ENHANCEMENT IN BIOGAS PRODUCTION FROM COW DUNG USING MICROBIAL ELECTROCHEMICAL CELL (MEC) AND SCALE UP OF THE PROCESS

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LIST OF ABBREVIATION

AcoD- Anaerobic Co-Digestion

AD- Anaerobic Digestion

ASS- Atomic absorption spectrometry

BES- Bioelectrochemical system

BSA- Bovine Serum Albumin

BSP- Biogas Support Programme

BTU -British thermal unit

CMC- Carboxymethyl cellulose

CNG- Compressed natural gas

DET- Direct Electron Transfer

DNS- 3, 5-dinitrosalicylic acid

ERDG- Energy Research and Development Group

GHG- Green House Gas

HRT- Hydraulic retention time

IEA- International energy Agency

IET- Indirect Electron Transfer

L- Liter

LNG- Liquefied Natural Gas

LPG- Liquid Petroleum Gas

MEC- Microbial Electrochemical Cell

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

NBSP- Nepal- Biogas Sector Programme

OECD- Organization for Economic for Cooperation and Development

OLR- Oxygen Loading Rate

RECAST - Research Center for Applied Science and Technology

RES- Renewable energy system

RT- Room Temperature (20-25°C)

TS- Total Solids

TVS- Total Volatile Solids

TWh- Terawatt hours

V- Voltage

VFA- Volatile Fatty Acids

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ABSTRACT

Biogas, a renewable energy source produced through the anaerobic digestion of organic materials, has great potential for meeting energy needs and addressing environmental concerns. In anaerobic processes, acetate-utilizing methanogens dominate methane production, while H_2 or CO_2-utilizing methanogens are often limited by the availability of electron donors, resulting in a high CO_2 content in biogas. Traditional biogas production is often limited in colder climates because of reduced microbial activity, resulting in lower biogas yields. Researchers have demonstrated that methanogens can use external electron to produce methane from CO_2, a process known as electromethanogenesis. Microbial electrochemical cells (MECs) offer a solution to enhance biogas production, particularly in colder regions. MECs are bio-electrochemical systems that use electroactive bacteria to convert organic matter into methane through electromethanogenesis. Unlike conventional digesters, MECs can operate efficiently at lower temperatures, making them suitable for cooler climates such as mountainous or high-altitude areas. In this study, the voltage was optimized at 1.5V using a 3.5 L tubular MEC with carbon felt electrodes serving as both the anode and cathode. Gas production was observed at low temperatures of 18°C and 28°C. Prior to setup, cow dung was diluted at a ratio of 1:9 and 0.2% $NaHCO_3$ was added as a buffer to maintain a pH of 7±0.02. Furthermore, pilot-scale scaling up of the MEC was carried out using a 17L digester. Various parameters were measured, and a reduction in COD and reducing sugar was observed after 15 days of digestion. The biogas produced consists mainly of methane, carbon dioxide, hydrogen, and trace other gases. The implementation of MEC technology led to a 5% and 10% decrease in CO_2 levels, with methane concentrations rising to 8% and 14% at 28°C and 18°C, respectively. Biogas production increased significantly by 80 times and 9.27 times in the 3.5 L and 17 L digesters, respectively, compared to the control setup at a temperature of 18°C. Similarly, biogas production in the 3.5 L digester and 17 L digester increased by 2.8 and 1.20 times, respectively, at 28°C and room temperature (20-25°C). In a 3.5 L digester at 28°C, the COD reduction was 31.64% in the control group, while the MEC showed a reduction of 52.23% at 1.5V. At pilot scale, the COD reduction was 36.06% in the control group and 42.18% in the MEC. At low temperatures, the control group achieved a COD reduction of 8.48%, while the MEC setup achieved 18.70% reduction. In the pilot-scale study, the control achieved a COD removal rate of 9.76%, and the MEC achieved 18.85% reduction. In a 3.5 L digester at 28°C, the control group achieved a reducing sugar reduction of 13.15±6.79%, while the MEC setup showed a reduction of 41.40±16.24% at 1.5V. At pilot scale, the control group had a reducing sugar reduction of 14.32±9.24%, and the MEC setup achieved 21.92±6.97% reduction. At low temperatures, the control group achieved a reducing sugar reduction of 6.66±8.00%, while the MEC setup achieved a reduction of 34.50±6.68% in the 3.5L digester. In the pilot-scale study, the control group had a reducing sugar removal rate of 3.15±8.69%, and the MEC setup achieved a reduction of 17.71±3.42%. Four bacteria (SK1, SK2, SK3, and SK4) were isolated from the production media. Subsequent 16s rRNA gene sequencing identified them as *Acinetobacter cumulans, Pseudomonas aeruginosa, Exiguobacterium profundum,* and *Pseudomonas flexibilis*, respectively. This research concludes that using MEC to produce biogas is more efficient than conventional digesters at both normal and low temperatures (18°C). This

study recommends investigating the durability of electrodes, reducing costs, optimizing substrates, and diluting them for large-scale biogas production.

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

INTRODUCTION

1.1 Background

Nepal is a developing nation where 61% of people live in rural areas and 66% of workers are employed in the agricultural sector. The majority of people rely on agriculture for their livelihood, and it accounts for 23.9% of the country's GDP. However, the majority of the manufacturing is for domestic use, and this industry needs to grow (National Population and Housing Census, 2021). Animal farming is a common livelihood in Nepal, and the waste produced by these animals is often neglected and improperly disposed of due to a lack of knowledge and cost constraints. This improper disposal can lead to serious environmental and health issues such as pathogen contamination, foul odors, air and waterborne diseases, and greenhouse gas emissions (Harikis han and Sung, 2003). To address these concerns, sustainable solutions for recycling animal manure and organic waste are needed. Biogas production through technologies such as anaerobic co-digestion and pre- or post-treatment plays a crucial role in achieving these objectives and meeting the requirements of the Kyoto agreement (Holm-Nielsen et al., 2009).

Nepal, being an agricultural country, generates a large amount of agricultural waste and cattle manure annually. Unfortunately, most of this waste is disposed of in open land, and cattle manure is used as a cooking fuel or fertilizer due to a lack of knowledge and technology. During the winter season, the production of gas from digesters decreases, forcing people to rely on cattle manure as a cooking fuel, which can contribute to environmental pollution. Therefore, there is a pressing need for new biogas production technology to address this issue and generate clean secondary energy even in low temperatures. Biogas is an important component of a fully renewable energy system (RES) as it can be stored in the gas network, providing flexibility to buffer the fluctuating energy supply from secondary sources such as wind and sun, and can also be used as a fuel for transport (Hamelin et al., 2014). The process of anaerobic digestion is an intriguing way to produce renewable energy, but for large-scale commercial production, spontaneous biological reactions require a thorough understanding of the relevant processes. It is currently of utmost importance to build suitable models for use in control theory in order to optimize fermentation processes and address key issues in order to produce renewable energy from biodegradable organic waste (Fedailaine et al., 2015).

Animal dung contains complex organic molecules such as cellulose, hemicellulose, lignin, and sometimes pectin. The degradation of cellulose and hemicellulose can produce fermentable sugars like glucose, arabinose, and xylose. These sugars can be processed to create valuable byproducts such as biogas and bioethanol through anaerobic digestion (AD) and fermentation. These processes transform the sugars into useful compounds.

Anaerobic digestion is a process in which complex organic molecules from organic materials are broken down into simpler forms by microbial metabolism in the absence of oxygen. This process results in the formation of methane, carbon dioxide, hydrogen sulfide, hydrogen, and microbial biomass. An aerobic digestion has been considered as a waste-to-energy technology that is widely used for treating various organic waste types, such as the organic fraction of municipal solid waste, sewage sludge, food waste, and animal manure, etc (Pol et al., 2004).

Anaerobic digestion considerably reduces nitrate leaching into groundwater, nitrate and pathogen discharge into surface waterways, and odor emissions from storage lagoons. Elements of anaerobic digester systems have been used for decades in municipal wastewater facilities and more recently for digesting industrial and agricultural waste (Alatriste-Mondragón et al., 2006). These systems promote the growth of methane-forming bacteria that produce methane. Typically, organic waste is used as the primary input, and the systems produce biogas containing 55% to 70% methane and 30% to 45% carbon dioxide (Feng et al., 2015).

The state of the digester, the existence of microbial consortia, pH, temperature, trace elements, nutrients, the C/N ratio, and ammonia levels are some of the variables that affect the production of biogas. For the best biogas generation, these factors must be carefully maintained in a dynamic balance to encourage the growth of acetogenic and methanogenic bacteria. Maintaining the pH of the digester between 6.6 and 7.6 is good for promoting the growth of methanogenic bacteria. The availability of nutrients is also essential for improving the production of biogas. Global research is now being done to investigate how temperature affects the production of biogas. Previous studies have concentrated on mesophilic environments; however, because environmental parameters can fluctuate, researchers are also looking into thermophilic and psychrophilic situations. (Bouallagui et al., 2004) claim that anaerobic digestion can take place at temperatures below 20°C that are psychrophilic. Cow dung and other inhibition factors found in the substrate are significant players. Overuse of inhibitors can stop methanogenic bacteria from growing, which reduces the quantity of methane produced. Ensuring sufficient availability of nutrients and trace elements is crucial for the appropriate growth of microorganisms in the digester.

Bioelectrochemical systems (BES) are an emerging technology in which microorganisms act as catalysts on electrode surfaces (Hamelers et al., 2010). According to (Logan et al. 2008), applying a small voltage in specially designed microbial electrolysis cells (MECs) with electrochemically active microorganisms can lead to a high yield of hydrogen gas at the anodic side. In anaerobic digestion (AD), CO_2 gas is produced as a byproduct, which can be captured and utilized by electromethanogens to convert it into CH_4 (Cheng et al., 2009). This coupling of CH_4 production to CO_2 capture through microbial electrochemical cells holds promise for industries seeking to reduce their greenhouse gas emissions. This process involves water addition to support the hydrolysis reaction and acetogenesis stage. During the hydrolysis stage, hydrolytic microbes degrade complex organic compounds into smaller, insoluble compounds (RR, 2012). The MEC, a modified form of microbial fuel cell (MFC), has been successfully employed to efficiently store electrical energy as a biofuel, namely hydrogen (Logan et al., 2008). The study by (Villano et al., 2010) elucidated the performance of a microbial biocathode, capable of reducing carbon dioxide to methane, at rates of 0.055 ± 0.002 mol per gram of volatile suspended solids.

1.2 Current studies

1.2.1 Status of Biogas in Nepal

In Nepal, biogas technology has grown in popularity as a sustainable energy source with positive social and environmental effects. Women in rural regions have always cooked with wood, which contributes to deforestation and health problems. The demand for

firewood has decreased and interior air quality has improved as a result of the adoption of biogas systems, which provide a cleaner energy source. Significant CO_2 emission reductions, increased energy security by lowering dependency on imported fuels like liquefied petroleum gas, and improved soil quality thanks to the generation of organic fertilizers have all resulted from Nepal's successful biogas project implementation. These biogas systems use anaerobic digestion to turn organic waste, such cattle dung, into biogas, which is then used for heating and cooking. The biogas technology has been aggressively promoted by the Nepal government as a substitute renewable energy source to tackle energy shortages, especially in rural communities. However, a number of digesters have ceased to produce methane gas for a variety of reasons, most likely because the amount of gas produced is insufficient to meet energy requirements. Fixed dome below-ground biogas plants, which are adapted from Chinese and Indian fixed dome types, have been utilized in Nepal (Henderson and Eng, 1997; Khoiyangbam et al., 2004). The biogas sector has been beneficial for the country, but only 9% of its potential has been realized (Gautam et al., 2009).

Research on anaerobic co-digestion has been helpful, allowing the use of agricultural and kitchen waste along with cattle manure. (Subedi and Baral, 2021) have shown that biogas can be produced even at psychrophilic conditions in Nepal, but the enhancement of biogas production needs to be increased. The main challenge is generating biogas at cold temperatures and during the winter season. More efforts are needed to reach people in remote areas and provide them with the necessary financial and technical support. Furthermore, in order to increase the technology's adaptability across the nation, it is essential to tackle the issues related to methane production in colder climates.

1.2.2 Global improvement of biogas

A wide variety of source materials either mixed or not, can be used to produce biogas. Biogas production has great potential for sustainable energy, but current techniques are insufficient to meet demand. Biogas is a clean energy source that can reduce carbon footprints, but its full potential is not yet utilized. New methods are needed to enhance production. The concentration of methane is crucial for efficient biogas production and increased yield. Methane is essential for the potential applications and value of biogas. The energy yield from biogas depends on factors such as feedstock, pretreatment, and digestion system model. Additionally, contaminants such as corrosion, uncontrolled emissions, and health risks can have negative effects on the utilization system. Policies to regulate and promote biogas production are essential for its development (Sun et al., 2015).

Recent research has identified various techniques such as co-digestion and pretreatment as alternative methods to increase biogas production in biodigesters (Adelard et al., 2015). Various methods can be used for pretreatment in biogas production, such as physical pretreatment, rapid decompression, auto-hydrolysis, acid or alkali pretreatments, solvents targeting lignin or cellulose, leaching, supercritical, oxidative, or biological pretreatments. Other techniques include combined gasification and fermentation, integrated biogas production, innovative biogas digester design, co-digestion, and bio-augmentation (Patinvoh et al., 2017). Co-digestion involves the use of different substrates such as municipal waste, food and animal waste, and crops and animal manure, with a focus on the latter to increase biogas generation (Wangliang et

al., 2016). Co-digestion offers benefits by optimizing nutrient balances in a mixture of nitrogen-rich and carbon-rich substrates, resulting in a higher methane yield (Esposito et al., 2012). This innovative and effective strategy aims to reduce ammonia inhibition during the AD process by promoting synergies, diluting harmful compounds, improving substrate quality, and enhancing biogas production (Labatut et al., 2011). However the pretreatment process for household biogas production is energy-intensive and primarily used for industrial biogas production.

Digesters have traditionally been designed for simple digestion processes, but recent research suggests the need to upgrade designs to optimize digestion and biogas production. The goal is to create an environment that allows for efficient contact between microorganisms and the substrate. Although some compounds may effectively release dissolved intermediates for the process, it is crucial to remember that they may also hinder the anaerobic digestion process (Bolado-Rodríguez et al., 2016). Using two- or multi-stage digesters is another creative anaerobic digestion design. By doing this, the processes of methanogenesis and acidogenesis are kept apart, creating ideal conditions for the many microorganisms involved. In comparison to single digesters, this leads to improved process control and the capacity to manage higher oxygen loading rates (OLRs) (Demirel and Yenigün, 2002).

Microbial electrochemical cells (MECs) are a new technology based on microbial fuel cells (MFCs), with the addition of a small external voltage supply. Many researchers may be unfamiliar with the construction and factors affecting MEC performance. MECs share similarities with MFCs in terms of anode design and electrogenic reactions. MECs function in oxygen-free conditions and facilitate the growth of non-exoelectrogenic fermentative or methanogenic microbes as well as obligate anaerobic bacteria, such as exoelectrogenic *Geobacter* spp., *Pseudomonas* spp., and *Shewanella* spp (Liu et al., 2008).

Under the same conditions, other substrates such as glucose, starch, sodium propionate, sodium butyrate, and sodium acetate were used in MEC anodic culture experiments. With the benefits of a high hydrogen conversion rate and a large supply of substrate supplies, MEC hydrogen production is environmentally friendly. All kinds of organic wastes, including livestock manure, home sewage, activated sludge, and industrial wastewater, can be utilized as perfect substrate supplies for MEC hydrogen generation, according to several studies (Shao et al., 2019).

