0.244 V under more biologically relevant conditions at a pH 7, by the reaction:

$CO_2 + 8H^+ + 8e^- \longrightarrow CH_4 + 2H_2O$ (Verstraete & Rabaey, 2006)

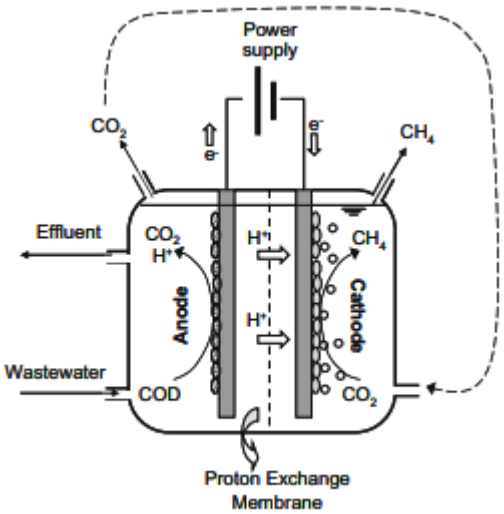


Figure 2. 4: Methane gas production from bioelectrochemical carbon dioxide reduction (Villano et al., 2010)

2.10 Advantages of anaerobic digestion

2.10.1 Environmental benefits

Controlled anaerobic digestion of organic material can results in environmental beneficial by containing the decomposition processes which prevent potentially damaging methane from entering the atmosphere, and subsequent burning of the methane gas will release carbon-neutral carbon dioxide back to the carbon cycle. Biogas production is a competent way of managing organic waste that can provide an environmentally sound and economically sustainable solution by reducing carbon dioxide to methane gas (Cecchi & Cavinato, 2015). Around 18% global warming is thought to be caused by anthropogenically derived methane emissions and both CO_2 and CH_4 are potent of green house gases (GHG) (Viéitez & Ghosh, 1999). Hence anaerobic digestion helps to recycle the carbon cycle and reduces emission of GHG in the environment by providing methane gas as byproduct.

2.10.2 Medical benefits

According to Bendixen, (1994) animal digested contain large group of pathogens which can pollute the environment as well as human health is also in great risk. To control this pathogen, anaerobic digestion at mesophilic and thermophilic condition can be useful to reduce the growth of pathogen. The animal wastes usually contain E. coli and Enterococci and intestinal parasites which can be harmful for the health of human mankind if disposal of the wastes are not done properly hence anaerobic digestion can inhibit the growth of these harmful pathogens (Larsen et al., 1994).

2.10.3 Digested as fertilizer

Biogas is discrete technology because it has potentials of using organic material to generate both energy and superior nutrient compost or digestate as fertilizer (Chibueze et al., 2017). The slurry produced after digestion is an improved fertilizer in term of its availability to plants (Tafdrup, 1995)

2.10.4 Energy production

Anaerobic digestion is also known as waste-to-energy technology and is widely used in the digestion of different organic wastes, for example: organic material of municipal solid waste, sewage sludge, food waste, animal manure, etc (Li et al., 2009).

2.11 Enhancement method of anaerobic digestion

Biogas production from anaerobic digestion can be done by various methods like anaerobic co- digestion of different substrate, pretreatment of substrate before digestion, two stage digestion of the substrate.

2.11.1 Anaerobic co-digestion (Acod)

Animal manures content less carbon (C) content than other organic wastes. So, to fulfill the carbon content, nowadays anaerobic co-digestion process is becoming popular among many people. This method ensure a biogas production safeguarding the economic sustainability of the production (Hamelin et al., 2011). Besides this, Acod further benefit by various method like dilution of the potential toxic compounds eventually present in any of the co-substrates involved, adjustment of the moisture content and pH, supply of the necessary buffer capacity to the mixture, increase of the biodegradable material content, widening the range of bacterial strains taking part in the process.

2.11.2 Pretreatment

Animal manure is complex organic molecule, which is compose of cellulose hemicelluloses, lignin etc. Due to this, it is hard to digest by microorganism during digestion process. Hence, for the compete digestion of cattle manure, microorganism take longer time. Due to this long hydraulic retention time, biogas production becomes costly in term of economy. The goal of the “pretreatment” is to facilitate the digestion process by removing these barriers and to make the organic content of the substrate easily accessible and utilizable by the microbial community. There have been several approaches toward pretreatment, which can be classified as physical, chemical, physicochemical, and biological (Taherzadeh & Jeihanipour, 2012.)

2.11.3 Two stage digestion

According to Comino and co-worker (2009) two-stage anaerobic digestion could provide great advantages over the single-stage digestion due to a more rapid and more stable treatment achieved. Two stage digestion processes is divided in two parts. Firstly, the substrate is digested for hydrolysis and acidogenesis phase in 1 chamber. After the digestion process completion the slurry is transferred in second chamber for acetogenesia and methanogenesis phase. Blonskaja and co-worker (2003) used a two-stage system for digestion to process distillery waste and observed a higher growth rate of methanogenic populations with increased biogas production. In practice however, it is argued that the two-stage digestion

has not been able to validate its claimed advantages in the market, and the added benefits in increasing the rate of hydrolysis and methanization have not been confirmed (Pohland & Ghosh, 1971).

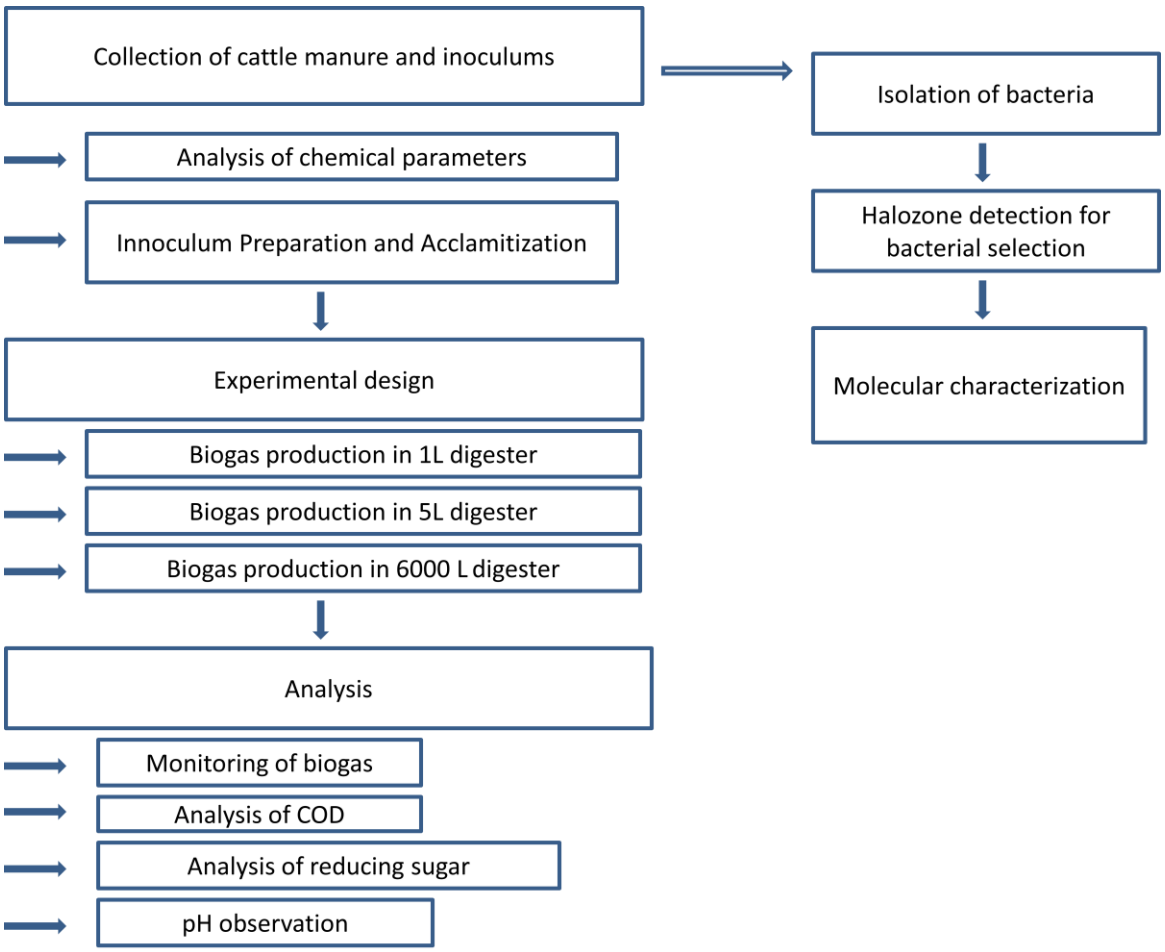
2.12 Problem

Anaerobic digestion is the complex method, in which four different stage need to complete for production of methane gas. During this process of digestion, various obstacles can hinder the process. Among them, hydraulic retention time, temperature, pH, micro flora of the system, ammonium accumulation etc are important problem faced by different part of the world. However, I had discussed some major problem faced in our country, Nepal.

- The hydraulic retention time of substrate play important role in energy production from complex organic. To fulfill the energy demand of the future generation commercialization of this method should be done. Due to the high retention time of substrate, cost for energy production also increased. According to Yadvika and co-worker (2004) hydraulic retention time is increased during winter season and as a result biogas production is decreased. Therefore, more efforts are need to be done in order to remove various limitations.
- As the temperature of environment drop during winter season, production of biogas is also decrease. According to Kalia and Singh (1996) stated that, biogas production reduced from 1700 L/day in May–July to about 99l/d in January–February. So for the winter season, better enhancing technology should be discovered which can benefit the peasant of rural area even in winter season.

3 MATERIALS AND METHODS

3.1 Flow chart of materials and methods



3.2 Laboratory setting

This study was conducted in the laboratory of Central Department of Biotechnology, Tribhuvan University and National Academy of Science and Technology (NAST). All the experiments were done in triplicate.

3.3 Substrate and inoculum collection

In this research, cow dung was used as substrate for biogas production. Fresh and formed cow dung was collected from Shailendra Koirala's cow shed, Tribhuvan University premises, Kirtipur and Swastika agro and animal farm, Dhapasi, since June to August, 2019. Cows were fed with grass, hay, grains and legumes.The inoculum for digestion experiment was collected from NAST

anaerobic digester which was in full operation. The digester may contain high amount of mature isolates which can help in enhancement of biogas in laboratory experiment.

3.4 Analysis of chemical parameter

Cow dung was subjected to analysis of chemical parameters like pH, reducing sugar, total soluble solid (TSS), volatile soluble solid (VSS), chemical oxygen demand (COD). The phosphorus content was determined according to standard methods for the Examination of Water and Wastewater (APHA, 1998). Trace elements (lead, iron, copper, zinc, manganese and nickel) which can affect the growth of microbial biota in the experiment were also analyzed using AAS method after digestion.

3.4.1 Determination of total suspended solid and volatile suspended solids (Valo, et al., 2004)

To determine 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 overnight drying, the sample was again weighed and TSS was determined according to the formula given in the Appendix I. To determine volatile suspended solids (VSS) 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, TU, Nepal (RECAST).

3.4.2 Determination of reducing sugar

The stock solution of glucose was prepared (1000 µg/mL). Glucose concentration ranging from 10 µg/mL to 500 µg/mL was prepared from the stock. A 0.02 mL of sample was taken along with 0.18 mL of water to make final volume of 200 µL. Then 200 µL of each standard and sample was taken into which 0.2 mL DNS reagent was added. After the addition of DNS the solution was kept in boiling water bath for 10 minutes. It was allowed to cool down and 2 mL of distilled water was added. The absorbance was read in 540 nm for both sample and the standard (Lorenz, et al., 1961). Same procedure was followed for the sample from the digester as in the standard curve preparation with appropriate ratio and reducing sugar was determined by using the linear regression equation of the standard.

3.4.3 Determination of chemical oxygen demand

The phthalate stock solution (1000 mg/L) was prepared in 50-mL volumetric flask. Then series of phthalate working solution of concentration 20 mg/L, 50 mg/L 100 mg/L, 200 mg/L, 600 mg/L and 900 mg/L were prepared by pipetting suitable volumes from stock solution. Then 10 mL of each working solutions were transferred in different culture tube with proper lebelling followed by 6 mL of digestion solution (Appendix-I) were added to each of the working solution and mixed thoroughly. Then, 14 mL of catalyst solution was added to each of the working solution and it was capped tightly and was shaken to mix the layers. The culture tubes were placed in an oven at 150 °C for 2 hours. The tubes were cooled and precipitate was allowed to settle. Background correction was performed with the blank solution (without sample) and the absorbance of the solutions were taken at 600 nm using the spectrophotometer from Shimaju (Henze et al., 2002).

Finally 2 mL of the sample was taken into a culture tube from the digester 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.

3.4.4 Digestion of sample in flask with H_2SO_4-salicyclic acid-H_2O_2

The cow dung sample was weighed 0.6g in digital weighing balance and was transferred to a 50mL volumetric flask. 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 were introduced and swirled carefully until the sample was moistened. It was then allowed to stand overnight. In addition, two blank digestions were 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 placed 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 next 10 min which led to appearance of white vapors. The process was repeated until the digest turned colorless. The flask was removed from the plate and kept at room temperature until it cooled down. About 10 mL of water was added and mixed; swirled until most of the precipitate had dissolved. The mark was made up with distilled water, mixed well. The digest was filtered to remove any SiO_2 that will otherwise dissolve gradually and then interfere in the determinations. The calibration solution for the 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 had 0.8 M H_2SO_4 (Temminghoff & Houba, 2004)

3.4.4.1 Determination of iron, copper, zinc, nickel, manganese and lead

The prepared digested solution was sent to National Academy of Science and Technology (NAST) to estimate the amount of a Fe, Cu, Zn, Ni, Mn and Pb.

3.4.4.2 Determination of phosphorus

Standard curve of phosphorus

A series of working solution ranging from 0.01 mg/L, 0.03 mg/L, 0.05 mg/L, 0.1 mg/L, 0.2 mg/L, 0.3 mg/L, 0.4 mg/L, 0.5 mg/L of phosphorus was prepared by suitable volume of phosphorus stock solution. The standard graph for the phosphorus determination is shown in Appendix II.

Determination of phosphorus

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

3.5 Enrichment of the collected cowdung sample in methanogen bacterium II medium (MMII)

Enrichment of culture for the methane production was carried out providing sugars through DSMZ 825 methanogen enhancement media (Appendix-I) in anaerobic state in air tight bottles. The anaerobic state was created by bubbling in N_2 gas in the jar. The culture was allowed to acclimatize for 1 month. Exactly 25 gram of cow dung sample from digester from NAST was mixed in 5000ml of the MMII media (Atlas, 2005)

Preparation of the inoculums

The culture after 2-3 subcultures was used as inoculums for the electromethanogenesis in the MEC. The inoculums were incubated for 7 days.

3.6 Scale up process

Digestion of sample was carried out in the different 1L, 5L and 6000 L anaerobic digesters. The digestions in 1L and 5L were done at Central Department of Biotechnology as batch digester whereas the digestion in 6000 L digester continuous digestion method was done in NAST. Daily about 20 Kg of sample with equal volume of water was added in the digester for 42 days. The water and cow dung concentration in the digester was in the ratio 1:1 (v/w) in all digester (Abubakar & Ismail, 2012).