1.3 Rationale

Nepal's rural people rely largely on biomass fuels for cooking and heating, which contributes to indoor air pollution, deforestation, and health problems. Nepal's biogas production holds great promise for socioeconomic development, environmental sustainability, and improved energy access as it is cost-effective and environmentally friendly alternative energy source. However, because of Nepal's varied topography and climate, there are difficulties in maximizing biogas production in cold climates. The nation's diverse climate, which includes cold, high-altitude locations, can reduce the effectiveness of conventional anaerobic digestion procedures. Enhancing biogas production in colder climates or weather can improve livelihoods, lessen dependency on biomass fuels, give rural communities a clean, renewable energy source, and increase energy security.

1.4 Research hypothesis

Using renewable energy sources like biogas from animal dung is a reliable and sustainable way to produce energy that helps preserve environmental resources. Biogas technology produces a clean-burning methane-rich gas that burns with a blue flame as LPG. In areas with limited alternative fuels, enhancing biogas production can be beneficial in challenging environmental conditions. By adopting new technologies and optimizing processes, biogas output can be increased in low-temperature settings, improving yields and sustainability in Nepal.

1.4.1 Null hypothesis (H_0)

There will be no significant increase in biogas production with the use of microbial electrochemical cell in normal and cold temperature.

1.4.2 Alternative hypothesis (H_1)

There will be significant increase in biogas production with the use of microbial electrochemical cell in normal and cold temperature.

1.5 Research objectives

1.5.1 General objectives

To investigate and optimize the use of MEC to enhance biogas production efficiency in laboratory and pilot-scale settings.

1.5.2 Specific objectives

- Study the chemical composition of cow dung.
- Observation of biogas production using cow dung in lab and pilot-scale anaerobic digesters.
- Observation of biogas generation under cold conditions.
- Create a biological process that uses naturally occurring microbes to produce methane while utilizing a voltage source.
- Design electrodes setting for biogas production which mimics with existing biogas plant.
- Compare the levels of soluble reducing sugar and COD after biogas formation.
- Identify the predominant microbes in anaerobic digesters.

1.6 Research Scope

Climatic conditions have been a major factor disrupting continuous biogas production throughout the year. However, advancements in microbial electrochemical systems (MEC) have shown promise in enhancing biogas production even at low temperatures, making it a viable option in critical temperature conditions. This could provide a cheap and reliable household energy solution, especially during adverse climatic conditions when cooking fuel may be scarce. By utilizing biogas, the shortage of cooking fuel during winter and cold environmental conditions can be overcome, reducing dependence on forest products in rural areas. This sustainable use of renewable resources, such as biogas, can lead to economic benefits for the nation, reducing reliance on petroleum products. In Nepal, where agriculture and cattle rearing are predominant, utilizing byproducts such as biogas can help address fuel scarcity and benefit farmers throughout the year, regardless of climatic fluctuations. Furthermore, biogas can be used for electricity generation and as a clean fuel for vehicles, potentially displacing petroleum products. Prioritizing renewable resources like biogas can contribute to sustainable natural resource management and energy security.

LITRATURE REVIEW

2.1 Global energy demand

In 2023, the total world primary energy consumption was 178,899 terawatt hours (TWh). Approximately 86% of the energy comes from non-renewable sources, with only 13% from renewable energy. Currently, the energy demand has been met, but it is expected to increase in the coming years. This overdependence on fossil fuels will lead to a rise in atmospheric carbon dioxide level (Vaclav 2017; EIA, 2023).

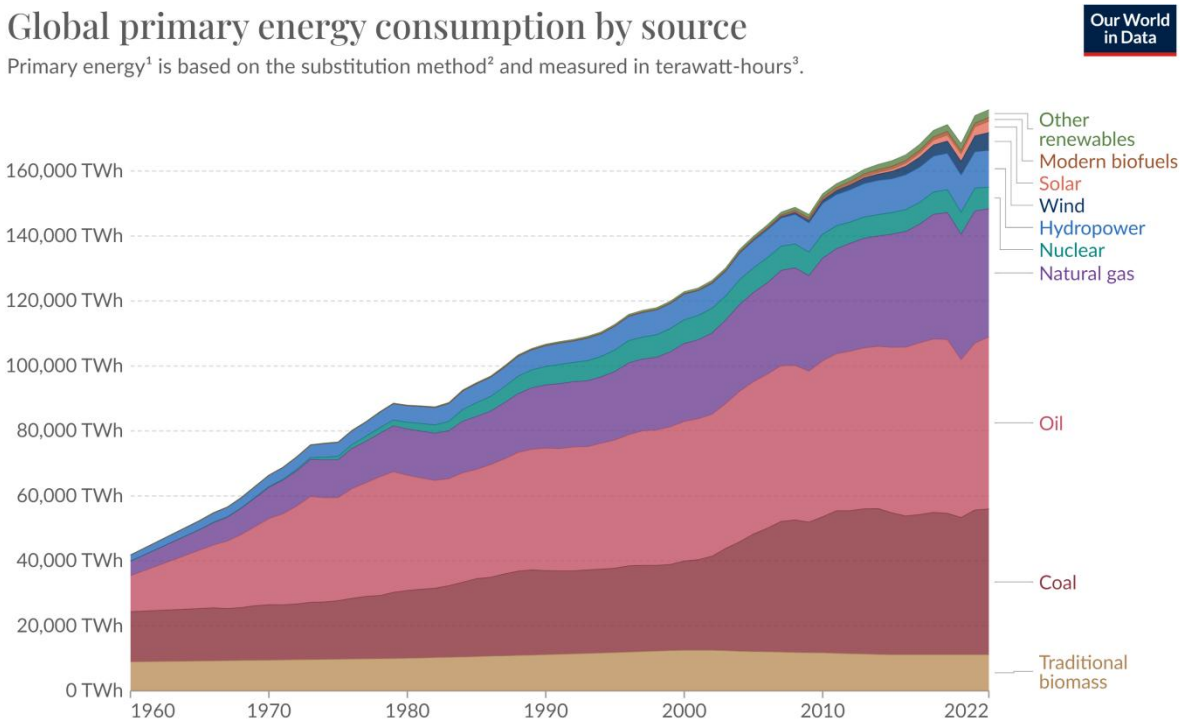

Figure 1: Energy Institute - Statistical Review of World Energy (2023)

If the rising demand for energy is not offset by enhanced energy efficiency measures, global energy consumption will keep increasing. This poses a challenge for transitioning from fossil fuels to low-carbon energy sources, as the new low-carbon energy must meet the extra demand and replace existing fossil fuels in the energy mix. Although global energy consumption is still on the rise, the growth rate seems to be slowing down, averaging about 1% to 2% annually (Ritchie et al., 2024).

2.2 Energy demand of Nepal

Nepal, classified as one of the least developed countries globally, heavily relies on imported fossil fuels despite having significant hydroelectric power potential. Traditionally, Nepal has met its energy demands primarily through sources like fuelwood, agricultural residue, and animal dung. The annual per capita demand for biomass (dry matter) in "fuelwood equivalent" is highest in mountain districts at 653 kg, followed by 660 kg in hill districts, and 435 kg in lowland districts. However, only a small percentage of households in each district have surplus biomass supply: 57% in lowland districts, 50% in hill districts, and 3% in mountain districts. The fuelwood equivalent values for various biomass sources vary, with crop residues having the lowest equivalent at 0.40 kg,

followed by dung at 0.93 kg, LPG at 23 kg, and biogas at 4.57 kg (Adhikari, 2017). Approximately 70% of the energy supply comes from fuelwood, followed by 13% from petroleum products, 3% from electricity, 1% from biogas, 4% from dung, and 6% from coal. Projections indicate a decrease in fuelwood dependency to 43% by 2030, driven by a 6% GDP growth rate, with electricity, petroleum products, and renewable energy sources poised to fill the gap (NEC, 2015).

As predicted, the supply of electricity has increased significantly due to the high implementation of hydropower. However, there is still a high dependency on fuel wood in Nepal, leading to deforestation and serious environmental damage. To address this issue, simply increasing the electricity supply is not enough. It is crucial to enhance the use of renewable energy sources to meet future energy demands and to protect the forests for the sustainable development of Nepal (Vaclav, 2017).

Nepal's economy is heavily reliant on agriculture, with 64% of the labor force engaged in this sector. Agriculture is expected to contribute 27.6% to the GDP in 2021. The major cereal crops produced in Nepal are rice, wheat, maize, and barley. Agricultural land area has grown from 37,020 sq. km in 1971 to 41,210 sq. km in 2020, at an average annual rate of 0.22%, comprising 28.7% of total land area (Malla, 2022). Livestock farming is an important part of this sector, providing energy, food, raw materials for pharmaceuticals and industrial products, and serving as a source of manure for various purposes (Walingo, 2010).

The estimated number of households in Nepal with cattle or buffaloes is 2,784,583, and the potential for biogas is in 1,937,006 households. A household with biogas can save about 250 kg of firewood per month, resulting in an annual saving of about three tons of firewood per household. This reduction in firewood consumption contributes to forest conservation. Additionally, the direct burning of cow dung is reduced by 48 kg per month. According to (Wong, 1978), non-sustainable fuelwood emits 1.5 tons of CO2 per ton, while kerosene emits 2.5 tons per 1000 liters. Based on these figures, a rural household using biogas produces approximately 4.5 tons of CO2 annually. This means each biogas system in Nepal helps prevent nearly 4.5 tons of carbon emissions per year by reducing firewood usage for cooking. Research indicates that households relying on biogas collect 1200 to 1400 kg of firewood annually, a significant reduction compared to households primarily using firewood as their cooking fuel (Somanathanet al., 2014).

2.3 Biomass as renewable energy source

According to the U.S. Energy Information Administration (EIA), biomass refers to renewable organic material derived from plants and animals. It stores the chemical energy produced by plants during photosynthesis. Biomass can be converted into liquid and gaseous fuels using various techniques, or it can be burned directly for heating. When biomass materials are burned, they emit carbon dioxide, although in significantly lower amounts compared to fossil fuels. The carbon released during their combustion is theoretically offset by the carbon absorbed during the growth of new biomass, integrating them into the carbon cycle. Biomass is used to generate heat, electricity, and liquid biofuels, making it the most widely used source of renewable energy. By using organic waste for energy production, we can achieve two important goals. Firstly, it helps to generate clean energy. Secondly, it provides a solution for disposing of large

amounts of waste that would otherwise end up in landfills, which can have serious environmental consequences (Margo, 2021).

Biomass encompasses a diverse range of materials such as wood sawdust, straw, seed waste, manure, paper waste, household waste, and wastewater. Historically, biomass resources have been utilized, but their importance has grown steadily due to their economic viability. Significant amounts of agricultural by-products are generated annually, presenting an opportunity for energy production and prompting their promotion as "energy crops" tailored for this purpose (Somanathan et al., 2014).

2.3.1 Biogas

Biodegradation of organic matter, like food waste, plant material, animal manure, and sewage, produces biogas. Biogas typically contains 40-70% methane, along with carbon dioxide and other gases (margo, 2022). Biogas, produced by the anaerobic digestion of organic matter by microorganisms, is an alternative energy source that has been used in rural and industrial settings since 1958. It offers a promising way to partially meet energy needs by utilizing specific types of biomass. Unlike other renewable energy sources, biogas is not limited by geography, and the technology required for its production is not complex or monopolistic (Balat and Balat, 2009). Biogas, also known as green energy, burns cleanly without emitting unpleasant odors when ignited, similar to the combustion of LPG or CNG (Abbasi et al., 2012). Methane gas is naturally formed through the decomposition of biomass in the absence of oxygen. This process is facilitated by a group of naturally occurring microorganisms called methane bacteria, which actively metabolize biomass (margo, 2022). The production of biogas is determined by the biodegradable organic matter content of the raw materials, as well as the C/N ratio, temperature, substrate, pH, and other factors (Akintokun et al., 2017).

Cellulose, which is found in biomass, is a polysaccharide made up of a linear chain containing hundreds to thousands of repeating D-glucose units. These units are linked together via (1-4) β glycosidic linkages. Cellulose is the most common natural polymer in the environment and is known for its biocompatibility, biodegradability, and high chemical reactivity (Ciolacu et al., 2012; French and Santiago Cintrón, 2013). Cellobiose and soluble cellulodextrin are examples of cellulose breakdown products that can be transformed into methane and carbon dioxide via a sequence of processes (Bhadra et al., 1986).

Methane can be produced naturally by microorganisms during anaerobic digestion, or it can be produced artificially by capturing carbon dioxide from fuel combustion and using renewable energy to produce electrolytic hydrogen. The hydrogen is then reacted with the captured carbon dioxide to synthesize methane. Methane (CH_4) can be synthesized from CO_2 and H_2 through an exothermic reaction and is the primary component of liquefied natural gas (LNG) (Hashimoto et al., 2001). The integration of biological and electrochemical techniques for methane production shows significant potential as it eliminates the need for precious metals in the final product. However, the use of certain inexpensive metals could further improve the efficiency of methane production(Spinner et al., 2012; Siegert et al., 2014). Electromethanogenesis is a novel approach to produce

methane by directly transferring electrons from a carbon electrode to microorganisms in the Archaea domain. These microorganisms have the ability to convert CO_2 into methane (Siegert et al., 2014). Carbon felts are eco-friendly, porous carbon electrodes commonly used for the development of electroactive biofilms in bioelectrochemical systems (Bajracharya et al., 2015).

2.3.2 Biogas plants in Nepal

The first mention of biogas in Nepal dates back to 1960 when Reverend B.R. Saubolle designed and built a biogas plant at St. Xavier's School to demonstrate how fuel could be obtained from waste (Saubolle & Bachmann, 1983). A significant turning point for biogas development in Nepal occurred in the fiscal year 1975/76, when the Biogas Development Committee (BDC) was established as part of the Energy Research and Development Group (ERDG) under Tribhuvan University. This fiscal year was also designated as "Agriculture Year" by the Ministry of Agriculture of Nepal, with biogas being a special program due to its effectiveness in controlling deforestation and preventing the burning of animal dung, which could otherwise be used as fertilizer (World Bank, 2010).

According to AEPC (2021), more than 400,000 household-level biogas plants have been installed in Nepal. However, approximately 10% of the biogas digesters are non-functional due to technical issues(Lohani et al., 2021). The biogas sector in Nepal has made significant progress thanks to the collaborative efforts of various stakeholders. The Biogas Support Program (BSP), an independent non-profit organization, has been a key contributor to this advancement, with financial support from the Netherlands (Bajgain and Shakya, 2005; Nakarmi and Karki, 2005).

2.3.3 Biogas production

A wide range of organic wastes can be utilized for methane production through anaerobic digestion. These include crop residues, animal manure, food waste, paper and cardboard waste, yard waste, organic sludge, brewery waste, energy crops, sewage sludge, and algal biomass (Farghali et al., 2020). The use of these substrates not only leads to methane generation but also contributes to waste minimization. As a result, waste treatment plants that utilize various organic substrates to produce biofuel and electricity are prevalent in many countries (margo, 2021). Table 2.1 presents various substrates alongside their respective methane content ranges.

Table 1: Different substrate and their methane content

Substrate	Methane content range	References
Crop Residues	30-60%	(Angelidaki & Ahring, 2000)
Animal Manure	50-70%	(Zhang et al., 2019)
Food Waste	50-70%	(Rajagopal et al., 2013)
Paper/Cardboard	40-60%	(Rafieenia & Karimi, 2017)
Yard Waste	30-50%	(Ma et al., 2015)

Organic Sludge	50-70%	(Mata-Alvarez et al., 2000)
Food Processing Waste	40-60%	(Li et al., 2011)
Brewery Waste	60-80%	(Chandra et al., 2012)
Maize/Sugar Cane	50-70%	(Weiland, 2010)
Miscanthus/Switchgrass	40-60%	(Kiesel et al., 2017)
Sewage Sludge	50-70%	(Appels et al., 2008)
Algal Biomass	40-60%	(Passos et al., 2014)

2.4 Anaerobic digestion

Anaerobic digestion (AD) is a biological process that involves the breakdown of carbonaceous matter, proteins, and lipids by various microbial consortia, resulting in the production of biogas. This process is similar to biological gasification, which also leads to methane (CH_4) production through the breakdown of biomass. The decomposition occurs in an oxygen-deprived environment, converting organic material into methane (CH_4), carbon dioxide (CO_2), and water. Prior to biogas generation, intermediary molecules such as carbohydrates, hydrogen, and acetic acid are used. Raw biogas normally includes 40 -75% CH_4 and 25 - 65% CO_2, however for best use as a fuel source, the methane percentage must be raised (Chynoweth et al., 2001).

Anaerobic digestion occurs in various natural environments, such as wetlands, landfills, and the digestive systems of animals, as well as in engineered systems like anaerobic digesters. It is a sustainable and environmentally friendly method for managing organic waste and producing renewable energy. Furthermore, the remaining material, called digestate, is nutrient-rich and can be used as fertilizer. Anaerobic digestion is widely applied in agriculture, wastewater treatment, and managing organic solid waste (Weiland, 2010).

The production of biogas through anaerobic digestion requires human labor for tasks such as sourcing, collecting, and transporting feedstock, manufacturing technical equipment, as well as constructing, operating, and maintaining biogas plants. This suggests that there is potential for the development and establishment of new ventures in biogas production within the country. These plants offer significant economic potential, contributing to income growth in rural areas and creating new employment opportunities(Comino et al., 2009).

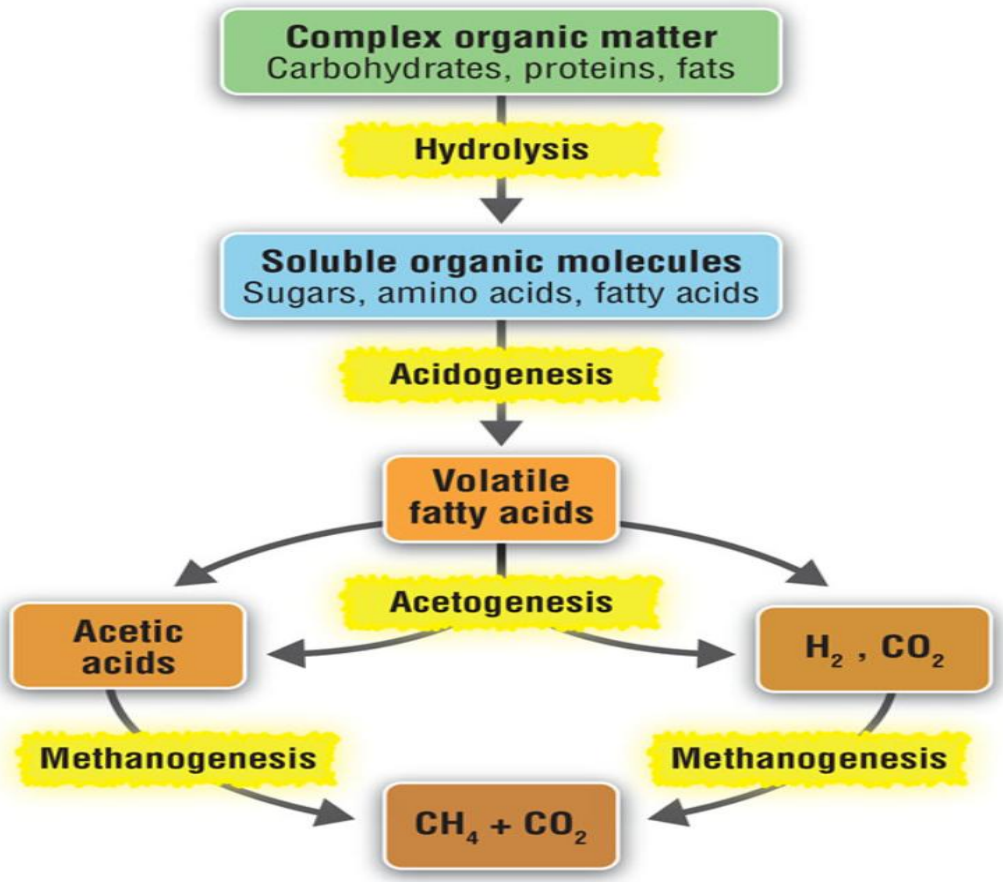

Figure 2: Flow chart of methane formation(Demirel, 2014).

The process of biogas production is complex and relies on various groups of microorganisms. According to (Demirel, 2014), anaerobic digestion involves several important stages, including hydrolysis, acidogenesis, acetogenesis, and methanogenesis. The main source of methane production comes from the conversion of acetate, accounting for 70%, while hydrogen conversion contributes only 30%. This may be due to the limited availability of factors such as hydrogen or other reducing agents, which restrict carbon dioxide reduction.

2.4.1 Phases in Anaerobic Digestion

Anaerobic digestion consists of four distinct stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis.

2.4.1.1 Hydrolysis

Hydrolysis of organic polymers within biomass is a rate determining step responsible for breaking down complex organic compounds into simpler units. This step is vital for both biogas production and the generation of various organic compounds like sugars and organic acids. These components, encompassing sugars, amino acids, and peptides, serve as accessible sources for bacteria. In anaerobic digestion, hydrolysis represents the initial and indispensable stage. Through this process, complex organic molecules are fragmented into simple sugars, amino acids, and fatty acids. Consequently, biomass residues yield hydrogen and volatile fatty acids (VFAs). Acetate and hydrogen serve as direct inputs for methane production, while volatile fatty acids undergo further processing in the acetogenesis phase(Sun and Cheng, 2002).

2.4.1.2 Acidogenesis

In the second stage of anaerobic digestion, known as acidogenesis, complex organic molecules like proteins, lipids, and carbohydrates are broken down into simpler organic substances like hydrogen, alcohols, NH_3, CO_2, hydrogen sulfide and volatile fatty acids (VFAs) by acidogenic bacteria. Enterobacter, Bacteroides, and Clostridium are a few of the bacteria that are active in this process (Angelidaki and Sanders, 2004).

2.4.1.3 Acetogenesis

The volatile fatty acids (VFAs) generated in the acidogenesis stage are further metabolized by acetogenic bacteria during acetogenesis. These VFAs are transformed into acetate and other basic organic acids, like acetic acid, butyrate and propionate. Since it supplies the substrates required for the final stage of anaerobic digestion to produce methane, this step is crucial.

Acetogenic bacteria, including Acetobacterium, Clostridium, and Syntrophobacter, are the main causes of acetogenesis. By interacting syntrophically with other microbes, these bacteria help break down complex organic materials into simpler molecules (Garcia-Peña et al., 2011).

2.4.1.4 Methanogenesis

Methanogenesis is a terminal stage in anaerobic digestion, where simple organic compounds like acetate, hydrogen, and carbon dioxide are converted into methane (CH4) and carbon dioxide (CO2) by methanogens, leading to biogas production. Methanogenesis is influenced by factors such as temperature, pH, substrate composition, and the microbial community within the anaerobic digester (Chandra et al., 2012). Methanogenesis is sensitive to pH levels between 6.5 and 8. The undigested material and dead bacterial remains make up the digestive waste. It is important to note that methanogenesis is sensitive to pH levels, with the optimal range for the process being between pH 6.5 and pH 8. Any remaining indigestible material and dead bacterial remains make up the digestate (Thauer et al., 1993).

2.5 Microrganisms in different phase of AD

Microorganisms play a crucial role in the multi-step process of anaerobic digestion, which produces biogas, including methane, from organic matter. In the initial stage, complex organic compounds such as proteins, lipids, and carbohydrates are broken down into simpler, chemically stable substances like sugars, amino acids, and fatty acids. This process is primarily driven by methane and carbon dioxide production through the symbiotic interactions of anaerobic and facultative anaerobic hydrolytic bacteria (Naik et al., 2010). These microorganisms initiate the breakdown of polymers and monomers, yielding acetate, hydrogen, and various volatile fatty acids like propionate and butyrate. Hydrolytic bacteria secrete enzymes such as cellulose, cellobiase, xylanase, amylase, lipase, and protease to facilitate this process. Strict anaerobes such as Bacteroides and Clostridium, as well as facultative bacteria like Streptococci and Enterobacteriacece, are primarily responsible for hydrolysis (Bryant, 1979). During the acidogenesis phase, the majority of bacteria are facultative anaerobes, utilizing any remaining oxygen inadvertently present. This creates a conducive environment for the growth of obligate anaerobes such as *Pseudomonas, Bacillus, Clostridium, Micrococcus, or Flavobacterium*. Studies have shown that actinomycetes and mixed consortia, which are cellulolytic

bacterial strains, can increase biogas production from cattle dung by 8.4–44% (Attar et al., 1998).

During the acetogenesis phase, hydrogen and carbon dioxide are converted into a mixture of methane and carbon dioxide. This process is primarily carried out by methanogenic bacteria such as *Methanosarcina spp.* and *Methanothrix spp.,* as well as hydrogen and formate-utilizing species like *Methanobacterium* and *Methanococcus*. Acetate bacteria, including genera such as *Syntrophomonas* and *Syntrophobacter*, play a key role in converting acid phase products into acetates and hydrogen, which can then be utilized by methanogenic bacteria (Schink, 1997). It was discovered that methanogenic bacteria that bind hydrogen are members of the *Methanobacteriaceae* family (Boone et al., 1993). Methanogenes differ morphologically in a significant way. Methanogenes are found in nearly every form found in bacteria, including cocci (*Methanococcus*), filiforms (*Methanothrix*), rods (*Methanobacterium*), short rods (*Methanobrevibacter*), and spirillaceae (*Methanospirillum*).

Methanogens, a specialized group of archaea, convert carbon dioxide, hydrogen, and acetic acid into methane and water during the final methanogenic phase. These anaerobic microbes thrive in oxygen-free environments and are sensitive to temperature, pH, and harmful compounds (Buan, 2018). Each microbial group in the anaerobic digestion (AD) process affects the system's stability and functionality. Maintaining a balance between methanogens and acidogens is crucial to prevent acidification, which can inhibit methanogens and reduce biogas yield (Cho et al., 2013). Understanding and managing the microbial communities within AD systems are key to maximizing biogas production and optimizing AD efficiency.

2.6 The impact of substrate types on digestion efficiency

Anaerobic digestion is a process that converts biomass into methane, and during this process, the rheological behavior of the sludge is crucial for heat and mass transport (Miryahyaei et al., 2019). According to (Triolo et al., 2011; Barakat et al., 2014; Monlau et al., 2012), the lignin content is a significant parameter that affects the methane production potential. The complex composition of lignocellulosic biomass poses challenges for the biodegradation of solid organic waste by microorganisms. The hydrolysis of organic matter into soluble compounds presents a bottleneck in anaerobic processes, particularly for high-solid-content wastes. The substrate's protein, fat, fiber, cellulose, hemicellulose, starch, and sugar content significantly influence methane production (Comino et al., 2009). Maintaining equilibrium between the methanogenic and acidification processes is essential for the efficient anaerobic breakdown of organic wastes. When the acidification process surpasses the methanogenic process, volatile fatty acids (VFAs) can accumulate in the reactor. This accumulation may lower the pH and impede the functioning of methanogenic archaea (Lerm et al., 2012; Li et al., 2013). Therefore, this process can be divided into two phases: acid formation and methane formation.

The products produced in different phases of anaerobic digestion are distinct, leading to the involvement of different microorganisms in each phase. At the end of the anaerobic digestion process, acetate, methane, CO_2, and H_2 gas are generated. These products can be harmful to the growth of initial digesting bacteria, particularly in the case of complex substrates like lignocellulosic biomass, where slow digestion is crucial to prevent a

buildup of acidic concentrations. High acidity can inhibit the growth of methanogenic bacteria, making the rate-limiting step dependent on the substrate used for biogas production.

Maintaining a delicate balance between acid-forming and methane-forming microorganisms is challenging and crucial for reactor stability and optimal methane yield. Physical separation of the two main groups of microorganisms has been explored as a strategy to leverage their different growth kinetics for maximizing methane production. Various techniques, including membrane separation, kinetic control, and pH control, have been employed to achieve phase separation and enhance biogas production efficiency (Demirel and Yenigün, 2002; Fernandes, 1986).

2.7 Role of various parameters in anaerobic digestion

The performance of anaerobic digestion (AD) is influenced by several key parameters, including temperature, pH, organic loading rate (OLR), carbon-to-nitrogen (C/N) ratio, nutrients, trace elements, and hydraulic retention time (HRT).

2.7.1 Role of temperature

Temperature plays a crucial role in the anaerobic digestion process for methane production, affecting the activity and efficiency of microbial communities responsible for organic matter degradation and methane generation. The temperature directly impacts microbial activity and methane production efficiency. Temperature fluctuations can disrupt microbial growth, potentially reducing methane production. Additionally, an imbalance in acidogenic and methanogenic microorganisms' availability can lead to system instability, influenced by the operating temperature (Hupfauf et al., 2018). Different microorganisms thrive within specific temperature ranges, with optimal temperatures for methane-producing bacteria like Methanobacterium and Methanosarcina typically falling within the mesophilic range of 25-40°C or the thermophilic range of 50-60°C.

The digestion process can occur at different temperature ranges: psychrophilic (<20°C), mesophilic (25-40°C), and thermophilic (50-60°C). The methane yield varies at these temperature conditions due to microbial growth effects. Mesophilic and thermophilic conditions are commonly utilized in commercial-scale anaerobic digestion (AD) systems to maximize methane production rates (Nie et al., 2021).

At lower temperatures, the microbial community's activity decreases, slowing down organic matter degradation and methane production. Conversely, higher temperatures can denature the enzymes involved in anaerobic digestion, reducing methane production efficiency (Buhr and Andrews, 1977). Thermophilic archaea stabilize cellular components by synthesizing heat shock proteins or related proteins at high temperatures (Lloyd et al., 2005). In contrast, cold-adapted psychrophiles often modify their membranes structurally by increasing the levels of unsaturated lipids to maintain normal membrane fluidity and function during cold exposure (Dev et al., 2019). (Ahring et al., 2001) found that raising the operational temperature from 55 to 65°C can disrupt the balance between acid-producing and acid-consuming microorganisms, leading to increased ammonia accumulation, acidic conditions, and higher levels of volatile fatty acids (VFAs). The acidic environment inhibits the growth of methanogenic organisms, resulting in decreased methane production (Angelidaki and Ahring, 1993). Therefore,

maintaining the ideal temperature within the digester is crucial for maximizing methane yield and ensuring the stability of the anaerobic digestion process.