3.7 Experimental design

A pair of graphite electrode (Nippon Electrode Co Ltd, Japan) of dimension 10cm×3cm×1cm was inserted into the 5 L reagent bottle to form an electric biological reactor referred as Microbial Electrochemical Cell (MEC) anaerobic reactor. This process was carried out in both working volumes of 1 L and 5 L, with supply of external 2 V. This amount of voltage was chosen from literature (Poudyal, 2018). The control for the experiment were conducted in common reactor which was same as other reactor but without applying voltage. Oxygen was removed from by bubbling nitrogen gas for 10 min before digestion. Wax was applied across the caps (cork) of reactor in which surgical glass pipes were inserted through the caps .The gas collection was done by downward displacement of water and 0.1 M KOH solution. The biogas in measuring cylinder was measured. The reactors were operated in batch method for 7 days. The experiments were operated at 15 ^{0}C and at room temperature. For the 6000 L digester, the digestion process was carried out in continuous process. About 20 kg of cow dung was poured every day at the same time and mixed well. The operation was carried out for 42 days at the open air temperature. The collected gas was used for the boiling of water which was measured by gas meter. Unfortunately the MEC method was not applied for digestion due to some technical problem.

3.8 Monitoring methane production with and without supply of electricity

Gas analyzer was used to monitor methane production rate from total biogas production by 6000 L digester. The gas analyzer can detect the possible various types of gas like oxygen, H_2S, CO_2, CH_4 etc. In case of 1 L and 5 L digester, monitoring was done by downward displacement of KOH solution.

3.9 Sampling from the digester and analysis of COD, reducing sugar, pH and biogas

Two milliliters of semisolid sample was taken out every day from the 1 L and 5 L digesters incase of 6000 L digester, each sample was taken after 7 days apart for the analysis of chemical oxygen demand (COD) and reducing sugar. The pH of the sample was measured using pH meter daily after sampling and noted. Total biogas production measurement was done by downward displacement of water only whereas methane concentration was determined by the 0.1 N KOH displacements.

3.10 Isolation and identification of bacteria

3.10.1 Isolation of methanogen

Hundred micro liter of the inoculums prepared in DSMZ 825 methanogen enhancement media were spread on the agar plate enriched with DSMZ 825 methanogen enhancement media. The plate was sealed with parafilm, labeled and kept in the anaerobic jar and placed in 37 °C kept in incubator for 24 hrs. 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).

3.10.2 Isolation of cellulose degrading bacteria

For the isolation of cellose degrading organism carboxymethyl cellulose (10g/L) was added to the DSMZ 825 methanogen enhancement media without glucose. After the incubation the colonies were transferred for the pure culture. These pure culture were test for the cellulase screening process from which higher rate of cellulose degrading organism was chosen for the further study (Sasaki, et al., 2012)

3.10.3 Genomic DNA extraction of screened organism

Around 1.5 mL of the culture was centrifuged and the supernatant was removed. The pellet was resuspended in 570 µL of TE buffer and vortex. Then 30 µL of 10% SDS was added along with 3 µL of 20m g/mL proteinase k solution. After proper mixing, it was incubated for 1 hour at 37 ^{0}C. After the incubation 100µl of 5 M NaCl was mixed. Later 80 µL of CTAB (10%) was added. The solution was

incubated for 10 min at 65 ^{0}C in a water bath. After incubation equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed well. Then it was centrifuged at 12,000 rpm for 5 min and aqueous solution was transferred into new tube. The interface was kept undisturbed. Next equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed properly. The tube was then centrifuged at the max speed (14000 rpm) for 5 min and the supernatant was transferred to a new tube. The process of extraction using chloroform: isoamyl alcohol was repeated again. To the supernatant extracted 0.6 ml of isopropanol was added and mixed well until the precipitation of DNA was observed. The solution was centrifuged to remove the isopropanol. The pellet was washed with 1 mL of 70% ethanol and centrifuged at 12000 rpm for 2 min. The supernatant was discarded and let pellet to dry at the room temperature. The pellet was resuspended in 50 µL of TE buffer and kept on -20 ^{0}C for further use.

3.10.4 PCR amplification of gDNA

The gDNA was amplified by using 16s rRNA primers from Geneamp kit (U.S. Biochemical's, Cleveland, Ohio). The sequence of the forward primer and reverse primer were 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-ACGGCTACCTTGTTACGACTT-3' respectively (Weisburg at al.,1991). 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	1
3.	Reverse Primer	1
4.	Nuclease Free Water	4.375
5.	Template	2.5
6.	BSA	2.5
7.	$Mgcl_2$	1
8.	Taq polymerase	0.125

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

Then after mixing the component (1, 2, 3, 4, 6, 7and 8) also added and performing the short spin, 2.5 µL templates 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	94°C	4 min
2.	Denaturation	94°C	40 sec
3.	Annealing	55°C	40sec
4.	Extention	72°C	1 min 10 sec
5.	Repeat step 2-4 (35×)		
6.	Final extension	72°C	7 min
7.	Hold	4°C	Storage

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.

3.10.5 Sequence analysis of the amplicons

The amplicons were sent to National Academy of Science and Technology, Khumaltar for the sequencing. The sequencing was only done by the forward primer for single strand DNA.

3.10.6 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 raw sequences was 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 primer end was delineated from the alignment matrix. Primer excluded barcode sequences were exported for further analysis.

3.10.7 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.

4 RESULTS AND DISCUSSION

4.1 Determination of chemical parameters of cow dung substrate

Chemical components/parameters which can affect the growth of different microbial biota in the fermentation process were analyzed. Components like phosphorus, iron, copper, lead, zinc, manganese; and parameters like TSS, VSS and moisture content was measured by using dry cow dung sample while other parameter COD, reducing sugar and pH was done by diluted sample. The measured chemical components in cow dung were given in Table (4.1).

Table 4. 1: Chemical parameters/component of collected cow dung substrate

Chemical parameters/components	Concentration
Chemical oxygen demand (mg/g)	4.85±0.25
Total phosphorus (mg/g)	2.35±0.012
Soluble reducing sugar (µg/g)	6.54±0.16
Iron (mg/g)	1.5
Copper (µg/g)	2.72
Nickel (mg/g)	0.0
Zinc (µg/g)	47.6
Manganese (mg/g)	0.357
Lead (µg/g)	0.85
pH	6.98±0.31
Total suspended solid (TSS)	17.46±1.5%
Volatile soluble sugar (VSS)	3.11±0.34%
Moisture content	82.52±1.5%

The value of TSS and VSS were also determined which was found to be 17.46±1.5% and 3.11±0.34% respectively. The concentration of COD and

phosphorous was found to be 4.85±0.25 mg/g and 2.35±0.012 mg/g whereas the soluble reducing sugar concentration was 6.542 µg/g. The analysis of trace element showed that the collected sample contain 1.5 mg/g iron, 47.6 µg/g zinc, 0.375 mg/g manganese and 0.85 µg/g lead, 2.72 µg/g copper. The pH and moisture contain of the cow dung sample was found to be 6.98±0.31 and 82.52±1.5% respectively.

The environmental parameter analyses were performed to get the biophysical and chemical status of the substrate. As various researches stated that the nature of the substrate determines the necessity if various up gradation is required or not. The nature of the substrate directly affects the microbial biomass although the lack of process stability, low loading rates, slow recovery after failure and other specific requirements like TSS, VSS, trace elements and nutrients from waste composition are some of the other limitations associated with it (Van den Berg, 1983; Yadvika et al., 2004). According to Tomlinson et al., (1996) the value of the TSS and VSS could be in the range of 15%-20% and 10% - 15% respectively. Several researches have proved that TSS data is critical in order to determine the operational behavior of waste treatment system (Zehnder & Gujer, 1983). The presence of the P in the cow dung sample signifies the importance as fertilizer in crop. According to Lindorfer and co-worker (2012), the concentration of P and Fe is in the maximum level and other elements were found to be in minimum level.

4.2 Biogas production at room temperature and at 15°C in 1 L, 5 L and 6000 L digesters

In this research, biogas production was done at two different temperatures. One setup was maintained at room temperature (23-29°C) during June to August while, other was maintained at 15°C. Also, three sets of digesters (1 L, 5 L and 6000 L) were used for the production at each temperature. For the production of biogas external voltage of 2 V was supplied at 1 L and 5 L digester. However, 6000 L digester setup could not be supplied with external voltage. The efficiency of biogas production was monitored by analyzing change in chemical oxygen demand (COD), soluble reducing sugar, gas produced and pH of the solution during the digestion process. The COD, reducing sugar, biogas and pH of the solution in the test setup (2 V supplied) was compared with the control setup, in case of 1 L and 5 L digester.

4.2.1 Chemical oxygen demand of different digesters during biogas production

During the biogas production process, COD was measured at 24 h interval for 7 days in 1 L and 5 L digester while in 6000 L digester sample was collected at 7 days interval. The measured COD at room temperature is given in Figure (4.1). Figure (4.1A) shows the measured COD during the digestion process in 1 L digester and Figure (4.1B) show COD in 5 L digester. The standard curves for the COD analysis is given in APPENDIX (II).

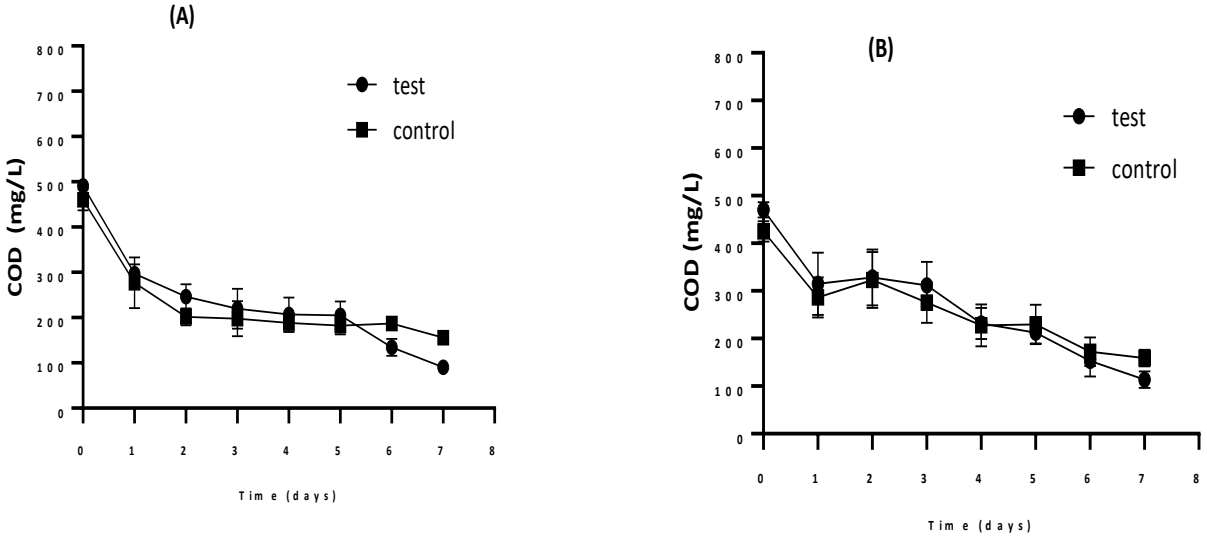
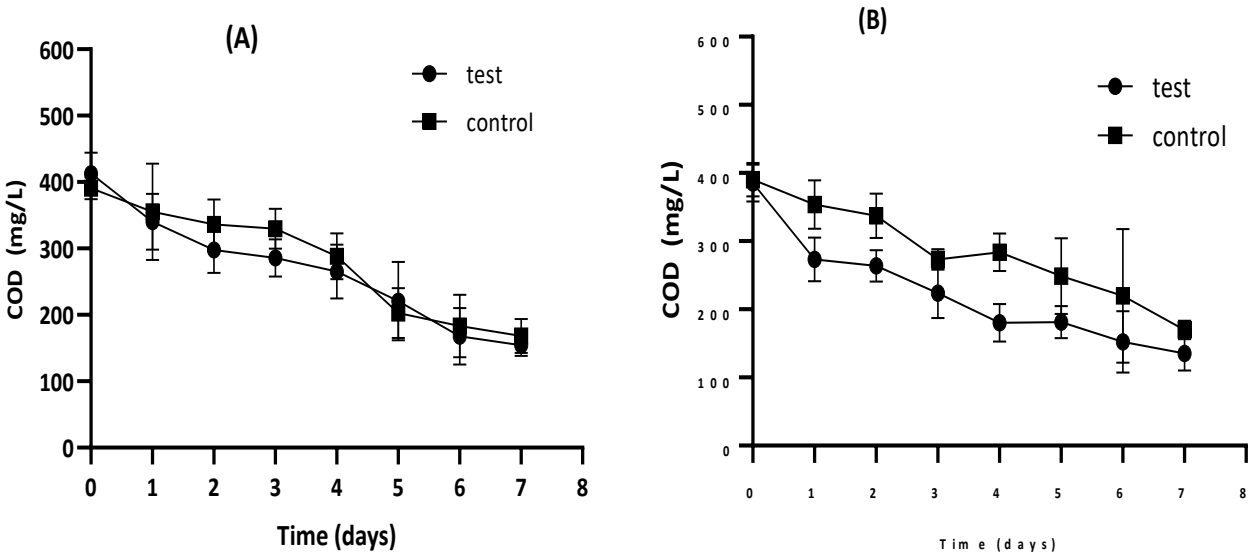


Figure 4. 1: Chemical oxygen demand (COD) measured at room temperature in different digesters, (A) in 1 L digester supplied with 2 V, (B) in 5 L digester supplied with 2 V.

*Note: test is a setup with 2 V external voltage control setup is setup without external voltage

Figure (4.1A), showed initial concentration of COD was 497.39±10.88 mg/L and 426.833 ±23.82 mg/L in test and control respectively in 1 L digester. After 7 days of digestion, final COD was 90.27±12.98 mg/L in test digester while the COD of control digester was reduced to 155.583±7.63 mg/L. Similarly, Figure (4.1B), showed initial concentration of COD was 470.166±16.31 mg/L and 426.833±21.26 mg/L in test and control respectively in 5 L digester. After 7 days of digestion, final COD was reduced to 135.166±17.63 mg/L in test digester and the COD of control was found to be reduced to 169.75±17.44 mg/L.

During the biogas production process, the measured COD at 15^{o}C is given in Figure (4.2). Figure (4.2A), shows the measured COD during the digestion process in 1 L digester and Figure (4.2B), gives the measured COD in 5 L digester. It was difficult to maintain 15 ^{o}C and supply external voltage for 6000 L digester. So, only 1 L and 5 L setup were analyzed at 15 ^{o}C.



.

Figure 4. 2: Chemical oxygen demand (COD) measured at 15 °C in different digester, (A) in 1 L digester supplied with external 2 V, (B) in 5 L digester supplied with external 2 V.

*Note: test is a setup with 2 V external voltage control setup is setup without external voltage

Figure (4.2A), showed the initial COD was 412.666±31.77 mg/L and 432.5±24.56 mg/L in test and control digester respectively at 15 °C in 1 L digester. After 7 days of digestion, the final COD concentration was reduced to 154.333±16.26 mg/L in test digester while in control digester final COD was reduced to 173.416±25.81 mg/L. Similarly, Figure (4.2B) showed the initial COD was 385.206±27.23 mg/L and 390.166±16.78 mg/L in test and control digester respectively at 15 °C in 5 L digester. After 7 days of digestion, the final COD concentration was reduced to 135.166±16.26 mg/L in test digester while in control digester final COD was reduced to 169.75±25.81 mg/L.

The comparison of COD reduction among different digester volume and type, during the production process is shown in Table (4.2). The table showed

reduction in COD after 2 V supplied, is higher compared to the control set at both 15°C and RT setup and in both 1 L and 5 L digester type. At 15°C, the COD reduction percentage was found to be 62.61±0.19% and 64.91±0.17% in 1L and 5L digester respectively with 2 V whereas, the reduction percentage of COD in control set at the both temperature setup were found to be 59.9±0.19% and 56.53±0.19% in 1L and 5L digester respectively. This concludes that reduction of COD is increased with application of 2 V by about 2.7% and 8.3% in 1L and 5L digester respectively compared to control setup. Also, at RT the COD reduction was increased with application of 2 V externally in both digester volume types. At RT, the reduction of COD was found to be 81.96±0.06% and 71.25±0.1% respectively which is about 11% and 18.42% reduction in 1L and 5L digester.

Table 4. 2: Comparison of COD during digestion process among different digesters. (Summary)

Digester volume (L)	Digester type	temperature	Initial COD (mg/L)	Final COD (mg/L)	COD reduction (%)
1	test	15°C	412.666±31.77	154.333±16.26	62.61±0.19
1	control	15°C	432.5±24.56	173.416±25.81	59.9±0.19
1	test	RT	497.39±10.88	90.27±12.98	81.96±0.06
1	Control	RT	426.833±23.82	155.583±7.63	63.54±0.12
5	test	15°C	385.206±27.23	135.166±16.26	64.91±0.17
5	control	15°C	390.583±16.78	169.75±25.81	56.53±0.19
5	test	RT	470.166±16.31	135.166±17.63	71.25±0.1
5	control	RT	426.833±21.26	169.75±17.44	60.23±0.15

*Note: test is a setup for 2 V externally supplied in digester and control setup is without external voltage supply.

The analysis of COD can be done to get idea about the presence of soluble protein, soluble polysaccharides and VFAs. Higher the concentration of these materials higher will be the concentration of COD. The generation of electricity during the chemical reaction can coupled to a fall in chemical oxygen demand from over 1,700 mg/L down to 50 mg/L (Kim et al., 2004). Hence the introduction of small amount of electricity can enhance the reduction in COD concentration. This research shows that, at 15°C digester reduction of COD was found to be

62.61% and 64.91% in 1L and 5L digester respectively provided with 2 V of externally whereas the reduction of COD in control of same temperature was found to be 56.53±0.19% and 59.9±0.19%. This shows that the reduction of COD reduction was 2.7% and 8.3% greater in 1L and 5L digester respectively. As for the RT the reduction of COD is even higher in compared to the low temperature digester. In 1L and 5L digester at RT the reduction of COD was found to be 81.96±0.06% and 71.25±0.1% respectively which is around 11% and 18.43% higher in comparison to controlled digester. According to Castrillón and co-workers (2002) stated the average COD reduction efficiency from cattle manure is 51-79%. According to Sakalis et al.,(2005), 94% of dye can be removed using a pilot plant electrochemical reactor for textile wastewater treatment. At pH 7, about 82% of chemical oxygen demand (COD) was solubilized and the maximum volatile fatty acid (VFA) concentration of 36 g/L was achieved on the fourth day (Hamelin et al., 2010). Hence, these results suggest that, efficient COD removal can be done even at 15 °C with the supply of 2 V externally.

4.2.2 Soluble reducing sugar in different digesters during biogas production

During the biogas production process, soluble reducing sugar was measured at 24 h interval for 7 days in 1 L and 5 L digester while in 6000 L digester sample was collected in 7 days interval. The measured reducing sugar at room temperature is given in Figure (4.3). Figure (4.3A), shows the measured reducing sugar during the digestion process in 1 L digester and Figure (4.3B), shows reducing sugar in 5 L digester. The standard curves for the reducing sugar analysis is given in APPENDIX (II).

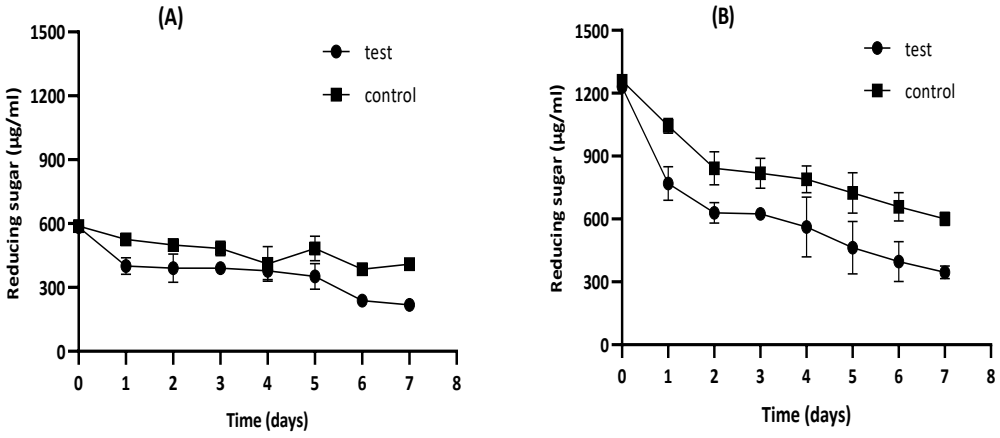


Figure 4. 3: Soluble reducing sugar measured at room temperature in different digesters, (A) in 1 L digester supplied with 2 V , (B) in 5 L digester supplied with 2 V.

*Note: test is a setup with 2 V external voltage control setup is setup without external voltage

Figure (4.3A) showed initial concentration of soluble reducing sugar was 582.5±21.21 µg/L and 588.33±27.1 µg/L in test and control respectively in 1 L digester. After 7 days of digestion, reducing sugar was reduced to 217.79±25.92 µg/L, in test digester, and in control set it was reduced to 409.16±5.18 µg/L. Also, Figure (4.3B) showed initial concentration of reducing sugar was 1229.55±10.71 µg/L and 1254.16±14.43 µg /L in test and control respectively in 5 L digester. After 7 days of digestion, reducing sugar was reduced to 345.83±30.13 µg/L in test digester, and the reducing sugar of control was reduced to 600.833±31.22 µg/L.

During the biogas production process, soluble reducing sugar was measured at 24 h interval for 7 days in 1 L and 5 L digester while in 6000 L, digester sample was collected in 7 days interval. The measured reducing sugar at 15 °C is given in Figure (4.4). Figure (4.4A) shows the measured reducing sugar during the digestion process in 1 L digester and Figure (4.4B) shows reducing sugar in 5 L digester.

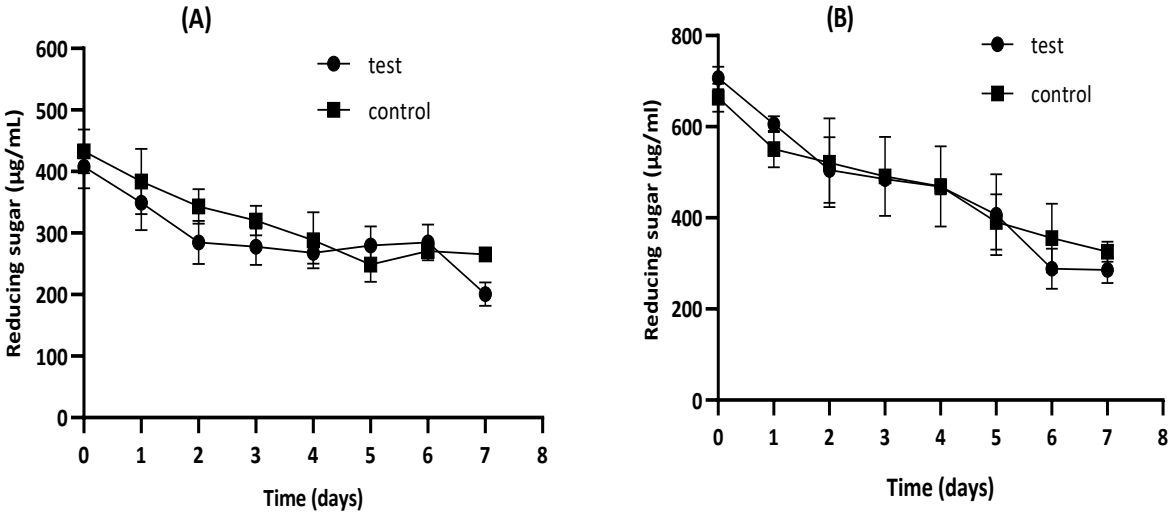


Figure 4. 4: Soluble reducing sugar measured at 15 °C in different digester, (A) in 1 L digester supplied with 2 V, (B) in 5 L digester supplied with 2 V

*Note: test is a setup with 2 V external voltage control setup is setup without external voltage

Figure (4.4A) showed the initial reducing sugar was 447.499±35.19 µg/L and 390.583±35.62 µg/L in test and control digester respectively at 15 °C in 1 L digester. After 7 days of digestion, the final reducing sugar concentration was

reduced to 154.583±19.22 µg/L and 265.27±9.76 µg/L in test and control digester setup respectively. Similarly, Figure (4.4B) showed the initial reducing sugar was 706.94±25.01 µg/L and 663.61±30.74 µg/L in test and control digester respectively at 15 °C in 5 L digester. After 7 days of digestion, the reducing sugar concentration was reduced to 285.38±29.27 µg/L and 325.83±22.42 µg/L in test digester control digester respectively.

Table 4. 3: Comparison of reduction of reducing sugar in different digesters

(Summary)

Digester volume (L)	Digester type	temperature	Initial reducing sugar (mg/L)	Final reducing sugar (mg/L)	Reducing sugar (%)
1	test	15°C	447.499±35.19	154.583±19.22	65.45±0.19
1	control	15°C	390.583±35.62	265.27±9.76	32.08±0.36
1	test	RT	582.5±21.21	217.79±25.92	62.66±0.13
1	control	RT	588.33±27.1	409.16±5.18	30.45±0.18
5	test	15°C	706.94±25.01	285.38±29.27	59.63±0.13
5	control	15°C	663.61±30.74	325.83±22.42	50.9±0.16
5	test	RT	1229.55±10.71	345.83±30.13	71.87±0.05
5	control	RT	1254.16±14.43	600.833±31.22	52.09±0.07

*Note: test is a setup for 2 V externally supplied in digester and control setup is without external voltage supply

The Table (4.3), showed the reduction in soluble reducing sugar is higher in test digester compared with the control digester. The reducing sugar at 15 °C in test digester was reduced by 65.45±0.19% and 59.63±0.13% in 1 L and 5 L setups respectively. However, the reduction of reducing sugar in control digester was 32.08±0.36% and 50.9±0.16% at same temperature of 15°C. The difference in reduction percentage is 33% in 1L digester and 9% in 5 L digester. Likewise, the reduction of soluble reducing sugar in RT of 1 L and 5 L were 62.66±0.13% and 71.87±0.05% in test and control respectively which is 32% higher in 1L and 19% higher in 5L digester with compared to the control digester.

The degradation of lignocellulosic biomass produces soluble sugar which can be further used as a substrate for biogas production with the help of microbial consortium (Andri & Sriariyanun, 2017). The reduction of the soluble reducing sugar at both 15°C and room temperature were observed. The reduction was increased with the supply of external voltage. The acidogenic bacteria utilized the soluble sugars as a sub layer and produced another sub layer for the next bacteria groups. Acetogenic bacteria utilize this sub layer as substrate and give rise to acetate, hydrogen, and CO_2. The product from different stages of digestion was utilize by hydrogenotropic and acetolastic methanogenic bacteria to produce methane (Letters, 2014). According to Andri & Sriariyanun, (2017) the degradation of lignocellulosic biomass produces soluble sugar which can be further use as a substrate to biogas production with the help of microbial consortium.

4.2.3 Biogas production at room temperature (RT) in different digesters

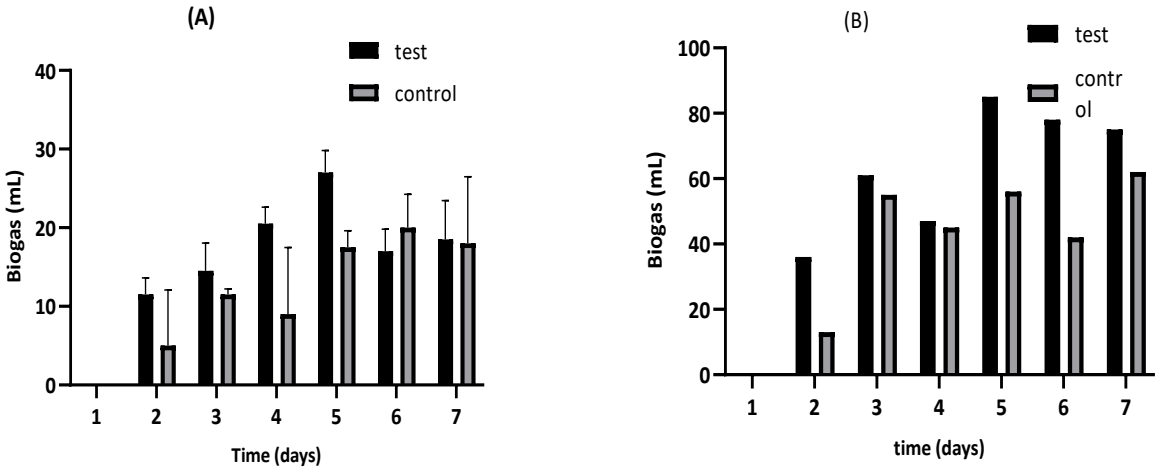


Figure 4. 5: Biogas productions at RT in different digesters, (A) biogas production in 1 L digester, (B) biogas production in 5 L digester.

*Note: test is a setup with 2 V external voltage control setup is setup without external voltage

During the biogas production process, biogas production was measured at 24 h interval for 7 days in 1 L and 5 L digester while in 6000 L digester, biogas production was measured for 42 days interval. The biogas production at room temperature is given in Figure (4.5). Figure (4.5A) shows the measured biogas during the digestion process in 1 L digester and Figure (4.5B) shows reducing sugar in 5 L digester.

Biogas production was measured by the downward displacement of water in 1 L and 5 L digester while in 6000 L digester, pressure gauze was used. The unit of biogas production in 1 L and 5 L digester was mL however in 6000 L biogas was measured in K Pa unit. The digesters 1 L and 5 L was operated in batch method while 6000 L digester was operated in continuous method with the daily addition of 20 kg of cow dung after proper mixing with 20 L of water.

Figure (4.5A) showed biogas production at room temperature in 1 L digester setup was an average of 27±2.82 mL biogas was produced with an application of 2 V externally. At same condition, biogas production in controlled digester was an average of 17.5±2.12 mL. Similarly, Figure (4.5B) showed biogas production at 5 L digester in which an average of 85 mL biogas was produced with an application of 2 V externally. At same conditions, biogas production in controlled digester was an average of 50 mL.