2.7.2 Role of pH

The pH level in anaerobic digestion is critical for microbial communities and their metabolic activities, impacting digester stability and performance. The ideal pH range for methane production is typically 5.5 to 8.5, but can vary depending on microbial composition. The optimal pH range for methanogenic bacteria is 6.5 to 8 (Boe, 2006). At higher pH levels, there is a risk of ammonia toxicity, which may be caused by excessive methanogenesis. This can inhibit the activity of methanogenic microorganisms. Another greatest risk of digester failure arises from excessive acid buildup, which is characterized by a rapid reduction in pH brought on by an increase in volatile fatty acids (VFAs). Because of the increased levels of organic acid produced by this spike in acidogenic bacterial activity, the pH falls below 5.0, which is lethal to methanogens, resulting in a decrease in methane production. However, this can be counteracted by increasing the amount of fresh feedstock (Ostrem et al., 2004).

2.7.3 Role of oxygen loading rate (OLR)

Microorganism's growth rate is determined by their oxygen loading rate (OLR), which also affects how starved they are. A high OLR results in fast growth of microorganisms in anaerobic digestion, while a low OLR inhibits their growth due to starvation. Higher OLRs can increase biogas production, but they may reduce specific biogas and methane yields due to the accumulation of volatile fatty acids (VFAs) that can inhibit the methanogenic phase of anaerobic digestion. Oxygenation can help stabilize anaerobic digesters during start-up or unstable periods with high VFA accumulation, preventing digester souring, a common issue in anaerobic digestion. Oxygenation can help stabilize anaerobic digesters during start-up or unstable periods with high VFA accumulation, preventing digester souring, a common issue in anaerobic digestion. Reactor temperature and substrate feeding rate determine OLR rate (Jiang et al., 2020; Liu and Tay, 2004).

2.7.4 Role of C/N ratio

The relative amounts of carbon and nitrogen in the feedstock are measured by the C/N ratio, which can have an impact on the anaerobic digestion process's overall performance as well as microbial activity. The C/N/P ratio is crucial for microbial growth as these elements are essential for protein synthesis, amino acids, and ammonia production. Ammonia also acts as a buffer to neutralize acidity in anaerobic digestion. Therefore, feedstock must have the correct C/N ratio. (Rajeshwari et al., 2000) suggest a C/N/P ratio of approximately 100:3:1 for optimal growth of methanogenic bacteria. A low C/N ratio can lead to ammonia production and a pH increase above 8.5, which is detrimental to methanogenic bacteria. Conversely, a high C/N ratio can reduce methane production due to nitrogen deficiency, which is essential for methanogen digestion (Azizt et al., 2015).

2.7.5 Role of nutrients and trace element

For more than three decades, deficiencies in trace elements within anaerobic digestion (AD) processes have been acknowledged, affecting the growth of microorganisms involved. The decision to supplement nutrients and trace elements in the substrate used for AD depends on the specific substrate being utilized . Organic waste and manure contain adequate amounts of micro and macro nutrients, making deficiencies irrelevant. Nutrients like nitrogen, phosphorus, sulfur, calcium, and magnesium are crucial for the growth and metabolism of microorganisms in anaerobic digestion (Rorat et al., 2019). (Lindorfer et al., 2012) reported that the macronutrients, salt and phosphorus, exhibited the greatest variance, while the micronutrients, molybdenum, nickel, and cobalt, showed the least deviation. But there was also a significant amount of variation in iron, manganese, zinc, selenium, tungsten, boron, and selenium.

2.7.6 Role of $NaHCO_3$

Sodium bicarbonate acts as a pH buffer to stop the buildup of volatile fatty acids, which is necessary for the anaerobic digestion process that produces methane gas (Valença et al., 2021). But in order to prevent changing the dominant methanogens and bacteria and preventing methane production, its concentration needs to be closely monitored (Lin et al., 2013). It may significantly increase biogas production and stable pH levels when used as a pretreatment, like with corn stalk (He et al., 2019). Sodium bicarbonate aids co-digestion processes balance the acidity of substrates and provides the ideal pH environment for bacteria that produce methane (Ghaleb et al., 2020). The presence of sodium bicarbonate in anaerobic digestion facilitates the formation of calcium carbonate. Calcium carbonate then acts as a catalyst, promoting the conversion of carbon dioxide into methane, consequently enhancing methane yield (Valença et al., 2021).

2.8 Biochemistry and microbial background

2.8.1 Microbiology of methanogens

Microbiology in AD plants is often poorly understood, with limited research on the actual microorganisms present in bioreactors. Recent studies show that AD plants in close proximity can have different microbial communities. Certain microbial strains can enhance reactor performance, particularly methanogens, which are crucial for methane production. Methanogens lack catalase and superoxide dismutase, making them highly sensitive to oxygen. This sensitivity poses challenges in understanding their biochemistry, physiology, and ecology.

Methanogens are a class of archaea that are essential to many ecosystems because they can produce methane as a metabolic byproduct in anaerobic environments (Thauer, 2007). Their metabolic processes have a major role in the generation of biogas, the cycling of carbon, and the emission of greenhouse gases (Conrad, 2009). The ecological relevance and adaptability of methanogens are highlighted by their habitat diversity, which includes wetlands, animal digestive tracts, and anaerobic wastewater treatment systems (Ferry, 2010). Methanogens are indispensable components within microbial food chains, comprising at least three interconnected metabolic groups of strictly anaerobic bacteria. These bacteria collaborate to transform complex organic biomass into carbon dioxide (CO_2) and methane (CH_4). Initially, the intricate organic compounds

undergo conversion into a mixture of volatile fatty acids, alcohols, carbon dioxide, and hydrogen (H_2) (Abbanat et al., 1989). In anaerobic reactors, the primary carbon exchange takes place between fermentative microorganisms and methanogens, resulting in the generation of methane and CO2. In this process, essential components include enzymes like methyl coenzyme M reductase (MCR) and coenzymes such as coenzyme F420 and coenzyme M, which play vital roles in facilitating electron transfer and carbon fixation reactions (Dhakephalkar et al., 2019). Approximately 20 to 30% of the carbon undergoes conversion into intermediate products throughout this process (Mackie and Bryant, 1981). In addition to methanogens, anaerobic methane oxidizers utilize methane as a substrate to reduce sulfate and nitrate. These microorganisms help to oxidize methane in anaerobic environments (Welte et al., 2016).

Methanogenic bacteria can be classified into two groups: acetoclastic methanogens, which use acetate to produce methane and carbon dioxide, and hydrogenotrophic methanogens, which use carbon dioxide from hydrogen to produce methane (Demirel, 2014).

Methanogen carry out the following reactions.

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, classified within the Archaea domain, are a unique group of microbes. They belong to the phylum Euryarchaeota and are known for their ability to produce methane using metabolic processes that involve hydrogen and carbon dioxide. They possess unique membrane lipids and enzymes crucial for methane production, such as methyl-coenzyme M reductase, a pivotal enzyme in the methanogenesis pathway (Dhakephalkar et al., 2019). Factors such as cell wall composition, sensitivity to antibiotics, translation and transcription machinery, and strict requirements for anaerobic culture conditions contribute to their unique characteristics (Lange and Ahring, 2001). Despite their vast evolutionary diversity, methanogens can only use a certain quantity of glucose as a substrate (Woese, 1987).

Methanobacterium and *Methanobrevibacter* are members of the Methanobacteriales family, whereas *Methanosarcina* belongs to the Methanosarcinaceae family. Methanogens are divided into several families depending on their morphological and physiological properties. Some methanogens grow at low temperatures, while others may live in high-salinity settings. Their habitat and substrate utilization vary according to their species. Marine methylotrophic genera include *Methanolobus*, *Methanococcoides*, and *Methanosarcina acetivorans* (Jones, Nagle, and Whitman., 1987). Further isolates have been acquired from extreme habitats, including geothermal springs.

Methanobacterium thermoautotrophicum and *Methanothermus fervidus* were isolated from terrestrial hot spring waters (Zeikus et al., 1980). However, acetotrophic methanogens are less frequently encountered in hot springs.

Methanogens are additionally found in artificial environments like biogas plants, landfills, and digesters. Depending on the type of substrate present, various habitats have different microbial communities. Complex polymers are hydrolyzed into sugars and amino acids in biogas plants, whereupon they undergo fermentation to yield acetate, hydrogen, and carbon dioxide, which serve as substrates for the methanogenic process. Consequently, mesophilic biogas plants typically contain hydrogenotrophic and aceticlastic methanogens, with *Methanosarcina* and *Methanoculleus* being the most prevalent species (Kern et al., 2016; Karakashev et al., 2005; Lucas et al., 2015; Sundberg et al., 2013). However, syntrophic acetate oxidation might be the main mechanism for methane synthesis in some circumstances (Schnürer and Nordberg , 2008; Westerholm et al., 2016).

2.8.3 Electromethanogenesis

Electromethanogenesis is an innovative process that converts carbon dioxide directly into methane using electricity as an energy source. This process is facilitated by electromethanogens, which are electroactive microorganisms that use electrical currents to reduce carbon dioxide to methane (Lovley and Nevin, 2013). In this case, methanogens can directly take electrons from electrodes to reduce CO_2 ($CO_2 + 8H^+ + 8e^- \rightarrow CH_4 + 2H_2O$) (Villano et al., 2010).

Acetogenic bacteria convert CO_2 and H_2 to acetate anaerobically using the reductive acetyl-CoA pathway (Ljungdhal, 1986). In the case of MES, reduction takes place at the biocathode using electric energy rather than an external supply of reductants like hydrogen. The bacteria in the biocathode can acquire electrons directly from the cathode or indirectly through mediators or hydrogen generated during water electrolysis. The first proof of principle of carbon dioxide reduction in microbial electrosynthesis was demonstrated by (Nevin et al., 2011) using the acetogen *Sporomusa ovate*. At a cathode potential of 0.6 V/Ag/AgCl, organism can directly reduce carbon dioxide by utilizing electrons from solid graphite electrodes, creating acetate and a small quantity of 2-oxobutyrate.

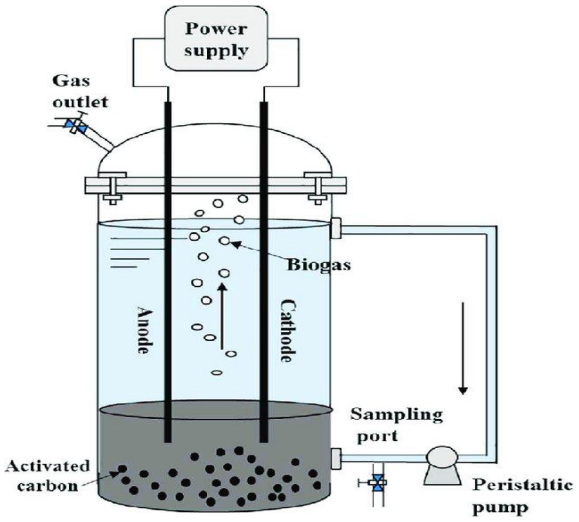

Figure 3: Schematic diagram of the MEC-AD combined system

At anode: $C_6H_{12}O_6 + 12H_2O \longrightarrow 6HCO_3^- + 30H^+ + 24e^-$

Anode potential E0 = -0.41V (Rozendal et al., 2006)

At cathode: $HCO_3^- + 8e^- + 9H^+ \longrightarrow CH_4 + 3H_2O$

Cathode potential E0 = -0.24V (Rozendal et al., 2006)

Bio-methanation has been presented as an option for smaller-scale applications (Götz et al., 2016). Several Archaea microorganisms are known to catalyze the conversion of carbon dioxide to methane by autotrophic methanogenesis in strictly anaerobic settings (Bapteste et al., 2005). Hydrogenotrophic methanogenesis, a well-known process, is frequently used in large-scale anaerobic digestion facilities as one of the primary metabolic pathways for methane synthesis from biomass (Premier et al., 2013). Furthermore, electrotrophic methanogenesis or electromethylogenesis has recently been identified as an alternate mechanism for carbon dioxide reduction (Cheng et al., 2009).

Several unique biotechnological applications of these biochemical pathways have been proposed for methane synthesis by lowering CO_2 via electrostimulation of methanogenic microbial communities in bioelectrochemical systems (Blasco-Gómez et al., 2017). One technique is to force electron flow from externally applied electrochemical potentials to encourage methane synthesis, even in the absence of favorable electron donors such organic molecules (Bajracharya et al., 2015). However, the system's thermodynamics are more favorable when the electron donor at the anode is an organic molecule (Jadhav et al., 2017; Logan and Rabaey, 2012). In this scenario, the oxidation reaction is facilitated by acetoclastic electroactive microbes that release electrons to the conductive surface of the anode (You et al., 2014). Acetoclastic methanogens are frequently observed in anodic biofilms of bioelectrochemical systems, where they compete with electroactive microorganisms for the same electron donor, acetate (Rago et al., 2018).

2.8.4 Electroactivity of methanogens

2.8.4.1 Electron transfer

When electrodes are placed into a reactor containing methanogens, the organisms can use them to create methane. An external potential causes electrolysis of water at the anode, which produces oxygen and protons. Electrons are transmitted to the anode, where extra electrons from metabolic reactions can also be delivered, much like in a microbial fuel cell. The electrons are subsequently sent to the cathode via an external connection. At the cathode surface, electrons are delivered to methanogens, allowing them to create methane. While the exact mechanism is unknown, three major concepts have been proposed: indirect electron transfer (IET), mediated electron transfer (MET), and direct electron transfer (DET). It is probable that at least one of these pathways aids in electron transport (Geppert et al., 2016; Sydow et al., 2014; Zhen et al., 2016).

Indirect Electron Transfer (IET): Electrons are transferred from the cathode to protons that have been generated at the anode and passed through the membrane between the anodic and cathodic chambers. This process results in the production of hydrogen at the cathode, which is subsequently utilized in methanogenesis. This Indirect Electron Transfer (IET) enables the conversion of hydrogen and carbon dioxide into methane (Villano et al., 2010).

Mediated electron transfer (MET): Mediator molecules may accumulate electrons on the cathode surface, convey them to methanogens, and finally deliver them to microbes. Methanogens make methane by transferring electrons, protons, and carbon dioxide (Choi and Sang, 2016). Flavins, phenazines, and quinones can operate as mediators, either naturally produced by organisms or introduced to the reaction medium (Patil et al, 2012; Sydow et al., 2014). MET in methanogens could be facilitated by the use of natural redox compounds as electron shuttles (Park and Zeikus, 1999).

Direct electron transfer (DET) happens when electrons are delivered directly from the cathode surface to methanogens, possibly via surface proteins or conductive filaments. Methanogens make methane by combining electrons, protons, and carbon dioxide (Cheng et al., 2009). Studies have revealed that direct electron transfer occurs in methanogens (Lohner et al., 2014; Zhen et al., 2016). In a research with a hydrogenase-deficient strain of M. maripaludis, electron uptake was shown to occur independently of hydrogenase, ruling out indirect electron transfer (IET) (Lohner et al., 2014).

2.9 Advantages of anaerobic digestion

Anaerobic digestion is a commonly used and preferred technique for treating organic waste and producing renewable energy due to its numerous advantages.

2.9.1 Energy source

Anaerobic digestion, sometimes referred to as waste-to-energy technology, is a common method for breaking down a variety of organic wastes, including animal manure, food waste, sewage sludge, and organic material from municipal solid waste (Li et al., 2009). Biogas can be used for heating properties, generating power, and fueling vehicles. It is considered a green energy source because it reduces reliance on fossil fuels and helps decrease greenhouse gas emissions (Westerholm et al., 2016).

2.9.2 Environmental benefits

Anaerobic digestions systems help reduce greenhouse gas emissions by capturing methane produced during the process and using it as a renewable energy source. This offsets emissions from fossil fuel combustion and promotes carbon sequestration, contributing to mitigating climate change (RA, 1995). Approximately 18% of global warming is attributed to human-generated methane emissions. Both CO2 and CH4 are potent greenhouse gases (GHG). Anaerobic digestion aids in recycling the carbon cycle and decreasing GHG emissions by producing methane gas as a byproduct (Viéitez and Ghosh, 1999).

2.9.3 Pathogen Reduction

According to (Bendixen, 1994), animal feces includes numerous pathogens that might damage the environment and harm human health. Anaerobic digestion in mesophilic and thermophilic environments can help manage these bacteria. Animal excrement often contains dangerous germs such as E. coli and Enterococci, as well as intestinal parasites. Improper waste disposal may damage human health, although anaerobic digestion can slow the growth of these pathogens (Larsen et al, 1994). The anaerobic digestion process operates at elevated temperatures, typically between 35°C and 55°C,

which helps inactivating pathogens present in the feedstock. This makes anaerobic digestion an effective method for sanitizing organic waste and reducing the risk of disease transmission (Hassen et al., 2001).

2.9.4 Low Operating Costs

Anaerobic digestion systems offer cost-effective waste treatment and energy generation, particularly when combined with biogas utilization. Proper system design and management can result in low operating costs compared to other waste treatment technologies (Al Seadi et al., 2013).

2.9.5 Digested as fertilizer

Biogas technology is a versatile solution that uses organic resources to generate electricity and high-quality compost or digestate for fertilizer. The slurry produced during the digestion process is an excellent fertilizer that is immediately available to plants (Tafdrup, 1995).

2.10 Prevalent challenges in existing biogas plant

Anaerobic digestion is a complex process involving four stages to produce methane gas. Various obstacles can hinder this process, including issues such as hydraulic retention time, temperature, pH, microbial flora, and ammonium accumulation. In Nepal, specific challenges related to anaerobic digestion have been discussed below.

1. Methane leakage: The biogas plant's design frequently causes significant methane leakage during the start-up phase. In addition, tiny leaks may occur as a result of construction materials such as sand, cement, or concrete (Lohani and K.C, 2021).

2. Technical limitations: Temperature is a critical factor affecting plant performance, with seasonal fluctuations potentially reducing biogas production by up to 50% (Araldsen L, 2016). This is why nearly one-third of users only receive sufficient gas during the summer months. Additionally, the climate restricts the spread of biogas technology to the mountainous regions of Nepal, with most installations concentrated in the southern districts (Bajgain and Shakya, 2005).

3. The substrate's hydraulic retention period is critical for producing energy from complex organics. Commercializing this method is essential to meet future energy demands. However, the high retention time of the substrate also increases the cost of energy production. (Sreekrishnan et al., 2004) reported that the hydraulic retention time increases over the winter months, resulting in a drop in biogas production. Therefore, more efforts are needed to address these limitations and optimize the process for efficient energy production.

2.11 Approach used to solve the challenge

2.11.1 Raising the temperature of the digester

Temperature variance is a crucial factor affecting gas production fluctuation. Gas production significantly decreases at temperatures below 15 °C. In some regions of northern China, gas production is halted during winter, and operations are limited to the peak gas production months (Paudel, 2019). Despite this, many bio gas users still rely on traditional fuels to some extent (Nakarmi et al., 2015), which is not ideal as the benefits of using bio gas are maximized when avoiding the use of firewood. Various techniques exist to raise digester temperature, but they are not widely adopted due to the need for

specialized knowledge, materials, and commitment.

2.11.2 Insulation

Insulating the digester is the most energy-efficient way to keep the digestion temperature stable and decrease energy loss to the environment. This can be accomplished at a low cost by utilizing locally available materials with high thermal conductivity, such as cereal straws, rice husks, sawdust, and shavings. These organic materials decompose to produce compost, which releases heat and raises the system temperature. A study discovered that covering the digester with compost increased biogas production by more than 50% throughout the winter. The compost pile, which was 0.7 to 0.8 meters tall, was covered with a plastic sheet, generating a greenhouse atmosphere (Lau et al., 2012). However, this method may produce an unpleasant odor due to the composting process not being fully contained.

2.11.3 External heating

Solar radiation serves as the principal external heating source for basic digesters. To maximize incoming energy, situate the digester in a cleared area facing the sun, preferably on a high side. Preheating feed ingredients by mixing and exposing them to the sun can also be effective. According to research, this approach can boost influent temperature by 4.5 to 9 °C, depending on daily conditions (Lau et al., 2012). Since solar radiation does not get very deep into the slurry, it is recommended to use a mixing tank with a clear plastic sheet, a high surface area, and a shallow depth. It has also been demonstrated that using sun reflectors or creating a greenhouse above the digester yields beneficial results. (MingErgy Pvt. Ltd., 2014).

Simple biogas systems usually do not require complicated heating systems. However, it is possible to raise the temperature of the digester by exploiting heat lost from an engine or generator via a heat exchanger. This strategy can also benefit the engine by delivering heat to the digester. The heat exchanger can be used to preheat feed materials or inside the digester, but the latter may cause corrosion damage (MingErgy Pvt. Ltd., 2014).

Other preheating options, such as using a portion of the biogas produced by industrial facilities to heat the digester, are sometimes expensive and inefficient for small-scale digesters. Small-scale plants cannot use 20-30% of the produced gas to heat the digester.

2.11.4 Anaerobic co-digestion (Acod)

Animal manures have a lower carbon (C) percentage than other organic wastes. To address this, anaerobic co-digestion processes are gaining popularity. This technology provides biogas production, which improves the economic sustainability of production (Hamelin et al., 2014). Anaerobic co-digestion also provides benefits such as dilution of potentially toxic compounds in co-substrates, adjustment of moisture content and pH, provision of required buffer capacity, increased biodegradable material content, and a broader range of bacterial strains participating in the process.

2.11.5 Pretreatment

Animal dung is a complex organic material made up of cellulose, hemicelluloses, lignin, and other components that are difficult for microorganisms to digest efficiently. This results in a longer digestion time for cattle manure, leading to higher costs for biogas production. Pretreatment methods aim to break down these obstacles and make organic matter more available to the microbial community, improving the efficiency of the digestion process. Various pretreatment approaches, including physical, chemical, physicochemical, and biological methods, have been developed to enhance the biogas production process (Taherzadeh and Jeihanipour, 2012).

2.11.6 Two stage digestion

Comino et al., (2009) suggest that two-stage anaerobic digestion outperforms single-stage digestion by delivering more rapid and stable treatment. The process consists of two chambers: the first for hydrolysis and acidogenesis, and the second for acetogenesis and methanogenesis. (Blonskaja et al., 2003) discovered that using a two-stage system to handle distillery waste boosted methanogenic population growth as well as biogas generation. However, in fact, the promised benefits of two-stage digestion, such as increased hydrolysis and methanization rates, have yet to be reliably confirmed (Viéitez and Ghosh, 1999).

MATERIALS AND METHODS

3.1 Materials

The present study was executed at the Central Department of Biotechnology, Tribhuvan University, Kirtipur, Nepal. The majority of required materials, equipments, bacteriological media (Hi-Media Company, India) and reagents were provided by department. All the chemicals were of reagent grade.

3.2 Collection of substrate and inoculum

For this experiment fresh cow dung was used as substrate and collected from cow of Sahiwal, Jersy cross and Holistein Friesian cross of Chovar Agro. Farm, Chovar premises, Kirtipur (2023-01-18). The feedstock for biogas digester was obtained from all irrespective of the categories. The cows were daily supplied with grass, hay, grains and legumes. All cows at the farm were de-wormed by oxfendazole tablet and vaccinated as per farm schedule.

For the inoculums, enrichment of microorganisms was done in 5 Liters reagent bottle maintaining anaerobic conditions for 20 days at 28 °C.

3.4 Compositional analysis of substrate:

For the chemical composition, cow dung was subjected to analyze pH, reducing sugar, total soluble solid (TSS), volatile soluble solid (VSS), moisture content. The phosphorus concentration was evaluated using standard methods for the examination of water and wastewater (APHA. (1998), 20th Ed.). Trace elements (iron, copper, zinc, cadmium, lead) that may influence the growth of microbial biota in the experiment were also analyzed using the AAS process after digestion.

3.4.1 Determination of total suspended and volatile suspended solids

To calculate the total suspended solids (TSS), cow dung sample was weighed. The sample was dried to a constant weight at 105±1 °C and left to dry overnight. After overnight drying the sample was weighed again, and the TSS value was calculated according to the formula given in the Appendix I. Moisture content percentage of the cow dung was calculated from the difference of mass between before and after drying. To calculate the volatile suspended solids (VSS), the TSS sample was transferred to a crucible and ignited at 550 °C for 1.5 hrs in muffle furnace. The VSS calculation was carried out at Central Department of Chemistry, TU (Prajapati, 2021).

3.4.2 Determination of reducing sugar

Reference solutions with concentrations from 10µg/mL to 200µg/mL were made using a 1000µg/mL glucose stock solution. Each reference solution's final volume was 3mL. A 3ml of deionized water and the sample was placed in separate test tubes. Then, 1ml of DNS (Di-Nitro Salicylic Acid) reagent was added to each test tube, and the tubes were boiled for 8 minutes. The change in color from yellow to orange indicated the presence of glucose. A UV-Spectrophotometer (UV-1800) was used to measure absorbance at 540nm, and background correction was carried out using the blank solution's absorbance value (Smith and Cresser, 2003).

3.4.3 Determination of Chemical Oxygen Demand

A standard curve was created using a range of reference solutions with concentrations from 20 mg/L to 600 mg/L to determine the chemical oxygen demand (COD). The solutions were prepared by diluting a stock of 1000 mg/L potassium hydrogen phthalate to a final volume of 2 mL. To each reference solution, blank, and sample, 1.2 mL of Digestion Solution was added, followed by 2.8 mL of catalyst solution (as outlined in Appendix II). The tubes were then shaken after each addition. Subsequently, the tubes were placed in a digester and heated in a hot air oven at 150°C for two hours. After digestion, the tubes were cooled, and background correction was performed by measuring absorbance at 600 nm using a UV Spectrophotometer (UV-1800). COD digestion is indicated by an increase in trivalent chromium ions, causing the digestion solution to turn green. A pale blue color indicates less COD digestion (Smith and Cresser, 2003).

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

The cow dung sample weighing approximately 0.6 g was placed in a 50 mL volumetric flask and allowed to come below the neck of the flask. Then, 3.3 mL of the digestion mixture was added, and four carborundum beads were introduced. The sample was carefully swirled until moistened. The flask was left to stand overnight, and two blank digestions were also prepared. The flask was then heated on a hot plate at 180 °C for about 1 hour. After cooling, 5 drops of H_2O_2 was added, and the flask was placed back on the hot plate and heated to about 280 °C for 10 minutes until the water had evaporated. This process was repeated until the digest turned colorless. After cooling, 10 mL of water was added and mixed well, and the mark was made up with distilled water. The digest was then filtered to remove any SiO_2 that could interfere with the determinations. The calibration solution for the analysis was prepared in the same final medium as the samples to ensure a consistent matrix. The final medium had 0.8 M H_2SO_4 . (Temminghoff, E., & Houba, V., 2004)

3.4.3.1 Determination of iron, copper, zinc, cadmium, and lead

The prepared digestive solution was submitted to the National Academy of Science and Technology (NAST) to determine the levels of iron, copper, zinc, cadmium, and lead.

3.4.3.2 Determination of phosphorus

Standard curve of phosphorus

A series of reference solutions with phosphorus concentrations of 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, and 0.5 mg/L were prepared by diluting the phosphorus stock solution to appropriate volumes.

Determination of phosphorus

The blank and digestive solutions were mixed in a 1:9 (v/v) ratio with deionized water. Then, 1mL of the diluted blank and digest sample was pipetted into a test tube. Next, 3.8mL of diluted mixed reagent (Appendix-I) was added and mixed. The solution was allowed to stand for an hour, and the absorbance was measured in a 1cm cuvette at a wavelength of 880 nm using a UV-spectrophotometer (UV- 1800) (Temminghoff, E., & Houba, V., 2004).

3.5 Scale up process

The digestion of sample was carried out in 1L, 3.5L, and 17L anaerobic digesters. The digestion was conducted at the Central Department of Biotechnology using batch digesters. The cow dung and water concentration in the digester was maintained at a ratio of 1:9 (v/w) in all digesters (Jeppu et al., 2022).

3.6 Optimization of electrode setting in MEC reactor

Carbon felt electrodes (11cm×3.5 cm×0.2cm) were used to make a 1 L MEC for the optimization of biogas production. The electrodes were set up in an inverted T shape in aspirator bottle, while erect parallel electrodes were set up in a conical flask. The shift was made from inverted T shape, erect to tubular electrodes.

3.7 Experimental design, MEC construction and operation

A single-chambered MEC constructed from plexiglass was utilized in the research, with a total capacity of 4 L. A pair of tubular carbon felt electrodes with diameters of 3.6 cm and 8.5 cm (Nippon Electrode Co Ltd, Japan) was used to construct a MEC anaerobic reactor. The electrodes were 24.5 cm in height and were inserted into a 3.5 L biogas digester with a height of 30 cm and a diameter of 10 cm. The experiment was conducted in working volumes of 3.5 L, and 17 L, with external electricity supplied at 1 Volt, 1.5 Volt, and 2 Volt. In the 17 L MEC digester, a 4 cm diameter anode electrode with a height of 37 cm was used, along with four cathode electrodes measuring 37 cm in height and 4.5 cm in width.

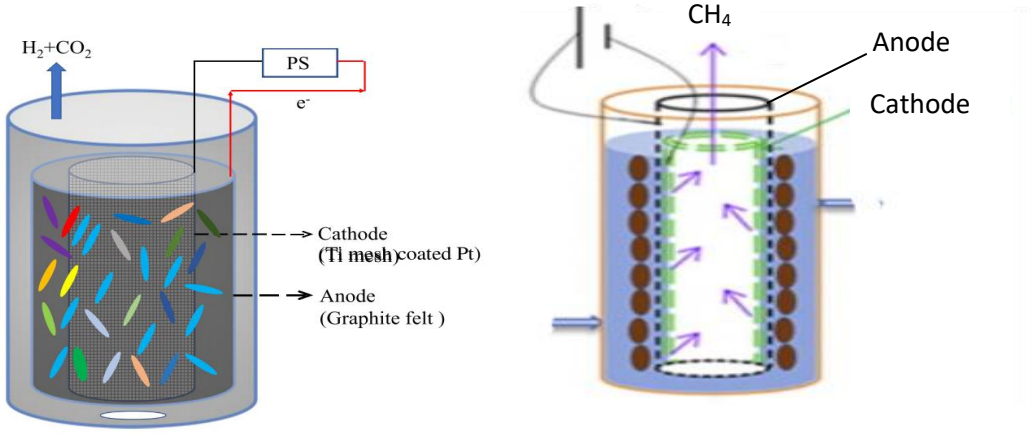

Figure 4: Figures A and B illustrate the fabrication process of the tubular MEC design (Kadier et al., 2016).