The biogas production at 15 °C temperature is given in Figure (4.6). Figure (4.6A) shows the biogas production during the digestion process in 1 L digester and Figure (4.6B) shows biogas production in 5 L digester setup.

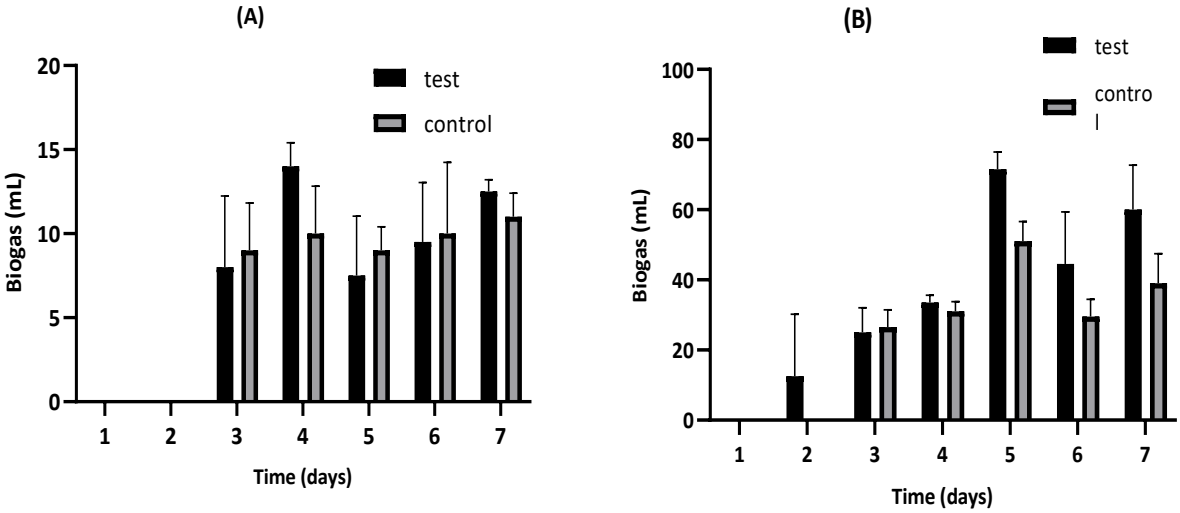


Figure 4. 6: Biogas productions at 15°C in different digesters, (A) 1 L digester with application of 2 V (B) 5 L digester with application of 2 V

*Note: test is a setup with 2 V external voltage control setup is setup without external voltage

Figure (4.6A), showed the biogas production at 15 °C. The highest biogas production was 14±1.41 mL at day 4 in 1 L test digester. At the same conditions, the gas production in controlled digester was 10±2.82 mL. Similarly, Figure (4.6B) showed biogas production at 15 °C. The highest biogas production was 71.5±4.95

mL at day 5 in 5 L test digester. At the same conditions, the biogas production in controlled digester was 51±5.65 mL.

Table 4. 4: Biogas comparison from different digester and temperature (Summary)

Digester volume (L)	Temperature (°C)	Biogas in test digester (ml)	Biogas in control digester (ml)	Biogas enhancement (%)
1	15	14±1.41	10±2.82	28.57±1.06
1	RT	27±2.82	17.5±2.12	35.18±0.52
5	15	71.5±4.95	51±5.65	28.67±0.54
5	RT	85	50	41.17

Table (4.4), shows the biogas enhancement in 1 L and 5 L digester at 15 ^{o}C and room temperature respectively. At 15 ^{o}C, in 1 L and 5 L digester about 28% enhancement of biogas was observed with 2 V external supply. Similarly, enhancement of biogas in 1 L digester and 5 L digester was 35.18±0.52% and 41.17% at room temperature respectively.

Methane production can be done in various range of temperature by the anaerobic digestion process. In which <20°C is psychrophlic digestion, 25°C-40°C mesophiliic digestion, 45°C-60°C thermoplilic digestion (Khalid et al.,2011; Kuruvilla et al.,2014). Zhen and coworker (2015), stated that considerable amount of methane production ratio was increased to around 60 mL/L after pure CO_2 was used in microbial electrochemical cell.

4.2.4 Determination of methane content in biogas from different digester at room temperature.

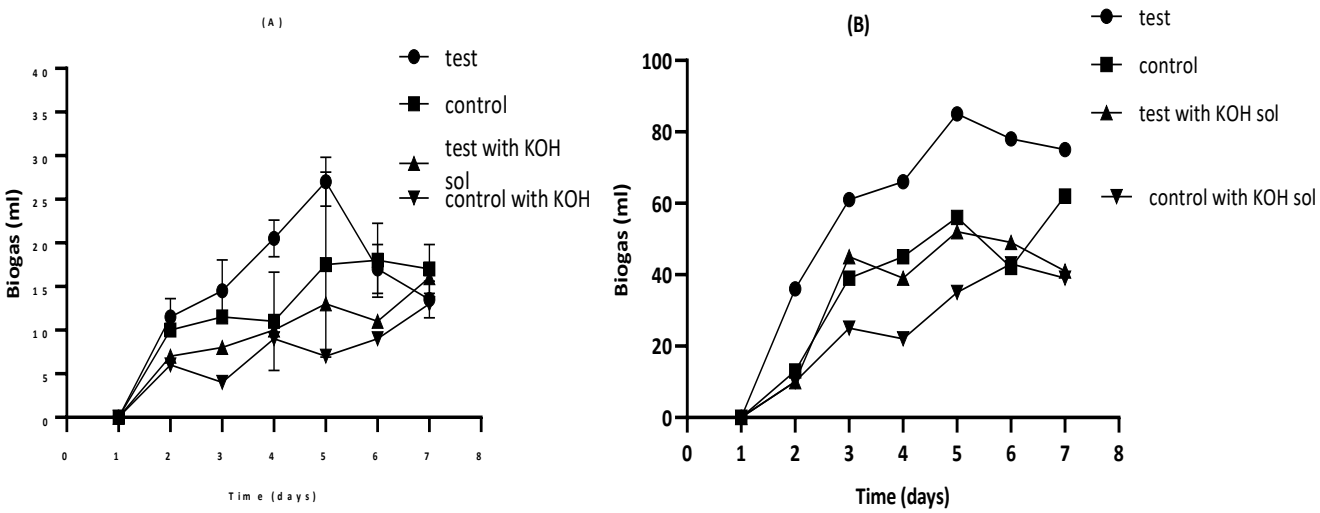


Figure 4. 7: Determination of methane content in biogas from different digester, (A) at 1 L digester and (B) at 5 L digester at room temperature.

p value=0.0321(between test and control)

p value=0.0034 (between test with KOH and control with KOH)

At 95% of level of confidence

*Note: test is a setup with 2 V external voltage control setup is setup without external voltage

Determination of methane content in biogas is done by downward displacement of KOH solution. KOH is alkaline solution which can absorb CO_2 and produce bicarbonate. Carbon dioxide present in biogas produced during digestion process dissolves in alkaline solution but not in water.

Figure (4.7A) shows methane content present in biogas mixture in 1 L digester. The average biogas at day 5 was 27 mL in test digester while 17.5 mL in control setup. At the same conditions, the biogas produced after absorbed by KOH solution was 13 mL in test digester and 7 mL in control set. From this data, it can be analyzed that, carbon dioxide was about 52% and 60% in test and control respectively. Similarly, Figure (4.7B) shows methane content in biogas in 5 L digester. It was determined by comparing with displaced water with displaced KOH solution. The average biogas at day 5 was 85 mL in test digester while 56 mL in control setup. At the same conditions, the biogas production after absorption with KOH solutions was 52 mL in test digester and 35 mL in control digester. This

suggests that, carbon dioxide absorbed was about 39% and 38% in MEC and control set respectively. The remaining gas can be taken as produced methane. At 95% of level of confidence, the graph between test and control give p- value 0.0321. At 95% of level of confidence, the graph between test and using KOH give p-value 0.0034 which are less than 0.05. Thus, the experiment was significant at 95% level of confidence.

Production of methane yield was increased with the use of hybrid GF biocathode to 80.9 mL/L at the potential of 1.4 V after incubation for 24 h having faradiac efficiency (C_E) of 194.4% (Zhen et al., 2015). Due to high yield of biogas production property of GF and it is one of the cheap and readily available in market than other electrode like platinum, nickel etc. made it more convenient to use in this experiment.

4.2.5 Observation of pH in different digester

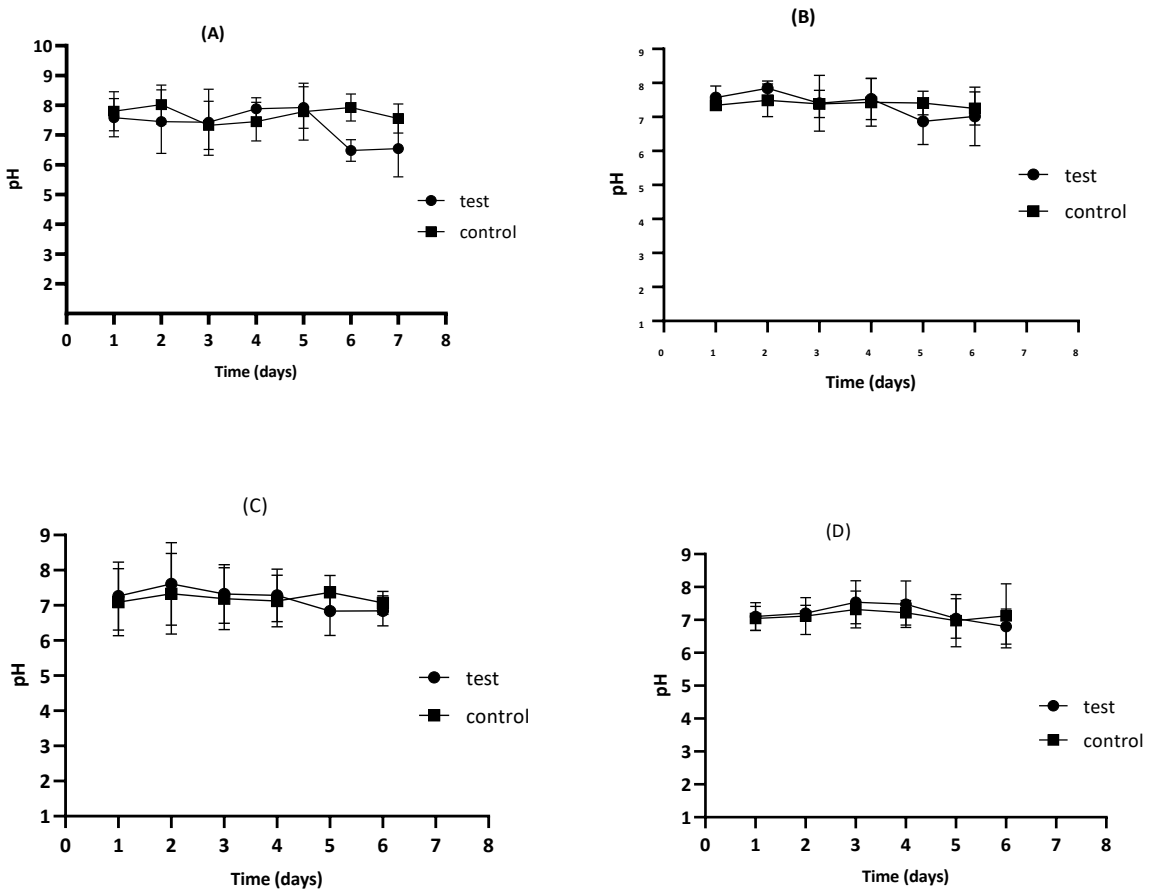


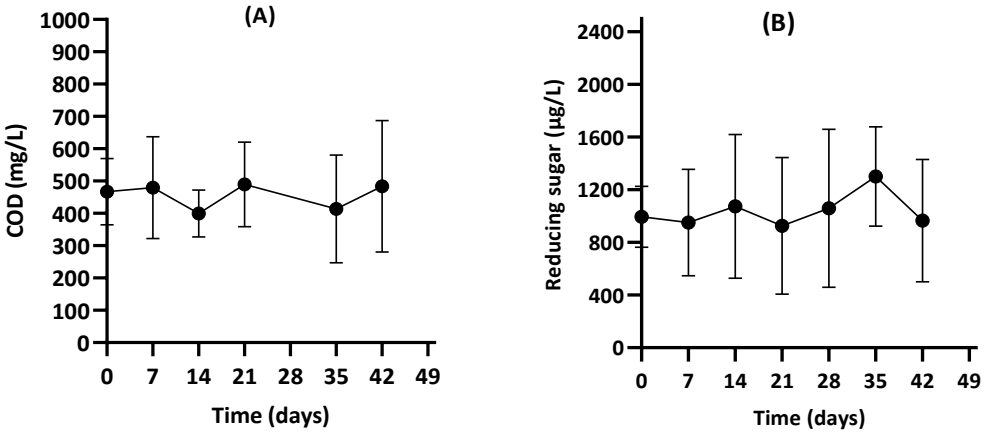
Figure 4. 8: pH observation at different digester, (A) at 15°C in 1 L digester, (B) at RT in 1 L digester, (C) at 15°C in 5 L digester, (D) at RT in 5 L digester

The observation of pH in different digester is given in Figure (4.8), which shows during the digestion process all the reactions were observed in the range of 6-8.5 pH. The digester provided with external 2 V show slightly more alkaline phase than control digester till day 5. After day 5, the pH of all test digesters was dropped to slightly acidic condition (6-7.5).

These results showed digestion process was operated at the pH range of 6-8.5 which is similar to the pH suggested by Boe, (2006). Methanogenic archaea can operate its function in pH interval from 5.5 to 8.5 with an optimal range of 6.5–8.0 (Boe, 2006). Acetoclastic methanogenesis is predominantly susceptible at low pH environment and quickly inhibited the process if the pH drops below 6.5 so methanogen which utilize acetate as substrate for biogas generation will be inhibited at low pH conditions (Van Lier, et al., 1996). The test (electricity provided) digester showed slightly more alkaline phase than the control one till day 5. After 5th day pH of the test was dropped to slightly acidic condition, this may be due to the inactivation of the MEC after 5 day. As various researches explain that during electrochemical cell activation, at cathode electrode CO_2 and H_2 was utilized and produces methane gas. During this period, absence of CO_2 and H_2 occurred which resulted in slightly alkaline condition than control digester. After the 5 day the inactivation of MEC, the reaction was returned to slightly acidic condition due to the accumulation of CO_2 and H_2 in the digester.

4.3 Biogas production and analysis of chemical oxygen demand and soluble reducing sugar in 6000 L digester

The biogas production in 6000 L digester was done at Nepal academy of Science and Technology. Different parameters like chemical oxygen demand, soluble reducing sugar and biogas content were analyzed for 42 days.



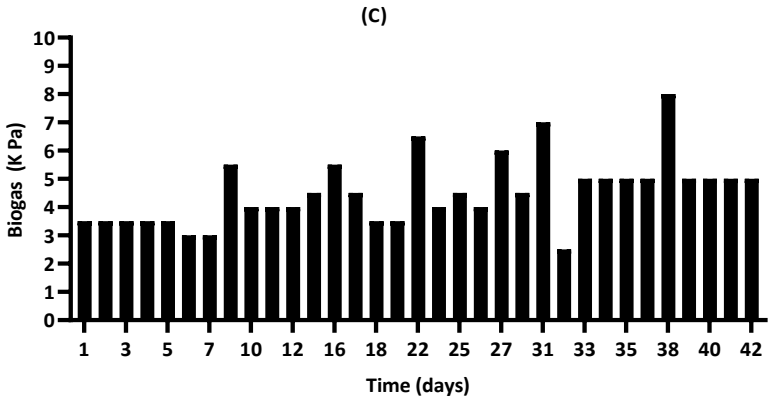


Figure 4. 9: Different parameter analysis in 6000 L digester, (A) chemical oxygen demand, (B) soluble reducing sugar, and (C) biogas production

Figure (4.9A), showed the COD concentration during the anaerobic digestion for 42 days in 6000 L digester. In this case, the COD concentration was measured in the range of 380 mg/L to 580 mg /L. Similarly, Figure (4.9B) showed the reducing sugar concentration during the anaerobic digestion in 6000 L digester. In this case, the reducing sugar concentration was measured in the range of 790 µg/L to 1200 µg/L. Also, Figure (4.9C), showed the biogas production at 6000 L digester. The biogas production at day 1, in the process was 3.5 Kelvin Pascal (K Pa). The gas production rate was constant during the first week. During the second week the biogas production was increase to 4.5 K Pa. The increasing pattern was continued up to 5^{th} week and reached to 5 K Pa at day 42. The highest methane percentage noted at 5^{th} week was 39%, which was measured using multi gas analyzer provided by the NAST.

We analyze the change in different parameters for already existing 6000 L plant at Nepal Academy of Science and Technology (NAST). While analysis of different parameters we observed that, there was very negligible change in COD, soluble reducing sugar. There was a provision of continuous feeding of substrate every day. Due to the leakage problem, biogas amount and integration of microbial electrochemical cell was not performed in 6000 L digester. As per their protocol we are suggested to add additional substrate. After that we observed the parameters again but could not see vast difference during 42 days of digestion process. Due to leakage of the system, we could not analyze biogas and methane content properly. Hope, my followers could be able to perform this process with electrochemical system after repair of the system.

4.4 Isolation and identification of microbes

4.4.1 Isolation of bacteria

The inoculums prepared in DSMZ 825 methanogen enhancement media was spread on the agar plate enriched with DSMZ 825 methanogen enhancement media and was kept into anaerobic jar and incubated at 37°C for 24 hrs. Then, from the plate after incubation several colonies were chosen and streaked separately in agar plate enriched with DSMZ 825 methanogen enhancement media and again incubated in anaerobic jar and incubated at 37°C for 24 hrs.

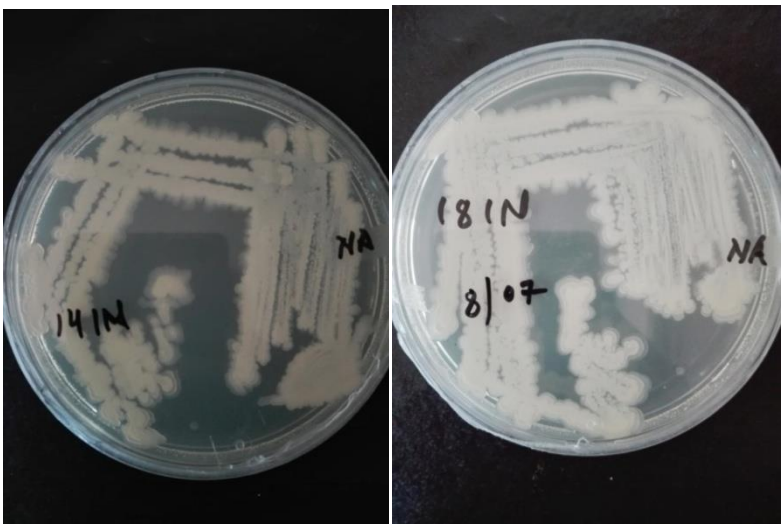


Figure 4. 10: (A) and (B) shows the colonies growth of 14IN and 18IN in agar medium enriched with DMSZ media after 24 hrs incubation respectively.

The morphological characteristic of 14IN colony is regular, creamy yellow, mucoid, convex elevation and sticky margin whereas colony of 18IN is regular, creamy white, convex elevation and mucoid.

4.4.2 Test of isolates for cellulolytic activity

The isolated bacterial samples were further investigated for the cellulose degradation test/ holozone test on carboxy-methyl cellulose (CMC) agar. The holozone test showed that both the samples 14IN and 18IN showed clear halo zone in CMC agar plate after flooding of congo red and NaCl. The formed holozone is shown in the Figure (4.11A) and Figure (4.11A). Since both the isolates formed clear transparent zone of hydrolysis both 14lN and 18lN were characterized morphologically.

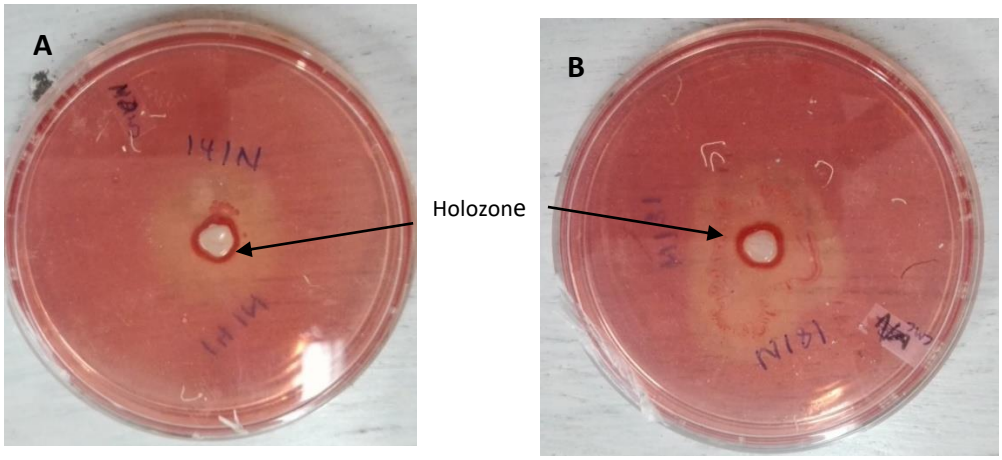


Figure 4. 11: test for cellulolytic activity by CMC degradation,(A) holozone shown by 14IN isolate and (B) holozone shown by 18IN isolates on CMC agar plate after flooding of congo red followed by NaCl.

These bacterial isolates utilized CMC as a source of energy to maximize biomass production and release cellulolytic enzyme to break cellulosic bonds. Major cellulolytic enzyme includes β 1-4 exoglucanase and β glucosidase. Release of cellulytic enzymes drives the attack on cellulosic fragments of cow dung and increase the sugar yield.

4.4.3 Genomic DNA extraction and PCR amplification of 14IN and 18IN isolates.

The genomic DNA of the two isolates was extracted by the CTAB method of extraction and PCR reaction was performed successfully and run on 1% agarose gel with 1 kb ladder from Sigma-Aldrich for 60 minutes in 90V. The 16s rRNA region of bacterial isolates were amplified. The size of the PCR product was found to be 1500 bp (Figure 4.11). PCR products were then sent for sequencing to Nepal Academy of Science and Technology.

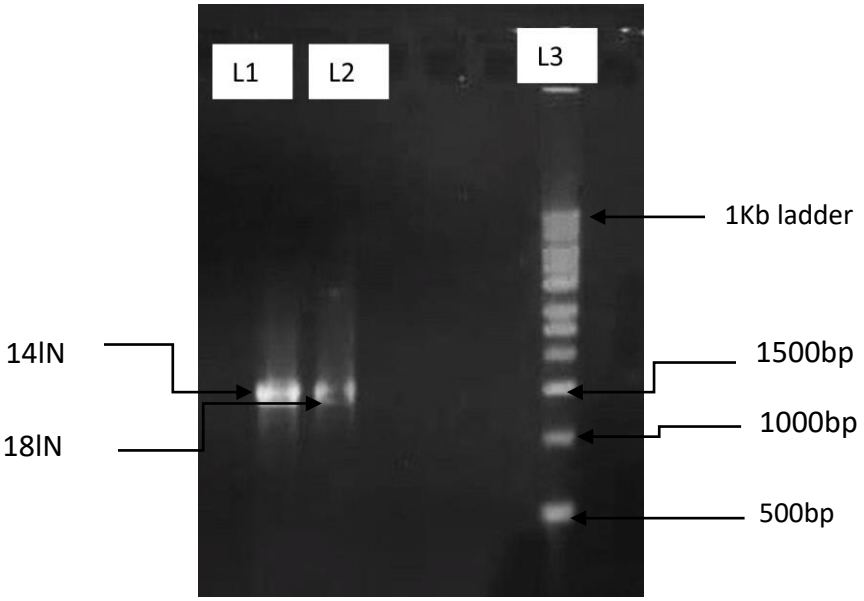
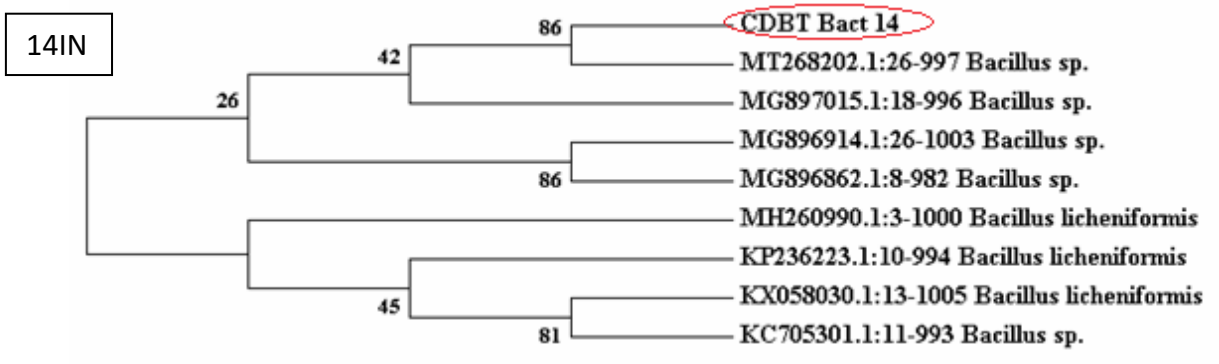


Figure 4. 12: 1% gel electrophoresis of PCR product where L3 contain 1Kb ladder and L1 and L2 contain products of 14 IN and 18 IN respectively

4.4.4 Sequencing of PCR products

The PCR product after the amplification was send to the NAST for sequencing. The sequencing of the products was done by Sanger's method.

4.4.5 Construction of phylogenetic tree



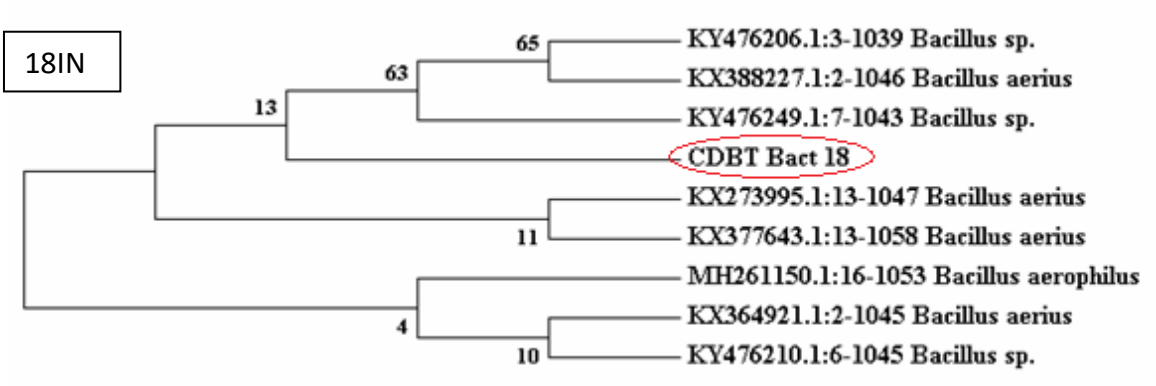


Figure 4. 13: Phylogenetic tree of 14IN and 18IN

The construction of phylogenetic tree provided the extent of biological diversity of the sequences of CDBT Bact 14 isolates with *Bacillus licheniformis* and the sequence of CDBT Bact 18 isolate with *Bacillus aerius.*

GeneBank Accession number

Table 4. 5: Genebank Accession number of 14lN and 18lN

Symbol	Microorganism	Submission number	Accession number
14Ln	*Bacillus licheniformis*	SUB10700707	OL588258
18Ln	*Bacillus aerius*	SUB10700683	OL588257

The isolate *Bacillus licheniformis* is a Gram positive and facultative anaerobic microorganism. It belongs to the phylum Firmicutis and family Bacillaceae. These species are mainly found in soil and feather of birds. *Bacillus licheniformis* produce varieties of enzyme like serine protease, amylase which can be utilize for industrial purpose and it is also used as a probiotic in animal feed. Due to the thermo tolerant nature of species it is also involved food spoilage. *Bacillus licheniformis* catabolizes L-arginine under anaerobic conditions through arginine deaminase pathway whiuch utilize arginine as energy source by coupling the phosphorylation of 1 mol of ADP into ATP per mol of arginine taken up from the medium (Broman, Lauwers, Stalon, & Wiame, 1978). The isolate *Bacillus aerius* also belongs to the phylum Firmicutis and family Bacillaceae. *Bacillus aerius* growth can occurs at 8–37°C temperature, but not at 40°C. The optimum pH for the bacteria is between 6 and 10. The first isolation of this bacterium was done in cryogenic tubes used to collect air samples at high altitudes of 24, 28 and 41 km as a novel strain (Shivaji et al., 2006).

5 SUMMARY

The growing need of renewable and ecofriendly energy for the future generation is hard to fulfill by the present energy plants. To meet the demand, the enhanced biogas production from the agricultural wastes, cattle manure, and waste water treatment can play important role particularly in country like Nepal. Biogas productions from these sources are ecofriendly and also known as clean energy which can be helpful for the sustainable development of the world in the next few decades. Renewable energy (biogas) production is a fast growing market in the world because it offers an economical alternative to the fossil fuels. MEC is new technology which has been used for production of bioethanol, hydrogen gas and methane. This energy produced from the lignocellulosic biomass and organic wastes have great potential. It is believed that in coming years, with more improvement in the technology and reduced prices which lead to a better and economical bioenergy.

This thesis work was carried out for the detection of biogas production in cold temperature using small voltage of energy. For the research cow dung was used as substrate. Firstly, the chemical parameters like pH, moisture content, total soluble sugar, volatile soluble sugar and chemical components (phosphorus, iron, copper, lead, zinc, and manganese) present in dry substrate were analyzed. Then setup for biogas was done in 1 L and 5 L digester each at 15 °C and room temperature, with supply of external voltage of 2 V. The level of substrate digestion during biogas production was monitored by analyzing COD and reducing sugar. The amount of gas produced was measured by downward displacement of water and KOH solution. The bacterial colonies present in the digester were isolated and subjected to molecular characterization.