A control experiment was carried out in a similar reactor without the application of voltage. Oxygen was removed from the reactor by bubbling nitrogen gas for 5 minutes before digestion. Wax and silicone were applied to the caps (cork) of the reactor to achieve an anaerobic state, and aquarium oxygen pipes were inserted through the caps for gas collection. Gas collection was done by downward displacement of water and 1 M KOH solution, and the biogas in the measuring cylinder was measured. The reactors were operated in batch mode for 15 days at temperatures of 18 °C, 28 °C, and room temperature (20 °C – 25 °C). For the 3.5 L and 17 L reactors, steel electrodes were also used, and the process was carried out at an optimized voltage of electricity.

3.8 Electrode treatment

The graphite electrode was subjected to ultra-sonication with 70% methanol for 15 minutes, followed by ultra-sonication with 70% acetone and distilled water, and treatment under UV light for 15 minutes to remove fine and organic particles. Finally, it underwent heat treatment at 80°C for 2 hours. Before use, the electrodes were treated with UV light for about 15 minutes (Liu et al., 2017).

3.9 Monitoring methane production with and without electrical supply

Methane gas monitoring was conducted by displacing KOH solution in each working volume, and a portable infrared syngas analyzer (Gasboard-3100p) from Hubei Cubic-Ruiyi Instrument Co., Ltd. for 17 L digester. The gas analyzer was capable of detecting various types of gases such as CH_4, CO_2, H_2, N_2, etc.

3.10 Liquid Sampling and Analysis of COD, Reducing Sugar, and pH

Each of 10 mL of liquid samples was taken from the 1 L, 3.5 L, and 17 L digesters for analysis of COD, reducing sugar, and pH. The pH of the samples was measured using a pH meter (HANNA PI2002) and recorded. Total biogas production was measured by displacing water, and methane concentration was determined by displacing 1 M KOH.

3.11 Isolation and Identification of Bacteria

3.11.1 Isolation of methanogen

A hundred microliters of the fresh inoculum were spread on an agar plate enriched with DSMZ 825 methanogen enhancement medium. The plate was sealed with parafilm, labeled, and placed in an anaerobic jar at 37°C in an incubator for 24 hours. Various bacterial colonies on the plate were selected, and pure cultures were isolated. Using aseptic technique, the bacteria were streaked on an agar plate containing DSMZ 825 methanogen enhancement media. The streaked agar plate was sealed and placed in an anaerobic jar at 37°C in the incubator for growth (Prajapati, 2021).

3.11.2 gDNA extraction from isolated organism

The sample pre-processing involved a series of various steps (William et al., 2004). Initially, a 30ml sample was placed in a Falcon tube and centrifuged at 1000rpm for 2 minutes to remove unwanted debris. Subsequently, the resulting supernatant was carefully transferred to a new Falcon tube and underwent further centrifugation at 3500rpm for 40 minutes. The pellet containing bacteria was then processed separately. To do so, it was re-suspended in 300µl TE buffer and subjected to another round of centrifugation at 5000rpm for 10 minutes. After centrifugation, the supernatant was discarded, and the pellet was re-suspended in 300µl CTAB buffer (10%) for further centrifugation process.

After centrifuging the pellet suspension at 1200rpm for 1 minute and discarding the supernatant, the pellet was re-suspended in 567µl TE buffer by repeated pipetting. Then, 30µl of 10% SDS and 3µl of 20mg/ml Proteinase K were added to the pellet, mixed thoroughly, and incubated at 37°C for 1 hour to induce viscosity due to cell lysis by the detergent. After that, 100µl of 5M NaCl was added and mixed thoroughly to maintain the salt concentration above 0.5M, which is crucial for preventing CTAB-nucleic acid precipitation.

Next, 80 µl of CTAB/NaCl solution was added to the mixture and thoroughly mixed before incubating at 65°C for 10 minutes. Then, an equal volume of Chloroform: Isoamyl alcohol (24:1) was added, mixed, and centrifuged at 13000rpm for 5 minutes to remove CTAB-Protein/Polysaccharides complexes.

The upper aqueous layer was collected and mixed with an equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) before centrifugation at 13000rpm for 5 minutes. The resulting supernatant was collected and mixed with 0.6ml chilled isopropanol for nucleic acid precipitation. After centrifugation at 13000rpm for 8 minutes, the supernatant was discarded, and the pellet was washed with 70% ethanol while centrifuging.

After the ethanol wash, the pellet was air-dried, re-suspended in 40 µl TE buffer, and quantitated using Nano-drop. Gel electrophoresis was then performed with 0.8% Agarose in TAE for 45 minutes at 90 volts, and the gel was visualized under a UV-transilluminator or Gel Doc for analysis.

3.11.3 PCR amplification of gDNA

The genomic DNA (gDNA) was amplified using 16s rRNA universal primer from the GenScript kit. The forward primer 27F, U1971-C070-27F-01, had the sequence 5'-AGAGTTTGATYMTGGCTCAG-3', while the reverse primer 515R, U1971-C070-515R-02, had the sequence 5'-TTACCGCGGCKGCTGGCAC-3' (S. H. Kim et al., 2012). Following are the ingredients used to create the PCR mixture in PCR tubes:

Table 2: 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.

After mixing the components (1, 2, 3, 4, 6, 7, and 8), and performing a short spin, 2.5 µL templates were added to each tube. The PCR solutions containing the relevant templates were thoroughly mixed and centrifuged. It was placed in a PCR machine that had already been programmed with the following conditions.

Table 3: 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	40 sec
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 (Prajapati, 2021).

3.10.5 Sequence analysis of the amplicons

The amplicons were sent to Center for Molecular Dynamics Nepal (CMDN), Thapathali, for the sequencing. The sequencing was performed using both forward and reverse primers for both DNA strands.

3.10.6 Sequence editing and alignment

The acquired 16S rRNA sequences were inputted into the MUSCLE algorithm for multiple sequence alignment using MEGA11 software. Subsequently, the data from the batch were subjected to phylogenetic analysis.

3.10.7 Construction of phylogenetic tree

Phylogeny tree was reconstructed by Neighbor-joining (NJ) method with bootstrap value 1000 in MEGA v.11 software. The NJ tree was created with K2P distance as the genetic measure and negative branch length set to zero using uniform distribution rates.

RESULTS AND DISCUSSION

The research involved collecting cow dung samples from Sahiwal, Jersey cross, and Holstein Friesian cross cows at Chovar Agro Farm in Chovar, Kirtipur (2023-01-18). The study aimed to optimize biogas production from cow dung at different temperatures (28°C and 18°C) with low energy input. The analysis included assessing the chemical parameters of the substrate such as pH, moisture content, soluble sugars, and other components. Biogas production experiments were conducted in digesters of varying sizes (1 L, 3.5 L, and 17 L) at different temperatures (15°C, 28°C, and room temperature) with different voltage inputs (1V, 1.5V, and 2V). The optimal voltage for biogas production was determined to be 1.5V, which was used to scale up the biogas production process. Four dominant facultative anaerobic bacteria were isolated from the MEC digester, which aid in biogas production. Subsequently, DNA extraction and amplification were carried out, followed by Sanger sequencing at CMDN, Thapathali, Kathmandu.

4.1 Determination of chemical and physical parameters of cow dung substrate

The study analyzed the chemical components and physical parameters of the substrate that are essential for anaerobic digestion. Physical parameters including TSS, VSS, moisture, and pH were immediately analyzed in fresh cow dung samples. Trace elements such as iron, copper, zinc, cadmium, and lead were also determined. Other parameters such as COD, reducing sugar, and phosphorus were measured after diluting the samples. The measured values of these parameters in cow dung substrates are presented in Table 4.

Table 4: Determination of chemical and physical parameters of substrate

Analytical parameters	Amount (Mean±S.D)
Chemical oxygen demand (mg/g)	13.51±1.61 mg/g
Soluble reducing sugar (mg/g)	2.52±0.81 mg/g
Total phosphorus (mg/g)	0.136±0.010 mg/g
pH	6.87±0.33
Iron	28.23 ppm
Copper	0.74 ppm
Zinc	1.917 ppm
Cadmium	0.05 ppm
Lead	0.2 ppm
TS (Total solids)	15.81±0.231%
TVS (Total Volatile solids)	80.66±0.577%
Moisture content	84.18±0.231%

TS, TVS and moisture content were analyzed, and found to be 15.81±0.231%, 80.66±0.577% and 84.18±0.231% respectively. The initial concentration of COD and soluble reducing sugar was found to be 13.51±1.61 mg/g and 2.526±0.81 mg/g respectively. The initial concentration of phosphorus was found to be 0.136±0.010 mg/g. The analysis of trace and heavy metals on a collected sample contains 28.23 ppm iron, 0.74 ppm copper, and 1.917 ppm zinc. According to (Irshad et al., n.d.), the limits of cadmium and lead in cattle dung manure were 0.5 ppm and 35.6 ppm, respectively. The amounts of these elements in the dung sample were found to be lower than the specified limits.

Environmental parameter evaluations were conducted to determine the substrate's biophysical and chemical state. According to research, the nature of the substrate is an important factor in determining the need for improvements. The substrate's nature directly impacts microbial biomass and can lead to issues such as process instability, low loading rates, slow recovery after failure, and particular needs such as total solids (TS), Total volatile solids (TVS), trace elements, and nutrients in waste composition (Sreekrishnan et al., 2004; Van den Berg, 1983). According to (Tomlinson et al., 1996), TS and TVS values should be between 15% and 20%, and 10% to 15%, respectively. In this study, the Total Solids (TS) fall within the expected range, but the Total Volatile Suspended Solids (TVS) exceed the range at 80.66%. The high TVS/TS ratio suggests a high organic content in the sample. TSS testing quantified the total concentration of suspended (non-soluble) solids in the sample. TS data is essential when analyzing the operational behavior of waste treatment systems (Gujer and Zehnder, 1983). TS values in a sample often correlate with excessive solid production induced by an increase in BOD loading, or they can signal bacterial concerns such as nutritional shortage (Feng et al., 2015). The existence of phosphorus (P) in cow manure samples emphasizes its importance as a fertilizer for crops. (Lindorfer et al., 2012) discovered that phosphorus and iron concentrations were highest, while other elements were at their lowest levels.

4.2 Comparison of electrode settings in different 1L apparatus

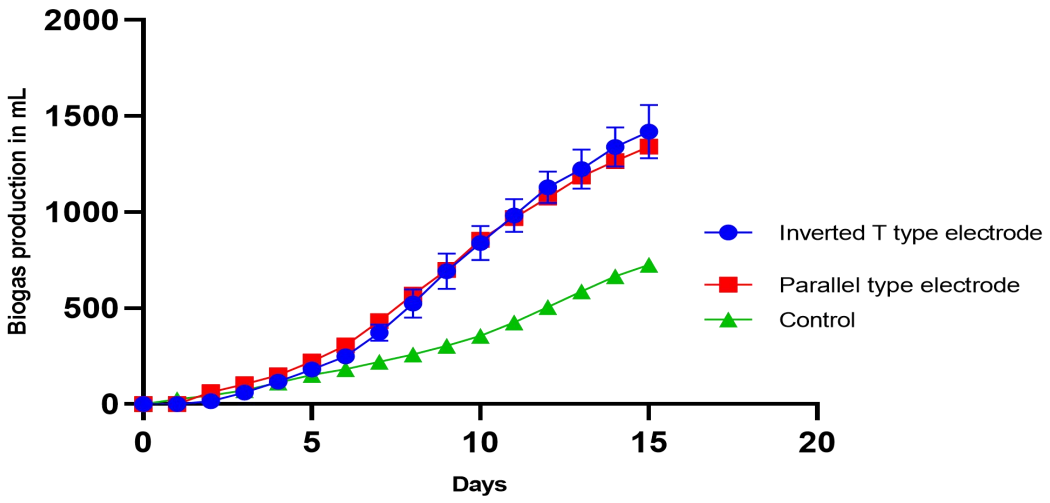

Figure 5: Comparison of electrode settings at 28°C in different 1L digesters supplied with a 2V potential difference (p.d).

Comparisons of electrode settings were conducted to produce methane gas in a 1L reactor using different types of reactors. An aspirator bottle and a conical flask were utilized as reactors. In the aspirator bottle, an inverted T-shaped electrode (11cm×3.5cm×0.2cm) was installed, while in the reagent bottle, the electrode was positioned in parallel at a distance of 2.5cm from each other. The experiment was conducted by setting up control reactors and supplying an external voltage of 2V to the MEC reactors, which were maintained at 28°C. The voltage level was selected based on a study by Poudyal, (2018). After 15 days, gas production measurements were taken, resulting in 725.5±9.192mL, 1322±137.885mL, and 1335±9.192mL of gas produced in the control, aspirator, and reagent bottle reactors, respectively. Gas production in the tests was found to be nearly the same in volume. Therefore, a parallel positioning of electrodes was chosen for further experiments as it was easier to maintain anaerobic conditions. Additionally, the parallel positioning of electrodes makes it easier to increase the electrode area inside the reactor, as the digester area increases. (Cheng and Logan, 2011; Yaqoob and Ibrahim, 2020) found that increasing the carbon electrode surface in MECs with external voltage can boost methane gas production by improving microbial colonization, electrochemical activity, and electron transfer efficiency.

Table 5: Comparison of gas production from 1 L batch setups with different electrode configurations and MECs operating at 2V. (Summary).

S.N	Electrode setting (1L)	Temperature	Voltage supplied(V)	Gas produced(mL) (Mean±S.D)
1	Control	28°C	-	725.5±9.192
2	MEC (Parallel type)	28°C	2 V	1335±9.192
3	MEC (inverted T type)	28°C	2 V	1322±137.885

4.3 Biogas production at 28°C and 18°C in 3.5L reactors

In this study, biogas production was conducted at two different temperatures: 28°C and 18°C. The experimental setup involved maintaining temperatures of 28°C and 18°C. External voltages of 1V, 1.5V, and 2V were applied to 3.5 L digester to facilitate biogas production. In all setups, a single-chamber glass tubular digester with a tubular carbon felt and steel electrode was used to operate the MEC system. Study conducted by (Kadier et al., 2016) found that single-chamber glass tubular was 33% more efficient than other types of MEC. The electrode size used in the MEC was a length of 24.5 cm with a diameter of 3.6 cm for the inner electrode (cathode) and 8.5 cm for the outer electrode (anode). During the digestion process, changes in gas production, soluble reducing sugar, chemical oxygen demand (COD), and pH levels were monitored in order to evaluate the efficiency of biogas production. The levels of COD, reducing sugar, biogas production, and pH in the experimental setup with varying voltages were compared to those in the control setup for 3.5 L digesters.