The finding of the research shows biogas production enhancement with voltage supply of 2 V. In both cases, 15 °C and room temperature, biogas production was increased. During the biogas production process, reduction in COD and reducing sugar reduction was observed. This signifies the digestion of substrate during the anaerobic digestion process. Also methane gas content was analyzed from the total biogas produced. The methane content of the biogas was found to be around 40-60% of total biogas which was similar to different literature. Methanogen, the prevalent organism was not isolated in the isolation part of this research because of the lack of maintenance of anaerobic condition. Though I

have isolated facultative microorganism which belongs to family Bacillaceae which may have play an important role in electron transfer as a biocatalyst.

Finally, this overall research concludes biogas production can be enhanced with small voltage supply in anaerobic digester.

6 CONCLUSION

Microbial electrochemical cell (MEC) is an innovative technique which utilizes microorganism as biocatalyst to degrade lignocellulosic biomass and organic wastes to generate biogas. Chemical parameters of the substrate can effect positively or negatively in the biogas production. In this experiment cow dung was degraded using graphite felt (GF) as electrode in MEC to reduce COD and soluble reducing sugar digester. With application of voltage of 2 V, biogas was enhanced at both 15 °C and room temperature. The analysis of change in different parameters for already existing 6000 L plant was done. While analysis of different parameters in 6000 L digester, we observed that, there was very negligible change in COD, soluble reducing sugar. Due to the leakage problem and biogas amount; integration of microbial electrochemical cell could not be perform in 6000 L digester. Molecular characterization by 16s rRNA sequencing showed that the isolated bacteria were *Bacillus licheniformis* and *Bacillus aerius* which show higher cellulytic activity than other bacterial isolates prevalently found in cow dung. Despite some technical problem, this experiment shows that MEC and electromethanogenesis can be performed even at the low temperature with a significant amount of methane yield and good substrate conversion.

7 Recommendation

- Durability of MEC should be investigated for commercial production.
- This technique is recommended for Clean energy Nepal, BSP Nepal and Alternative energy promotion center, Nepal for policy making and implementation to improve biogas production at hilly and mountainous region.
- Repeated work should be performed for validation of this method.
- Pure culture of hydrogenotropic methanogen should be isolated and investigate for the electromethanogenesis process of the organism.

REFERENCES

Abbanat, D. R., Aceti, D. J., Baron, S. F., Terlesky, K. C., & Ferry, J. G. (1989). *Microbiology and biochemistry of the methanogenic archaeobacteria*. *9*(6), 101–105.

Abbasi, T., Tauseef, S. M., & Abbasi, S. A. (2012). Biogas energy. *Biogas Energy*, 1–169. https://doi.org/10.1007/978-1-4614-1040-9

Abubakar, B. S. U. I., & Ismail, N. (2012). Anaerobic digestion of cow dung for biogas production. *ARPN Journal of Engineering and Applied Sciences*, *7*(2), 169–172.

Ahn, H. K., Smith, M. C., Kondrad, S. L., & White, J. W. (2010). *Evaluation of Biogas Production Potential by Dry Anaerobic Digestion of Switchgrass – Animal Manure Mixtures*. 965–975. https://doi.org/10.1007/s12010-009-8624-x

Ahring, B. K., Ibrahim, A. A., & Mladenovska, Z. (2001). Effect of Temperature Increase from 55 to 65^{o}C on Performance and Microbial Population Dynamics of an Anaerobic reactor Treating Cattle Manure. *35*(10), 2446–2452.

Andri, I., & Sriariyanun, M. (2017). Effect of Cellulase-producing Microbial Consortium on Biogas Production from Lignocellulosic Biomass. *Energy Procedia*, *141*, 180–183. https://doi.org/10.1016/j.egypro.2017.11.034

Angelidaki, I., & Ahring, B. K. (1993). *Applied Microbiology Biotechnology ©*. 560–564.

APHA. (1998). *Standard Methods for the Examination of Water and Wastewater* (20th, ed.). Retrieved from https://www.scirp.org/(S(lz5mqp453edsnp55rrgjct55))/reference/ReferencesPapers.aspx?ReferenceID=1909322

Atlas, R. . (2005). *Handbook of media for environmental microbiology* (2nd editio). https://doi.org/https://doi.org/10.1201/9781420037487

Attar, Y., Mhetre, S. T., & Dhawale, M. D. (1998). *Biogas production enhancement by cellulolytic strains of actinomycetes*. 11–15. Retrieved from https://www.osti.gov/etdeweb/biblio/635753

Bajgain, S., & Mendis, M. S. (2005). *The Nepal Biogas support programe: A successful model of public private partnership for rural household energy supply*.

Bajracharya, S., Ter Heijne, A., Dominguez Benetton, X., Vanbroekhoven, K., Buisman, C. J. N., Strik, D. P. B. T. B., & Pant, D. (2015). Carbon dioxide reduction by mixed and pure cultures in microbial electrosynthesis using an assembly of graphite felt and stainless steel as a cathode. *Bioresource Technology*, *195*, 14–24. https://doi.org/10.1016/j.biortech.2015.05.081

Balch, W. E., Fox, G. E., Magrum, L. J., Woese, C. R., & Wolfe, R. S. (1979). *Methanogens : Reevaluation of a Unique Biological Group*. *43*(2), 260–296.

Bendixen, H. . (1994). *Sfeguards against pathogens in Danish biogas plants*. 171–180. Retrieved from https://www.proquest.com/openview/50675b5eaab5bc75cb2373284003f469/1?pq-origsite=gscholar&cbl=2044520

Bhadra, A., Scharer, J. M., & Moo-Young, M. (1986). Anaerobic digestion of native cellulosic wastes. *MIRCEN Journal of Applied Microbiology and Biotechnology*, *2*(3), 349–358. https://doi.org/10.1007/BF00933476

Blonskaja, V., Menert, A., & Vilu, R. (2003). Use of two-stage anaerobic treatment for distillery waste. *Advances in Environmental Research*, *7*(3), 671–678. https://doi.org/10.1016/S1093-0191(02)00038-2

Bluffstone, R., & Toman, M. (2014). *Policy Brief Biogas Replacement of Fuelwood : Clean Energy Access with Low-Cost Mitigation of Climate Change*. 1–4.

Boe, K. (2006). *Online monitoring and control of the biogas process*.

Bok, F. A. M. De, Harmsen, H. J. M., Plugge, C. M., Vries, M. C. De, Akkermans, A. D. L., Vos, W. M. De, & Stams, A. J. M. (2005). *The first true obligately syntrophic propionate- oxidizing bacterium , Pelotomaculum schinkii sp . nov ., co-cultured with Methanospirillum hungatei , and emended description of the genus Pelotomaculum*. 1697–1703. https://doi.org/10.1099/ijs.0.02880-0

Bolado-Rodríguez, S., Toquero, C., Martín-Juárez, J., Travaini, R., & García-Encina, P. A. (2016). Effect of thermal, acid, alkaline and alkaline-peroxide pretreatments on the biochemical methane potential and kinetics of the anaerobic digestion of wheat straw and sugarcane bagasse. *Bioresource Technology*, *201*, 182–190. https://doi.org/10.1016/j.biortech.2015.11.047

Bouallagui, H., Haouari, O., Touhami, Y., Ben Cheikh, R., Marouani, L., & Hamdi, M. (2004). Effect of temperature on the performance of an anaerobic tubular reactor treating fruit and vegetable waste. *Process Biochemistry*, *39*(12), 2143–

2148. https://doi.org/10.1016/j.procbio.2003.11.022

Broman, K., Lauwers, N., Stalon, V., & Wiame, J. M. (1978). Oxygen and nitrate in utilization by Bacillus lichenifromis of the arginase and arginine deiminase routes of arginine catabolism and other factors affecting their syntheses. *Journal of Bacteriology*, *135*(3), 920–927. https://doi.org/10.1128/jb.135.3.920-927.1978

Bryant, M. P. (1979). Microbial Methane Production—Theoretical Aspects2. *Journal of Animal Science*, *48*(1), 193–201. https://doi.org/10.2527/jas1979.481193x

Buhr, H. O. (1976).Review Paper the Thermophilic Anaerobic Digestion Process

Castrillón, L., Vázquez, I., Marañón, E., & Sastre, H. (2002). Anaerobic thermophilic treatment of cattle manure in UASB reactors. *Waste Management and Research*, *20*(4), 350–356. https://doi.org/10.1177/0734247X0202000406

CBS, N. (2012). National Population and Housing Census 2011. *Texas Medicine*, *111*(3), 51–57.

Cecchi, F., & Cavinato, C. (2015). Anaerobic digestion of bio-waste: A mini-review focusing on territorial and environmental aspects. *Waste Management and Research*, *33*(5), 429–438. https://doi.org/10.1177/0734242X14568610

CES. (2001). *Submitted by : Centre for Energy Studies Institute of Engineering*.

Chen, Y., Cheng, J. J., & Creamer, K. S. (2008). Inhibition of anaerobic digestion process: A review. *Bioresource Technology*, *99*(10), 4044–4064. https://doi.org/10.1016/j.biortech.2007.01.057

Cheng, S., & Logan, B. E. (2007). Sustainable and efficient biohydrogen production via electrohydrogenesis. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(47), 18871–18873. https://doi.org/10.1073/pnas.0706379104

Cheng, S., Xing, D., Call, D. F., & Logan, B. E. (2009). Direct biological conversion of electrical current into methane by electromethanogenesis. *Environmental Science and Technology*, *43*(10), 3953–3958. https://doi.org/10.1021/es803531g

Chibueze, U., Okorie, N., Oriaku, O., Isu, J. &, & Peters, E. (2017). The Production of Biogas Using Cow Dung and Food Waste. *International Journal of Materials and Chemistry*, *7*(2), 21–24. https://doi.org/10.5923/j.ijmc.20170702.01

Chynoweth, D. P., Owens, J. M., & Legrand, R. (2001). *Renewable methane from*

anaerobic digestion of biomass. *22*.

Comino, E., Rosso, M., & Riggio, V. (2009). Development of a pilot scale anaerobic digester for biogas production from cow manure and whey mix. *Bioresource Technology*, *100*(21), 5072–5078. https://doi.org/10.1016/j.biortech.2009.05.059

Cusick, R. D., Bryan, B., Parker, D. S., Merrill, M. D., Mehanna, M., Kiely, P. D., … Logan, B. E. (2011). Performance of a pilot-scale continuous flow microbial electrolysis cell fed winery wastewater. *Applied Microbiology and Biotechnology*, *89*(6), 2053–2063. https://doi.org/10.1007/s00253-011-3130-9

De Graaf, D., & Fendler, R. (2010). Biogas Production in Germany; Federal Environment Agency. *Dessau Rosslau, Baltic Sea Region Programme. Dessau Rosslau, Baltic Sea Region Programme: Dessau-Rosslau, Germany,* 1–24. Retrieved from https://scholar.google.com/scholar?q=related:DoSPO_HdjvUJ:scholar.google.com/&scioq=Graaf+D,+Fendler+R+(2010).+Biogas+production+in+Germany.+Federal+Environment+Agency.+Dessau-Rosslau,+p.+29.&hl=en&as_sdt=0,5

Demirbaş, A. (2006). Global renewable energy resources. *Energy Sources, Part A: Recovery, Utilization and Environmental Effects*, *28*(8), 779–792. https://doi.org/10.1080/00908310600718742

Demirel, B., & Scherer, Æ. P. (2008). *The roles of acetotrophic and hydrogenotrophic methanogens during anaerobic conversion of biomass to methane : a review*. 173–190. https://doi.org/10.1007/s11157-008-9131-1

Demirel, B., & Yenigün, O. (2002). Two-phase anaerobic digestion processes: A review. *Journal of Chemical Technology and Biotechnology*, *77*(7), 743–755. https://doi.org/10.1002/jctb.630

Dev, S., Saha, S., Kurade, M. B., & Salama, E. (2019). Perspective on anaerobic digestion for biomethanation in cold environments. *Renewable and Sustainable Energy Reviews*, *103*(May 2018), 85–95. https://doi.org/10.1016/j.rser.2018.12.034

EPA. (2006). *Biosolids Technology Fact Sheet Multi-Stage Anaerobic Digestion*.

F.G.Pohland, & S.Ghosh. (1971). Development in anaerobic stabilization of organic wastes-The two-phase concept. *Environmental Letters, 1(4)*, 255–266.

Fantozzi, F., & Buratti, C. (2009). Bioresource Technology Biogas production from

different substrates in an experimental Continuously Stirred Tank Reactor anaerobic digester. *Bioresource Technology*, *100*(23), 5783–5789. https://doi.org/10.1016/j.biortech.2009.06.013

Fedailaine, M., Moussi, K., Khitous, M., Abada, S., Saber, M., & Tirichine, N. (2015). Modeling of the anaerobic digestion of organic waste for biogas production. *Procedia Computer Science*, *52*(1), 730–737. https://doi.org/10.1016/j.procs.2015.05.086

Fernandes, M. I. A. P. (1986). Application of porous membranes for biomass retention in a two-phase anaerobic process. *University of Newcastle upon Tyne*. Retrieved from https://ethos.bl.uk/OrderDetails.do?uin=uk.bl.ethos.374847

Fernandes, T. V., Klaasse Bos, G. J., Zeeman, G., Sanders, J. P. M., & van Lier, J. B. (2009). Effects of thermo-chemical pre-treatment on anaerobic biodegradability and hydrolysis of lignocellulosic biomass. *Bioresource Technology*, *100*(9), 2575–2579. https://doi.org/10.1016/j.biortech.2008.12.012

Ferry, J. G. (1993). Physiological Ecology of Methanogens. *Ecology*, *4*, 128–206. https://doi.org/10.2307/1939288

Gautam, R., Baral, S., & Herat, S. (2009). *Biogas as a sustainable energy source in Nepal : Present status and future challenges*. *13*, 248–252. https://doi.org/10.1016/j.rser.2007.07.006

Giordano, G. E. L. F. A. (2012). *Anaerobic co-digestion of organic wastes*. 325–341. https://doi.org/10.1007/s11157-012-9277-8

Hamelers, H. V. M., Ter Heijne, A., Sleutels, T. H. J. A., Jeremiasse, A. W., Strik, D. P. B. T. B., & Buisman, C. J. N. (2010). New applications and performance of bioelectrochemical systems. *Applied Microbiology and Biotechnology*, *85*(6), 1673–1685. https://doi.org/10.1007/s00253-009-2357-1

Hamelin, L., Naroznova, I., & Wenzel, H. (2020). Environmental consequences of different carbon alternatives for increased manure-based biogas. *Applied Energy*, *114*(2014), 774–782. https://doi.org/10.1016/j.apenergy.2013.09.033

Hamelin, L., Wesnæs, M., Wenzel, H., & Petersen, B. M. (2011). Environmental consequences of future biogas technologies based on separated slurry. *Environmental Science and Technology*, *45*(13), 5869–5877. https://doi.org/10.1021/es200273j

Hanafiah, M. M. (2016). *Biogas Production from Different Substrates under Anaerobic Conditions*. (January).

Harikishan, S., & Sung, S. (2003). Cattle Waste Treatment and Class A Biosolid Production Using Temperature-phased Anaerobic Digester. *Ad. Environ. Res.*, *7*(3), 701–706. Retrieved from http://ci.nii.ac.jp/naid/10024653872/en/

Hashimoto, K., Habazaki, H., Yamasaki, M., Meguro, S., Sasaki, T., Katagiri, H., … Akiyama, E. (2001). *Advanced materials for global carbon dioxide recycling*. *306*, 88–96.

Henderson, J. P. (1997). Anaerobic digestion in rural China. United States. *City Farmer, Canada's Office of Urban Agriculture*. Retrieved from http://www.cityfarmer.org/biogasPaul.html

Henze, M., Harremoës, P., Jansen, J. l. C., & Arvin, E. (2002). *Wastewater treatment:biological and chemical process* (2nd ed.). Springer.

Holm-Nielsen, J. B., Al Seadi, T., & Oleskowicz-Popiel, P. (2009). The future of anaerobic digestion and biogas utilization. *Bioresource Technology*, *100*(22), 5478–5484. https://doi.org/10.1016/j.biortech.2008.12.046

Horváth, S., & Taherzadeh, M. J. (2016). Innovative Pretreatment Strategies for Biogas Production. *Bioresource Technology*. https://doi.org/10.1016/j.biortech.2016.11.083

Hupfauf, S., Plattner, P., Wagner, A. O., Kaufmann, R., Insam, H., Podmirseg, S. M., … Podmirseg, S. M. (2018). Temperature shapes the microbiota in anaerobic digestion and drives efficiency to a maximum at 45 ° C. *Bioresource Technology*. https://doi.org/10.1016/j.biortech.2018.08.106

IEA. (2020). *World Energy Balances: Overview*. Retrieved from https://www.iea.org/reports/world-energy-balances-overview

Jones, W. J., Nagle, D. P., & Whitman, W. B. (1987). *Methanogens and the Diversity of Archaebacteria*. *51*(1), 135–177.

Joshi, J., Dhungana, P., Prajapati, B., Maharjan, R., Poudyal, P., Yadav, M., … Sreerama, L. (2019). Enhancement of Ethanol Production in Electrochemical Cell by Saccharomyces cerevisiae (CDBT2) and Wickerhamomyces anomalus (CDBT7). *Frontiers in Energy Research*, *7*. https://doi.org/10.3389/fenrg.2019.00070

Khalid, A., Arshad, M., Anjum, M., Mahmood, T., & Dawson, L. (2011). The anaerobic digestion of solid organic waste. *Waste Management*, *31*(8), 1737–1744. https://doi.org/10.1016/j.wasman.2011.03.021

Khoiyangbam, R. S., Kumar, S., Jain, M. C., Gupta, N., & Kumar, A. (2004). *Methane emission from fixed dome biogas plants in hilly and plain regions of northern India*. *95*, 35–39. https://doi.org/10.1016/j.biortech.2004.02.009

Kim, B. H., Park, H. S., Kim, H. J., Kim, G. T., Chang, I. S., Lee, J., & Phung, N. T. (2004). Enrichment of microbial community generating electricity using a fuel-cell-type electrochemical cell. *Applied Microbiology and Biotechnology*, *63*(6), 672–681. https://doi.org/10.1007/s00253-003-1412-6

Kuruvilla, A., Indranil, M., Sambhu, B., & Banerjee, N. (2014). *Biogas production from locally available aquatic weeds of Santiniketan through anaerobic digestion*. https://doi.org/10.1007/s10098-014-0877-6

Labatut, R. A., Angenent, L. T., & Scott, N. R. (2011). Bioresource Technology Biochemical methane potential and biodegradability of complex organic substrates. *Bioresource Technology*, *102*(3), 2255–2264. https://doi.org/10.1016/j.biortech.2010.10.035

Lange, M., & Ahring, B. K. (2001). *A comprehensive study into the molecular methodology and molecular biology of methanogenic Archaea*. *25*.

Larsen, H. E., Munch, B., & Schlundt, J. (1994). Use of indicators for monitoring the reduction of pathogens in animal waste treated in biogas plants. *International Journal of Hygiene and Environmental Medicine*, *195*(5–6), 544–555.

Letters, R. B. (2014). *Main factors affecting biogas production - an overview*. *19*(3), 9283–9296.

Li, R., Chen, S., & Li, X. (2009). Anaerobic co-digestion of kitchen waste and cattle manure for methane production. *Energy Sources, Part A: Recovery, Utilization and Environmental Effects*, *31*(20), 1848–1856. https://doi.org/10.1080/15567030802606038

Lindorfer, H., Ramhold, D., & Frauz, B. (2012). *Nutrient and trace element supply in anaerobic digestion plants and effect of trace element application*. 1923–1929. https://doi.org/10.2166/wst.2012.399

Liu, J., Liu, Y., Feng, C., Wang, Z., Jia, T., Gong, L., & Xu, L. (2017). Enhanced

performance of microbial fuel cell using carbon microspheres modified graphite anode. *Energy Science and Engineering*, *5*(4), 217–225. https://doi.org/10.1002/ese3.164

Liu, W. Z., Wang, A. J., Ren, N. Q., Zhao, X. Y., Liu, L. H., Yu, Z. G., & Lee, D. J. (2008). Electrochemically assisted biohydrogen production from acetate. *Energy and Fuels*, *22*(1), 159–163. https://doi.org/10.1021/ef700293e

Liu, Y., & Tay, J. H. (2004). State of the art of biogranulation technology for wastewater treatment. *Biotechnology Advances*, *22*(7), 533–563. https://doi.org/10.1016/j.biotechadv.2004.05.001

Lloyd, K. G., Edgcomb, V. P., Molyneaux, S. J., Bo, S., Wirsen, C. O., Atkins, M. S., & Teske, A. (2005). *Effects of Dissolved Sulfide , pH , and Temperature on Growth and Survival of Marine Hyperthermophilic Archaea*. *71*(10), 6383–6387. https://doi.org/10.1128/AEM.71.10.6383

Logan, B. E., Call, D., Cheng, S., Hamelers, H. V. M., Sleutels, T. H. J. A., Jeremiasse, A. W., & Rozendal, R. A. (2008). Microbial electrolysis cells for high yield hydrogen gas production from organic matter. *Environmental Science and Technology*, *42*(23), 8630–8640. https://doi.org/10.1021/es801553z

Lorenz, G., Slater, R. R., Birzgalis, B. R., Company, E. K., & Company, M. (1961). *Application of Different to Cellodextrins Coforimetric Tests blanks from absorbances of test samples ; those obtained in the tests using the iodine-potassium iodide test , by subtracting absorbances of test samples from absorbances of blanks . Absorbancy . 528*, 521–528.

Lozano, C. J. S., Mendoza, M. V., de Arango, M. C., & Monroy, E. F. C. (2009). Microbiological characterization and specific methanogenic activity of anaerobe sludges used in urban solid waste treatment. *Waste Management*, *29*(2), 704–711. https://doi.org/10.1016/j.wasman.2008.06.021

Lu, J., Gavala, H. N., Skiadas, I. V., Mladenovska, Z., & Ahring, B. K. (2008). Improving anaerobic sewage sludge digestion by implementation of a hyper-thermophilic prehydrolysis step. *Journal of Environmental Management*, *88*(4), 881–889. https://doi.org/10.1016/j.jenvman.2007.04.020

Lusk, P. (1999). Lastest progression in anaerobic digestion.Part I. *JG Press, Emmaus, PA*, *40*, 52–56. Retrieved from http://pascal-francis.inist.fr/vibad/index.php?action=getRecordDetail&idt=1911397

Mab, A., Angeles, L., & Lkpaitment, C. S. (1993). *Ecology and microbiology of biogasification*. *5*(3), 191–202.

Mac, S., & Llabr, P. (2000). *Anaerobic digestion of organic solid wastes . An overview of research achievements and perspectives*. *74*.

Marvin, P. B., & Mackie, R. I. (1981). Metabolic Activity of Fatty Acid-Oxidizing Bacteria and the Contribution of Acetate, Propionate, Butyrate, and CO2 to Methanogenesis in Cattle Waste at 40 and 60°C. *Applied and Environmental Microbiology*, *41*(0099-2240/81/061363-11$02.00/0), 1363–1373. https://doi.org/https://doi.org/10.1128/aem.41.6.1363-1373.1981

Miryahyaei, S., Olinga, K., Muthalib, F. A. A., Das, T., Aziz, M. S. A., Othman, M., … Eshtiaghi, N. (2019). Impact of rheological properties of substrate on anaerobic digestion and digestate dewaterability : New insights through rheological and physico-chemical interaction. *Water Research*, *150*, 56–67. https://doi.org/10.1016/j.watres.2018.11.049

MOF. (2012). *ECONOMIC SURVEY FISCAL YEAR 2011 / 12 GOVERNMENT OF NEPAL*.

Mursec, B. (2009). *Analysis of different substrates for processing into biogas*. *37*(2), 652–659.

Mussgnug, J. H., Klassen, V., Schlüter, A., & Kruse, O. (2010). Microalgae as substrates for fermentative biogas production in a combined biorefinery concept. *Journal of Biotechnology*, *150*(1), 51–56. https://doi.org/10.1016/j.jbiotec.2010.07.030

Mustafa, M. Y., Calay, R. K., & Román, E. (2016). Biogas from Organic Waste - A Case Study. *Procedia Engineering*, *146*(1877), 310–317. https://doi.org/10.1016/j.proeng.2016.06.397

Naik, S. N., Goud, V. V, Rout, P. K., & Dalai, A. K. (2010). *Production of first and second generation biofuels : A comprehensive review*. *14*, 578–597. https://doi.org/10.1016/j.rser.2009.10.003

Nakarmi, A., & Dhital, R. P. (2016). *Biogas as Renewable Source of Energy in Nepal . Theory and Development .*

NEC, 2015. (2015). Department of Electrcity Development Guidelines for Power System Optimization of Hydropower Projects .

Nie, E., He, P., Zhang, H., Hao, L., Shao, L., & Lü, F. (2021). How does temperature

regulate anaerobic digestion ? *Renewable and Sustainable Energy Reviews*, *150*(June), 111453. https://doi.org/10.1016/j.rser.2021.111453

OSTREM, M, K., MILLRATH, K., & THEMELIS, N. J. (2004). *Combining Anaerobic Digestion and Waste-To-Energy*. 1–7.

Perea-Moreno, M. A., Samerón-Manzano, E., & Perea-Moreno, A. J. (2019). Biomass as renewable energy: Worldwide research trends. *Sustainability (Switzerland)*, *11*(3). https://doi.org/10.3390/su11030863

Pitol-filho, D. C. L. (2012). *Studies concerning the accessibility of different allomorphic forms of cellulose*. 55–68. https://doi.org/10.1007/s10570-011-9620-1

Pobeheim, H., Munk, B., Lindorfer, H., & Guebitz, G. M. (2010). Impact of nickel and cobalt on biogas production and process stability during semi-continuous anaerobic fermentation of a model substrate for maize silage. *Water Research*, *45*(2), 781–787. https://doi.org/10.1016/j.watres.2010.09.001

Rajeshwari, K. V., Balakrishnan, M., Kansal, A., Lata, K., & Kishore, V. V. N. (2000). State-of-the-art of anaerobic digestion technology for industrial wastewater treatment. *Renewable & Sustainable Energy Reviews*, *4*(2), 135–156. https://doi.org/10.1016/S1364-0321(99)00014-3

Rasi, S., Veijanen, A., & Rintala, J. (2007). Trace compounds of biogas from different biogas production plants. *Energy*, *32*(8), 1375–1380. https://doi.org/10.1016/j.energy.2006.10.018

Saadullah, M. (2001). Smallholder dairy production and marketing: Opportunities and constraints. *Proceedings of a South–South Workshop Held at National Dairy Development Board (NDDB) Anand, India, 13–16 March 2001*, (March), 414–430.

Sakalis, A., Mpoulmpasakos, K., Nickel, U., Fytianos, K., & Voulgaropoulos, A. (2005). Evaluation of a novel electrochemical pilot plant process for azodyes removal from textile wastewater. *Chemical Engineering Journal*, *111*(1), 63–70. https://doi.org/10.1016/j.cej.2005.05.008

Sasaki, D., Morita, M., Sasaki, K., Watanabe, A., & Ohmura, N. (2012). Acceleration of cellulose degradation and shift of product via methanogenic co-culture of a cellulolytic bacterium with a hydrogenotrophic methanogen. *Journal of Bioscience and Bioengineering*, *114*(4), 435–439. https://doi.org/10.1016/j.jbiosc.2012.05.002

Schink, B. (1997). *Energetics of Syntrophic Cooperation in Methanogenic Degradation*. *61*(2), 262–280.

Shao, Q., Li, J., Yang, S., & Sun, H. (2019). Effects of different substrates on microbial electrolysis cell (MEC) anodic membrane: Biodiversity and hydrogen production performance. *Water Science and Technology*, *79*(6), 1123–1133. https://doi.org/10.2166/wst.2019.107

Shivaji, S., Chaturvedi, P., Suresh, K., Reddy, G. S. N., Dutt, C. B. S., Wainwright, M., … Bhargava, P. M. (2006). Bacillus aerius sp. nov., Bacillus aerophilus sp. nov., Bacillus stratosphericus sp. nov. and Bacillus altitudinis sp. nov., isolated from cryogenic tubes used for collecting air samples from high altitudes. *International Journal of Systematic and Evolutionary Microbiology*, *56*(7), 1465–1473. https://doi.org/10.1099/ijs.0.64029-0

Siegert, M., Yates, M. D., Call, D. F., Zhu, X., Spormann, A., & Logan, B. E. (2014). Comparison of nonprecious metal cathode materials for methane production by electromethanogenesis. *ACS Sustainable Chemistry and Engineering*, *2*(4), 910–917. https://doi.org/10.1021/sc400520x

Spinner, N. S., Vega, J. A., & Mustain, W. E. (2012). Recent progress in the electrochemical conversion and utilization of CO 2. *Catalysis Science and Technology*, *2*(1), 19–28. https://doi.org/10.1039/c1cy00314c

Stu, B., Schmid, E., & Eder, M. W. (2011). *Impacts of biogas plant performance factors on total substrate costs*. *5*. https://doi.org/10.1016/j.biombioe.2010.12.030

Subedi, A., & Baral, B. (2021). *Journal of Development Innovations*. *5*(1), 46–62.

Tafdrup, S. (1995). Viable energy production and waste recycling from anaerobic digestion of manure and other biomass materials. *Biomass and Bioenergy*, *9*(1–5), 303–314. https://doi.org/10.1016/0961-9534(95)00098-4

Taherzadeh, M. J., & Jeihanipour, A. (n.d.). *Recalcitrance of Lignocellulosic Biomass to Anaerobic Digestion*.

Temminghoff, E., & Houba, V. (2004). *Analysis Procedures* (Second edi). Boston,London: Kluwer Academic publishers.