4.3.1 Voltage optimization at 28°C in 3.5L reactors using carbon felt electrode

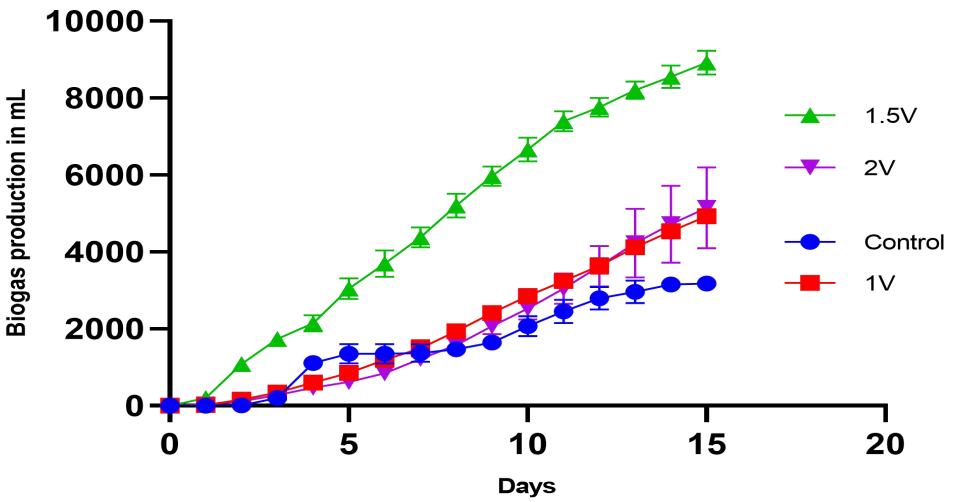

Figure 6: **Effect of voltage on biogas production at 28°C.**

The findings demonstrate that a voltage supply of 1.5 V yields the highest biogas production compared to the control, 1 V, and 2 V. When voltage was supplied in the range of 1-2 V, the production of gas was observed at 28 °C. The highest biogas yield was achieved at 1.5 V. The amount of gas produced on the 15th day was 3275±49.49 mL, 4925.75±32.88 mL, 8920±311.12 mL, and 5114±1052.17 mL for the control, 1 V, 1.5 V, and 2 V potential supplies, respectively. On average, biogas production was increased by 2.7 times when a MEC was supplied with 1.5 V.

Table 6: Comparing the effect of voltage on gas production in different 3.5 L batch setups. (Summary)

S.N	Digester volume (3.5L)	Temperature	Voltage supplied(V)	Gas produced(mL) (Mean±S.D)
1	Control	28°C	-	3275±49.49
2	MEC	28°C	1 V	4925.75±32.88
3	MEC	28°C	1.5 V	8920±311.12
4	MEC	28°C	2 V	5114±1052.17

4.3.2 Comparison of Biogas production at 18°C and 28°C

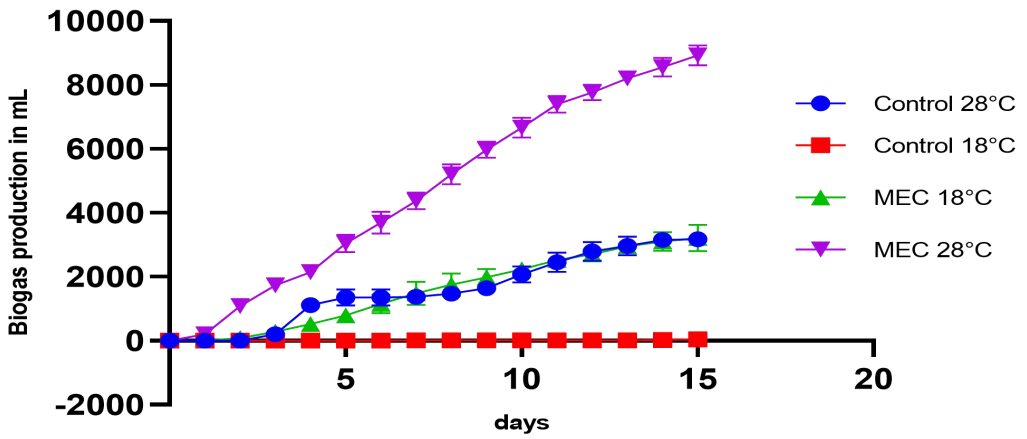

Figure 7: **Comparison of gas production at 18°C and 28°C between MEC and control.**

In the MEC, a voltage of 1.5 V was supplied, resulting in significantly higher gas production compared to the control at both temperatures. This study aims to compare the biogas production rates at two different temperatures, 18°C and 28°C. The experiment involved monitoring the biogas production over a period of time at each temperature to determine the impact of temperature on the rate of biogas production. The results showed that biogas production was higher at 28°C compared to 18°C, indicating that temperature has a significant effect on the efficiency of biogas production.

In this research, the main objective was to improve biogas production at low temperatures. It was found that biogas production at 18°C significantly increased when a specific voltage was applied. The biogas produced at 18°C with a 1.5 Volt electric potential was 80 times higher (3215mL) than the biogas produced in the control group without electric potential (40mL). At 28°C and 1.5 V, the gas production in the control was 3175±49.49 mL, while in the MEC it was 8920±311.12 mL. The biogas production in the MEC was 2.8 times higher than the gas produced in the control group without any electrical potential.

Table 7: Comparison of gas production at 18°C and 28°C between MEC and control. (Summary).

S.N	Digester volume (3.5L)	Temperature	Voltage supplied(V)	Gas produced(mL) (Mean±S.D)
1	Control	28°C	-	3175±49.49
2	MEC	28°C	1.5 V	8920±311.12
3	Control	18°C	-	40±14.14
4	MEC	18°C	1.5 V	3215±410.12

4.3.3 Comparative Analysis of Biogas Production Efficiency Using Carbon Felt and Steel Mesh Electrode

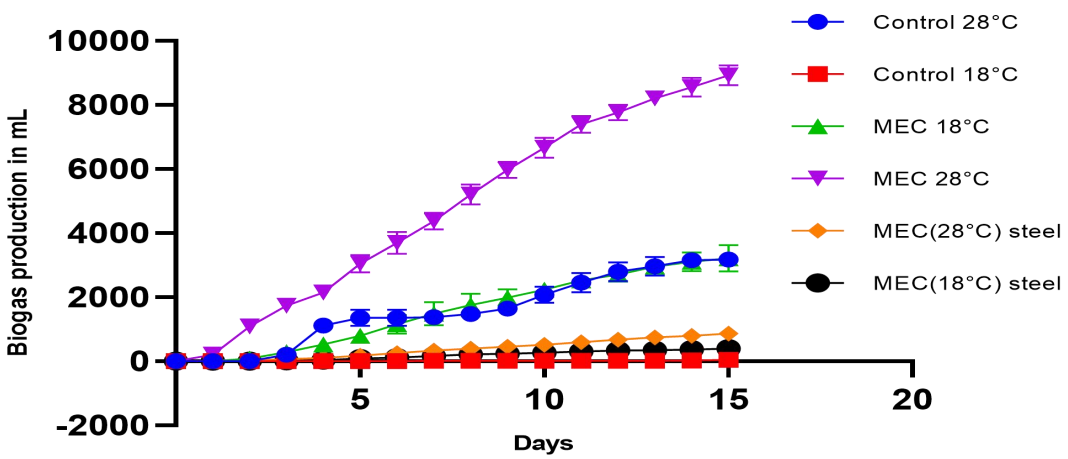

Figure 8: Comparison of gas production at 18°C and 28°C between carbon felt and steel mesh electrodes using a voltage of 1.5 V.

The graph illustrates the daily biogas production in a controlled experiment, showing a trend of higher production at higher temperatures. This aim to determine which electrode material is more effective in enhancing biogas production at 18°C and 28°C temperature.

Comparison of electrode types revealed that the carbon felt electrode outperformed the steel mesh electrode. At 28°C, the MEC with carbon electrode generated 8920±311.12mL of gas, while the MEC with steel electrode produced 862.5±38.89mL of gas, resulting in a 10 folds increase in biogas volume. Similarly, at 18°C, the MEC with carbon electrode produced 3215±410.12mL of gas compared to 390±42.42mL of gas produced by the MEC with steel electrode, resulting in 8 fold increase in biogas volume.

The biogas production from the MEC with steel electrode was lower than the control group at 28°C, but at 18°C, the MEC exhibited higher biogas production per day than the control group.

Carbon felt was chosen as the electrode in the Microbial Electrolysis Cell (MEC) due to its inert nature and high surface area for bacterial growth (Bajracharya et al., 2015). Carbon felt is known for its high electrical conductivity, chemical stability, and mechanical strength (Merkoçi et al., 2005). The electrochemical reduction of carbon dioxide to valuable chemical products is crucial for the carbon dioxide cycle. Research conducted in the central department of Chemistry by Thapa in 2024 shows that a steel carbon electrode is efficient for biogas production from $NaHCO_3$ solution without corrosion. So, to reduce the cost of biogas production, a steel mesh electrode was utilized because of its affordability and easy availability in the local market. While steel mesh electrodes offer high electrical conductivity and mechanical strength, they are less inert than carbon electrodes. Low gas production in MEC with steel electrode may be due to corrosion and a decrease in effective surface area and electrical conductivity. This reduction in efficiency can impact the overall gas yield in the electrolytic process used for biogas production. Additionally, the corrosion of steel electrodes can result in the release of iron into the electrolyte solution, which may have a toxic effect on the

microorganisms involved in biogas production, inhibiting their growth and metabolic activities (Logan et al., 2006). Also, steel electrodes provides a lower surface area compared to materials like carbon-based electrodes, potentially limiting microbial colonization and biogas production rates (Siegert et al., 2014). Due to these reasons, MEC with a steel electrode was not further investigated.

Table 8: Comparison of gas production at 18°C and 28°C between carbon felt and steel mesh electrodes using a voltage of 1.5 V. (Summary).

S.N	Digester electrode type (3.5L)	Temperature	Voltage supplied(V)	Gas produced(mL) (Mean±S.D)
1	Control	28°C	-	3175±49.49
2	MEC carbon	28°C	1.5 V	8920±311.12
3	MEC steel	28°C	1.5 V	862.5±38.89
4	Control	18°C	-	40±14.14
5	MEC carbon	18°C	1.5 V	3215±410.12
6	MEC steel	18°C	1.5 V	390±42.42

4.3.4 Gas collected after absorption of CO_2

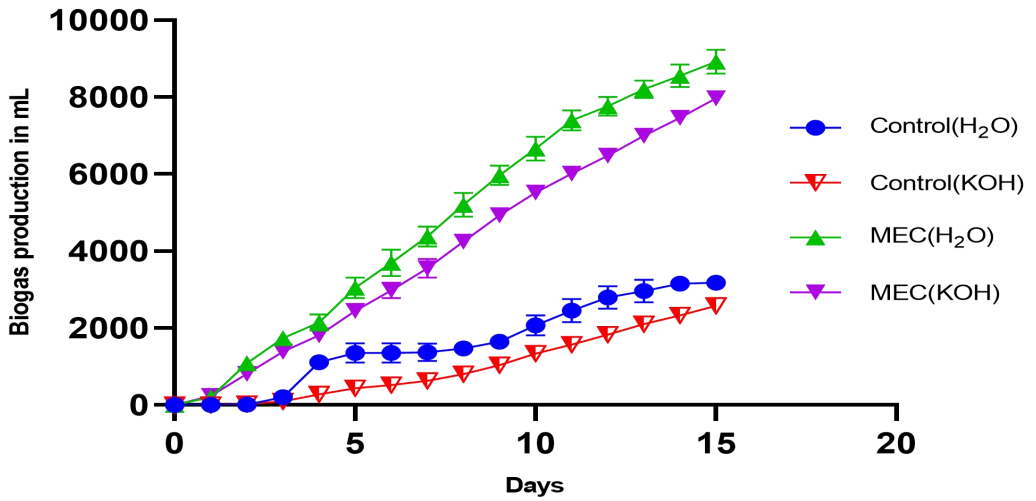

Figure 9: Gas collection with and without the displacement of KOH solution in control and test 3.5 L digester. In test a voltage of 1.5 V was supplied.

Amount of methane in biogas was determined by the downward displacement of a 1M KOH solution. CO_2 was physically absorbed through chemical absorption in the liquid solution, reacting with the alkaline compound to produce carbonate (CO_3) and bicarbonate (HCO_3) ions (Mani et al., 2008). The gas produced in the control without displacing KOH solution was 3175±49.49 mL, while with displacing KOH solution it was 2580±42.42 mL, showing that CO_2 accounted for approximately 18.7% of the total gas. In the MEC, the gas produced without displacing KOH solution was 8920±311.12 mL, and with displacing KOH solution it was 7967.5±310.12 mL, indicating that CO_2 made up only

about 10.7% of the total gas. The gas was collected by downward displacement of water and KOH solution. This method was used to remove the CO_2 content, as KOH rapidly absorbs CO_2 to form potassium carbonate and water. Potassium carbonate is a stable compound. KOH is a strong base, while CO_2 is an acidic oxide, leading to their reaction to form a salt and water. The rate of CO_2 reduction to CH_4 using MEC was found to be 8%. It was carried out due to the unavailability of specific instruments for detecting the amount of components in biogas. Study done by Nelabhotla and Dinamarca, (2019) showed that low concentrations of CO_2 (i.e., 7%) are present in the biogas. In contrast, biogas produced through traditional anaerobic digestion typically contains CO_2 concentrations of around 35-45%.

Table 9: Gas collection with and without the displacement of KOH solution in control and test 3.5 L digester (Summary).

S.N	Digester volume (3.5L)	Temperature	Voltage supplied(V)	Type of Liquid displacement	Gas produced(mL) (Mean±S.D)	% of CO_2 reduction
1	Control	28°C	-	Water	3175±49.49	18.7
2	Control	28°C	1.5 V	1M KOH	2580±42.42	
3	MEC	28°C	-	Water	8920±311.12	10.7
4	MEC	28°C	1.5 V	1M KOH	7967.5±310.12	

4.4 Large scale biogas production in 17 L digester

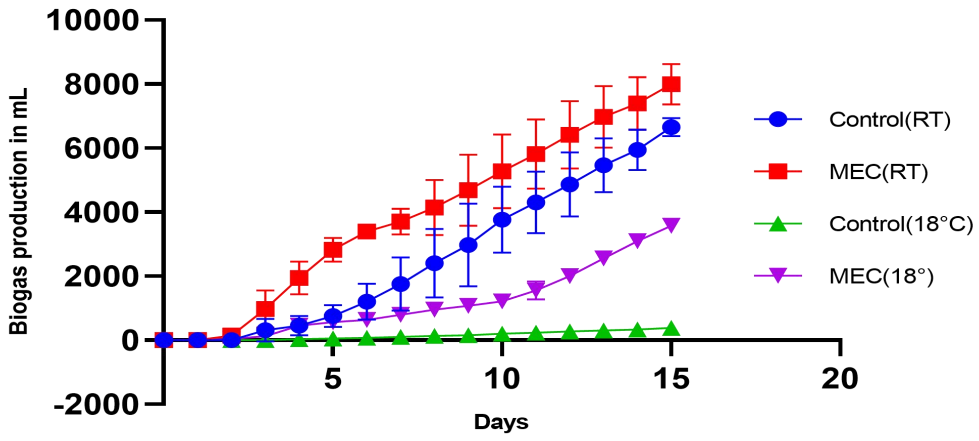

Figure 10: Comparison of gas production at 18°C and RT (20-25°C) between the 17L MEC and control digester

Figure 10: revealed that applying a voltage of 1.5 V in the MEC led to significantly higher gas production compared to the control at both temperatures.

The average biogas production on day 15 was 7997.5±637.10 mL in the MEC digester and 6655±282.84 mL in the control setup at room temperature (20-25°C). At 18°C, the average biogas production was 3572.5±187.38 mL in the MEC digester and 385±35.35 mL in the control digester. Gas production at RT was 1.20 times higher in the MEC

compared to the control group. At 18°C, the MEC produced 9.27 times more gas than the control group, demonstrating that MEC enhances biogas production compared to conventional biogas plants even at low temperatures.

Table 10: Comparison of gas production at 18°C and RT (20-25°C) between the 17L MEC and control digester (Summary).