Tomlinson, A. P., Powers, W. J., Van Horn, H. H., Nordstedt, R. A., & Wilcox, C. J. (1996). Dietary protein effects on nitrogen excretion and manure characteristics of lactating cows. *Transactions of the American Society of Agricultural Engineers*,

39(4), 1441–1448. https://doi.org/10.13031/2013.27637

US EIA. (2013). International Energy Outlook 2013 - DOE/EIA-0484(2013). *Outlook 2013*, 312.

Valo, A., Carrère, H., & Delgenès, J. P. (2004). Thermal, chemical and thermo-chemical pre-treatment of waste activated sludge for anaerobic digestion. *Journal of Chemical Technology and Biotechnology*, *79*(11), 1197–1203. https://doi.org/10.1002/jctb.1106

Van den Berg, L. (1983). Comparison of advanced anaerobic reactors. *In Proceedings of the 3rd International Symposium on Anaerobic Digestion*, 14–18.

Van Lier, J. B., Martin, J. L. S., & Lettinga, G. (1996). *Effect of temperature on the anaerobic thermophilic convertion of volatile fatty acids by dispersed and granular sludge*. *30*(1), 199–207.

Verstraete, W., & Rabaey, K. (2006). *Critical Review Microbial Fuel Cells : Methodology and Technology †*. *40*(17), 5181–5192.

Viéitez, E. R., & Ghosh, S. (1999). Biogasification of solid wastes by two-phase anaerobic fermentation. *Biomass and Bioenergy*, *16*(5), 299–309. https://doi.org/10.1016/S0961-9534(99)00002-1

Villano, M., Aulenta, F., Ciucci, C., Ferri, T., Giuliano, A., & Majone, M. (2010). Bioresource Technology Bioelectrochemical reduction of CO 2 to CH 4 via direct and indirect extracellular electron transfer by a hydrogenophilic methanogenic culture. *Bioresource Technology*, *101*(9), 3085–3090. https://doi.org/10.1016/j.biortech.2009.12.077

Walla, C. Ã., & Schneeberger, W. (2008). *The optimal size for biogas plants*. *32*, 551–557. https://doi.org/10.1016/j.biombioe.2007.11.009

Ward, A. J., Hobbs, P. J., Holliman, P. J., & Jones, D. L. (2008). Optimisation of the anaerobic digestion of agricultural resources. *Bioresource Technology*, *99*(17), 7928–7940. https://doi.org/10.1016/j.biortech.2008.02.044

Weiland, P. (2010). Biogas production: Current state and perspectives. *Applied Microbiology and Biotechnology*, *85*(4), 849–860. https://doi.org/10.1007/s00253-009-2246-7

Weisburg, W. G., Barns, S. M., Pelletier, D. A., & Lane, D. J. (1991). 16S ribosomal DNA

amplification for phylogenetic study. *Journal of Bacteriology*, *173*(2), 697–703. https://doi.org/10.1128/jb.173.2.697-703.1991

Woese, C. R. (1987). *Bacterial Evolution Background ..... 51*(2), 221–271.

Yadvika, Santosh, Sreekrishnan, T. R., Kohli, S., & Rana, V. (2004). Enhancement of biogas production from solid substrates using different techniques - A review. *Bioresource Technology*, *95*(1), 1–10. https://doi.org/10.1016/j.biortech.2004.02.010

Zehnder, A. J. B., & Gujer, W. (1983). Conversion Processes in Anaerobic Digestion. *Water, Science and Technology*, *15*(August), 127–167. Retrieved from https://doi.org/10.2166/wst.1983.0164

Zeikus, J. G., Ben-bassat, A., & Hegge, P. W. (1980). *Microbiology of Methanogenesis in Thermal , Volcanic Envirorunments*. *143*(1), 432–440.

Zhang, R., El-mashad, H. M., Hartman, K., Wang, F., Liu, G., Choate, C., & Gamble, P. (2007). *Characterization of food waste as feedstock for anaerobic digestion*. *98*, 929–935. https://doi.org/10.1016/j.biortech.2006.02.039

Zhen, G., Lu, X., Kobayashi, T., Kumar, G., & Xu, K. (2015). Promoted electromethanosynthesis in a two-chamber microbial electrolysis cells (MECs) containing a hybrid biocathode covered with graphite felt (GF). *CHEMICAL ENGINEERING JOURNAL*, 9–48. https://doi.org/10.1016/j.cej.2015.09.071

Ziemiński, K., & Frąc, M. (2012). *Methane fermentation process as anaerobic digestion of biomass : Transformations , stages and microorganisms*. *11*(18), 4127–4139. https://doi.org/10.5897/AJBX11.054

APPENDECES

APPENDIX I

A: Reagents

i: DMSZ 825 media

Table 9. 1: ComponentsofDMSZ825media

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 H_2O	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 H_2O	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 9. 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.	$KAl(SO4)_2$ 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 of 1000 mL was first dissolved in nitroacetic acid and then adjusted pH was to 6.5 with KOH, then other minerals were added. Final was adjusted o pH 7 with KOH.

iii: Vitamin solution:

Table 9. 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 $2H_2O$	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	l

B. Determination of chemical components

1. Digestion in flask with H_2SO_4- Salicylic acid – H_2O_2

2. Sulphuric Acid, 96 % (w/w), 18 mol/L (U = 1.84 g/cm^3)

3. Hydrogen Peroxide, 30 % (w/w).

4. Salicylic Acid, Powder.

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

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.1.

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, $KH2PO_4$ (see remark 2), in about 900 mL water in a volumetric flask of 1000 mL. Make up to 1000 mL with water.

3. Ammonium Molybdate Solution - Dissolve 40 g ammonium molybdate tetrahydrate, (NH4)6Mo7O24.4H2O, in ultra pure water and make up to 1000 mL. This solution should be stored in a bottle made of hard glass.

4. Potassium Antimonyl Tartarate Solution - Dissolve 0.274 g potassium antimonyl tartrate, KSbOC4H4O6.5H2O, in ultra pure water and make up to 100 mL with ultra pure water.

5. 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.

6. Anti-coagulation Agent - Wetting agent Aerosol 22, Merck nr 13908

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 min. Finally the graphite electrodes were treated with 70% acetone and ultrasonicated for 15 min 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 min.(J. Liu et al., 2017)

APPENDIX II

Standard graph of Chemical oxygen demand, soluble reducing sugar and Phosphorus

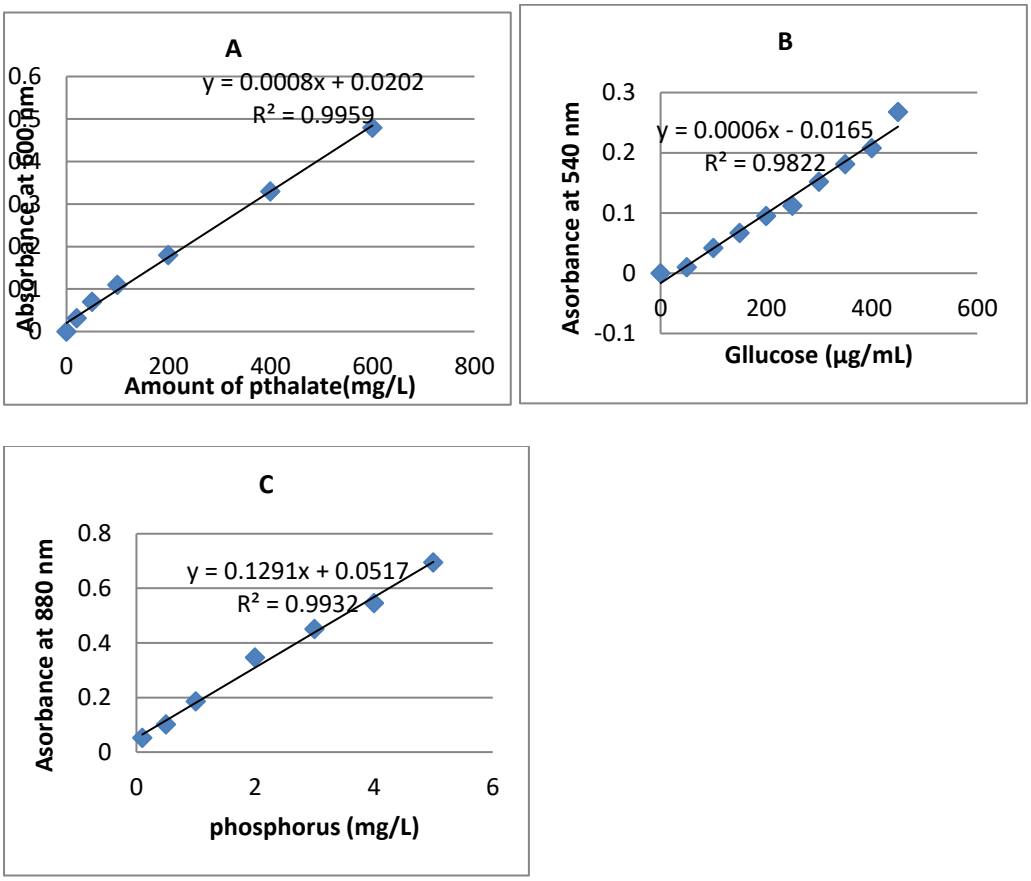


Figure 9. 1: Standard graph, (A) Chemical oxygen demand, (B) Soluble reducing sugar and (C) Phosphorus

APPENDIX III

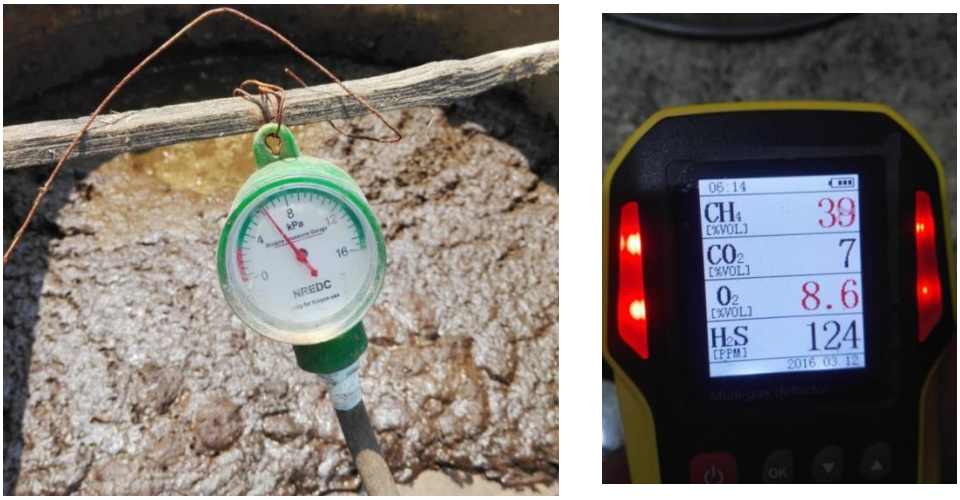


Figure 9. 2: Biogas production in 6000 L digester at NAST and analysis of different biogas composition by Multigas analyzer.

Figure 9. 3: Cow dung substrate collection from kritipur cowshed and from Dhapasi cow farm.

Figure 9. 4: Biogas production and collection from the cow dung in 5L digester at 15°C on BOD incubator using MEC with control.

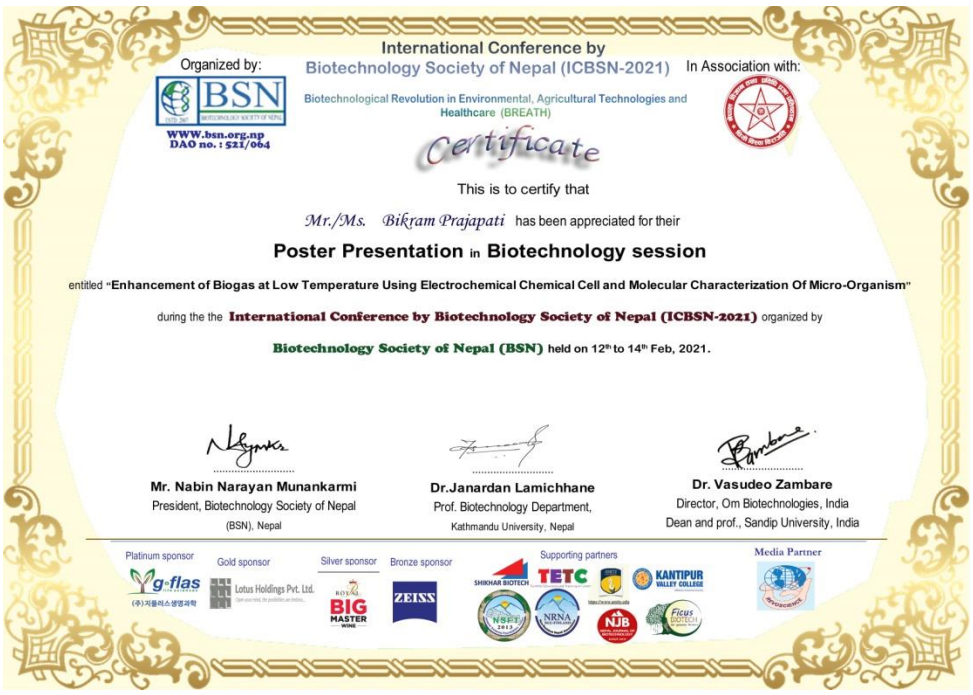


Figure 9. 5: Poster presentation certificate at International conference by Biotechnology society of Nepal (ICBSN 2021).

APPENDIX IV

>Bacillus licheniformis

GGGCGGGCTATCATGCAGTCGAGCGGAAGATGGGAGCTTGCTCCCTGATGTYAGCGGCG
GACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGG
GGCTAATACCGGATGCTTGATTGAACCGCATGGTTCAATTATAAAAGGTGGCTTTTAGCTA
CCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGC
RACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCA
GACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCA
ACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAG
TACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGT
GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGC
GCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCAT
TGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAA
TGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGCGACTCTCTGGTCTGTAACTGACGCT
GAGGCGCGAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC
GATGAGTGCTAAGTGTTAGAGGTTCCGCCCTTAGTGCTGCAGCAACGCATTAAGCACTCCG
CCTGGGGAGTACGGTCGCAGACTGAACTCAAGGATTGACGGGGCCGCACAGCGTGGAGC
ATGTGTTATCGAGCACGCGAGACCTTACAGTCTGACTCTCTGACACTAGAGAATAGGCTCT

>Bacillus aerius

GGGGGCTATCTGCAGTCGAGCGGACGAAGGGAGCTTGCTCCCGGATGTTAGCGGCGGAC
GGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGAGC
TAATACCGGATAGTTCCTTGAACCGCATGGTTCAAGGATGAAAGACGGTTTCGGCTGTCAC
TTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACG
ATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTC
CTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGC
CGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCA
AGAGTAACTGCTTGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAG
CAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCG
CAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAA
ACTGGGAAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCG
TAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAG
GAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGA
TGAGTGCTAAGTGTTACGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTC
CGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGATTTGACGGGGGCCCGCACAA
GCGGTRGAGCATGGTGGTTTAATTTCGAAGCACGCGAAGAACCTTTACCAGGTCTTGACAT
CTCTGACCACCCTAGAAGATAGGGCCTTCCCTCGGGGAMAGAGTGACAGTGTGCATGATG
TCGTCAGGCTCGTGTCCGTGGAGATGGTCATAAGTCCGCACCGARGCGCCA