S.N	Digester volume (17L) with carbon electrode	Temperature	Voltage supplied(V)	Gas produced(mL) (Mean±S.D)
1	Control	RT(20-25°C)	-	6655±282.84
2	MEC	RT(20-25°C)	1.5 V	7997.5±637.10
3	Control	18°C	-	385±35.35
4	MEC	18°C	1.5 V	3572.5±187.38

4.4.1 Analysis of biogas constituents produced from different 17L batch Setup

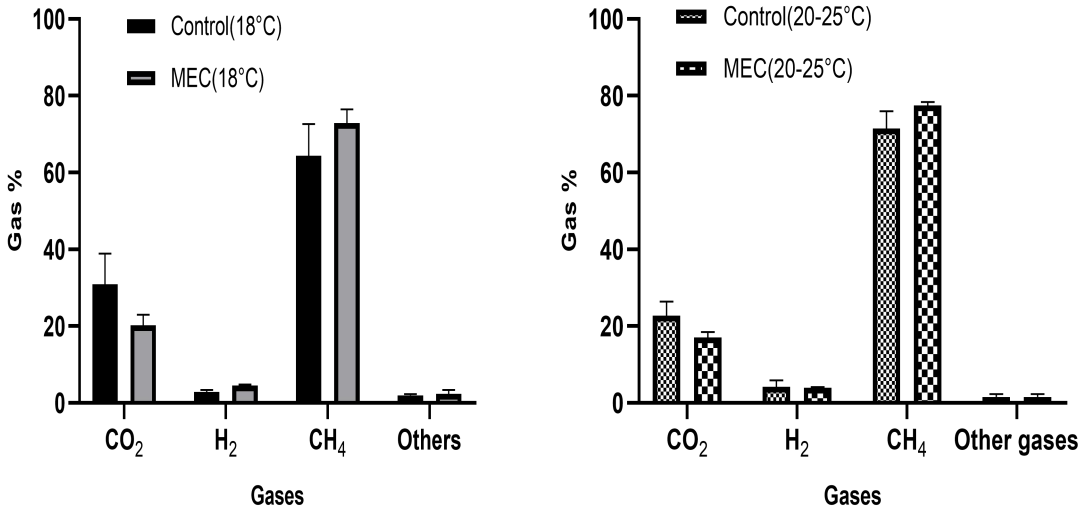

Figure 11: Analysis of the constituents of biogas produced from various 17-liter batches at room temperature (20-25°C) and 18°C

The biogas composition was determined using Protable Infrared Syngas Analyzer Gasboard -3100P (Hubei Cubic-Ruiyi instrument Co., Ltd, Japan). The gas analysis was done in Central Department of Environment Science (KU). The results showed that the biogas contained methane, carbon dioxide, Hydrogen and trace amounts of other gases (SO_2 and N_2). Methane was the predominant component, followed by carbon dioxide.

At RT (20-25°C), the control produced 22.72±3.64% of CO_2, 4.23±1.68% of H_2, 71.43±4.50% of CH_4, and 1.56±0.74% of other gases. In the MEC, the production was 17.02±1.44 of CO_2, 3.93±0.20% of H_2, 77.48±0.90% of CH_4, and 1.56±0.74% of other gases. The data shows a 23.59% reduction in CO_2 levels and a 8.87% increase in methane gas concentration.

At 18°C, the control produced 30.83±8.03% of CO_2, 2.83± 0.50% of H2, 64.38±8.20% of CH_4, and 1.94±0.33% of other gases. In the test, the production was 20.17±2.78% of CO_2,

4.54±0.21% of H_2, 72.89±3.54% of CH_4, and 2.40±0.97% of other gases. The data shows a 31.06% reduction in CO_2 levels and a 14.49% increase in methane gas concentration.

Table 11: Comparison of gas constituent produced from different 17 L batch setup (Summary).

Gases	At room temperature (20-25°C)			At 18°C		
	Control (%)	MEC (%)	% difference (Mean % ± S.D.)	Control (%)	MEC (%)	% difference (Mean % ± S.D.)
CO_2	22.72±3.64	17.02±1.44	23.59±18.62	30.83±8.03	20.17±2.78	31.06±27.01
H_2	4.23±1.68	3.93±0.20	2.06±45.45	2.83±0.50	4.54±0.21	62.01±21.20
CH_4	71.43±4.50	77.48±0.90	8.87±45.45	64.38±8.20	72.89±3.54	14.49±20.11
Others	1.56±0.74	1.56±0.74	0	1.94±0.33	2.40±0.97	20.87±29.51

4.5 Removal of COD in various digesters during biogas production

Table 12: Comparison of COD during digestion process among different digesters.

S.N	Samples	Temperature	Voltage supplied	Initial conc. (mg/L)	Final Conc. (mg/L)	% removal (mean % ± S.D.)
1	Control 1L	28°C	-	1351.75±161.91	1060.09±130.59	21.29±8.77
2	MEC 1L(inverted T)	28°C	2V		841.34±78.26	37.22±9.11
3	MEC 1L(parallel)	28°C	2V		854.21±58.96	36.55±3.25
4	Control 3.5L	28°C	-	952.86±45.18	648.33±80.35	31.64±11.43
5	MEC 3.5L	28°C	1V		523.948±94.87	44.71±11.63
6	MEC 3.5 L	28°C	1.5V		455.32±97.14	52.23±9.60
7	MEC 3.5 L	28°C	2V		605.44±99.94	36.69±7.70
8	Control 3.5 L	18°C	-	403.85±68.088	322.35±19.65	8.48±7.85
9	MEC 3.5 L	18°C	1.5V		223.70±80.35	18.70±6.61
10	MEC 3.5 L (Steel)	28°C	1.5V	952.86±45.18	849.92±70.86	10.53±10.60
11	MEC 3.5 L (steel)	18°C	1.5V	403.85±68.08	369.53±75.39	3.57±14.88
12	Control 17 L	RT	-	894.96±109.38	553.97±123.06	36.06±17.45
13	MEC 17 L	RT	1.5V		506.79±96.29	42.18±10.53
14	Control 17L	18°C	-		605.44±90.37	9.76±6.98
15	MEC 17 L	18°C	1.5 V		536.81±180.29	18.85±28.32

COD analysis was conducted to measure the levels of soluble protein, soluble polysaccharides, and VFAs in a sample. Elevated concentrations of these substances

indicate higher COD levels. The breakdown of organic matter by anaerobic microorganisms leads to a reduction in COD content and an increase in biogas production. The introduction of electricity during a chemical reaction can reduce COD levels from over 1,700 mg/L to 50 mg/L (Kim et al., 2004). Therefore, introducing a small amount of electricity can help reduce COD levels effectively. The MEC was operated for 15 days, along with the control, and the COD was measured by comparing the absorbance of the initial (before setup) and final (after 15 days) samples.

The study showed that at 28°C, the COD removal in the MEC digester was 37.22% with an inverted T carbon felt electrode and 36.55% with a parallel carbon felt electrode setup in the 1L digester. In comparison, the control group had a COD removal of 21.29%. This shows that the removal of COD was nearly equal in 1L digester having different setting of electrode. In 3.5 L digester at 28°C, the COD removal was 31.64% in the control. In comparison, the MECs containing tubular shaped carbon felt electrodes setting operated at power sources of 1V, 1.5V, and 2V showed COD removal of 44.71%, 52.23%, and 36.69% respectively. The MEC supplied with 1.5V had the highest COD removal, making it the optimal voltage option. However, the MEC with tubular steel electrodes achieved a 10.53% removal in COD. At 18°C, in a 3.5L digester, the COD removal rates were 8.48% and 18.70% in the control and MEC setups, respectively, using a 1.5V supply with tubular carbon felt electrodes. In contrast, the MEC with tubular steel electrodes at 18°C achieved a 3.57% removal in COD.

In the case of a 17L digester at RT, the COD removal was 36.06% in the control and 42.18% in the MEC. At 18°C, the COD removal rates were 9.76% in the control and 18.85% in the MEC. As for the RT or 28°C the rate of COD removal was found to be higher in comparison to the low (18°C) temperature. The average COD removal rate of cow dung during methane gas production is 47.2% (Wei et al., 2019). According to Castrillón Peláez et al., (2002) , the average COD removal efficiency from cattle manure is 51-79%. From above, removal of COD was achieved higher in MEC with the supply of 1.5 V than control both at 28°C and 18°C.

4.6 Removal of reducing sugar

Table 13: Comparison of reducing sugar removal in various digesters during digestion.

S.N	Samples	Temperature	Voltage supplied	Initial conc. (mg/L)	Final Conc. (mg/L)	% removal (mean % ± S.D.)
1	Control 1L	28°C	-	1173.88±50.11	143.38±15.62	14.97±13.35
2	MEC 1L(inverted T)	28°C	2V		125.39±12.03	25.16±15.24
3	MEC 1L(parallel)	28°C	2V		126.96±17.29	23.88±19.71
4	Control 3.5L	28°C	-	222.02±98.73	192.816±9.48	1315±6.79
5	MEC 3.5L	28°C	1V		138.69±17.19	37.53±17.71
6	MEC 3.5 L	28°C	1.5V		130.09±29.51	41.40±16.24
7	MEC 3.5 L	28°C	2V		144.94±25.37	34.71±13.45
8	Control 3.5 L	18°C	-	332.62±10.75	309.94±19.10	6.66±8.00
9	MEC 3.5 L	18°C	1.5V		217.67±20.45	34.50±6.68
10	MEC 3.5 L (Steel)	28°C	1.5V	271.63±11.72	159.02±4.06	41.42±1.41
11	MEC 3.5 L (steel)	18°C	1.5V	332.62±10.75	290.39±13.06	12.61±5.14
12	Control 17 L	RT	-	123.83±9.84	105.06±6.20	14.32±9.24
13	MEC 17 L	RT	1.5V		96.46±8.98	21.92±6.97
14	Control 17L	18°C	-		119.925±19.49	3.15±8.69
15	MEC 17 L	18°C	1.5 V		101.15±3.58	17.71±3.42

The MEC was operated for 15 days, and the absorbance of the sample was measured after performing the DNS test in both experimental and control setups. Table 7: shows a higher removal in soluble reducing sugar in the MEC digester compared to the control digester. At 28°C, the removal in soluble reducing sugar in the MEC digester was 25.16±15.24% with an inverted T carbon felt electrode and 23.88±19.71% with a parallel carbon felt electrode setup in the 1L digester. In comparison, the control group had a reduction of 14.97±13.35%.

In 3.5 L digester at 28°C, the reducing sugar removal was 13.15±6.79% in the control. In comparison, the MECs containing tubular shaped carbon felt electrodes setting operated at power sources of 1V, 1.5V, and 2V showed the removal of 37.53±17.71%, 41.40±16.24%, and 34.71±13.45% respectively. However, the MEC with tubular steel electrodes achieved a 41.42±1.41% removal in soluble reducing sugar. At 18°C, in a 3.5L digester, the reducing sugar removal rates were 6.66±8.00% and 34.50±6.68% in the control and MEC setups, respectively, using a 1.5V supply with tubular carbon felt electrodes. In contrast, the reduction of reducing sugar in the MEC with tubular steel electrodes at 18°C achieved a 12.61± 5.14% reduction.

In the case of a 17L digester at RT, the removal of reducing sugar was 14.32±9.24% in the control and 21.92±6.97% in the MEC. At 18°C, the reducing sugar removal rates were 3.15±8.69% in the control group and 17.71±3.42% in the MEC group. As for the RT or 28°C the rate of soluble reducing sugar removal was found to be higher in comparison to the low (18°C) temperature.

The removal of soluble reducing sugars was observed at both 18°C and room temperature, with an increase in removal observed with the application of external voltage. This shows that decrease in amount of reducing sugar in digester is directly proportional to the production of biogas. Facultative as well as anaerobic microorganisms utilize reducing sugar for their metabolism and growth. The metabolism carried out by microorganism results in the production of biogas. Toma et al., (2016) found that the sugar content in cow dung decreased by 26.19% after 15 days. The digestion process starts with the degradation of lignocellulosic biomass, results in the production of soluble sugars, which can serve as a substrate for biogas production via a microbial consortium (Tantayotai et al., 2017). Acidogenic bacteria utilize the soluble sugars as a substrate and produce a sub-layer like sugars and amino acid for subsequent bacterial groups. Acetogenic bacteria then use this sub-layer as a substrate to produce acetate, hydrogen, and CO_2. Hydrogenotropic and acetoclastic methanogenic bacteria create methane from the products of several phases of digestion (Dobre et al., 2014). According to Tantayotai et al., (2017), the degradation of lignocellulosic biomass generates soluble sugars that can be employed as a substrate for biogas production with the help of a microbial consortium.

4.7 Change in pH

Table 14: Comparison of pH levels in different digesters during digestion.

Digester volume (L)	Voltage supplied	Temperature (°C)	Initial pH	Final pH	Change
Control 1L	-	28	7.00±0.02	6.67±0.07	0.32±0.07
MEC 1L (inverted T)	2V	28	7.00±0.02	6.77±0.02	0.33±0.02
MEC 1L(parallel)	2V	28	7.00±0.02	6.75±0.02	0.25±0.02
Control 3.5L	-	28	7.00±0.02	6.52±0.28	0.33±0.06
MEC 3.5L	1V	28	7.00±0.02	6.75±0.02	0.25±0.02
MEC 3.5 L	1.5V	28	7.00±0.02	6.73±0.08	0.26±0.08
MEC 3.5 L	2V	28	7.00±0.02	6.49±0.20	0.50±0.20
Control 3.5 L	-	18	7.00±0.02	6.57±0.46	0.32±0.04
MEC 3.5 L	1.5V	18	7.00±0.02	6.80±0.07	0.2±0.07
MEC 3.5 L (Steel)	1.5V	18	7.00±0.02	6.82±0.03	0.17±0.03
MEC 3.5 L (steel)	1.5V	28	7.00±0.02	6.68±0.04	0.43±0.45
Control 17 L	-	28	7.00±0.02	6.66±0.08	0.33±0.08
MEC 17 L	1.5V	28	7.00±0.02	6.78±0.17	0.17±0.18
MEC 17 L steel	1.5V	28	7.00±0.02	6.76±0.13	0.23±0.13
Control 17L	-	18	7.00±0.02	6.71±0.05	0.28±0.05
MEC 17 L	1.5 V	18	7.00±0.02	6.89±0.01	0.11±0.01

In this research, the initial pH of each digester was maintained at 7±0.02, and the final pH was measured after 15 days of digestion. The pH observations in various digesters are shown in Table 4, indicating that all reactions occurred within the pH range of 6-7 during the digestion process, which is consistent with the range suggested by (Boe, 2006). Methanogenic archaea can function within a pH range of 5.5 to 8.5, with an optimal range of 6.5-8.0 (Boe, 2006). To balance the pH of substrates $NaHCO_3$ was used during dilution as it acts as pH buffer. Stable pH helps significantly to increase biogas production. Sodium bicarbonate aids co-digestion processes balance the acidity of substrates and provides the ideal pH environment for bacteria that produce methane (Ghaleb et al., 2020). The presence of sodium bicarbonate in anaerobic digestion facilitates the formation of calcium carbonate. Calcium carbonate then acts as a catalyst, promoting the conversion of carbon dioxide into methane, consequently enhancing methane yield (Valença et al., 2021).

4.8 Isolation of microbes from the inoculums

The inoculum was taken and spread on an agar plate enriched with DSMZ 825 methanogen enhancement media. The plate was then placed in an anaerobic jar and incubated at 37°C for 24 hours. After incubation, four different bacterial colonies were selected from the plate and isolated by streaking them separately on agar plates enriched with DSMZ 825 methanogen enhancement media. The plates were incubated in an anaerobic jar at 37°C for another 24 hours.

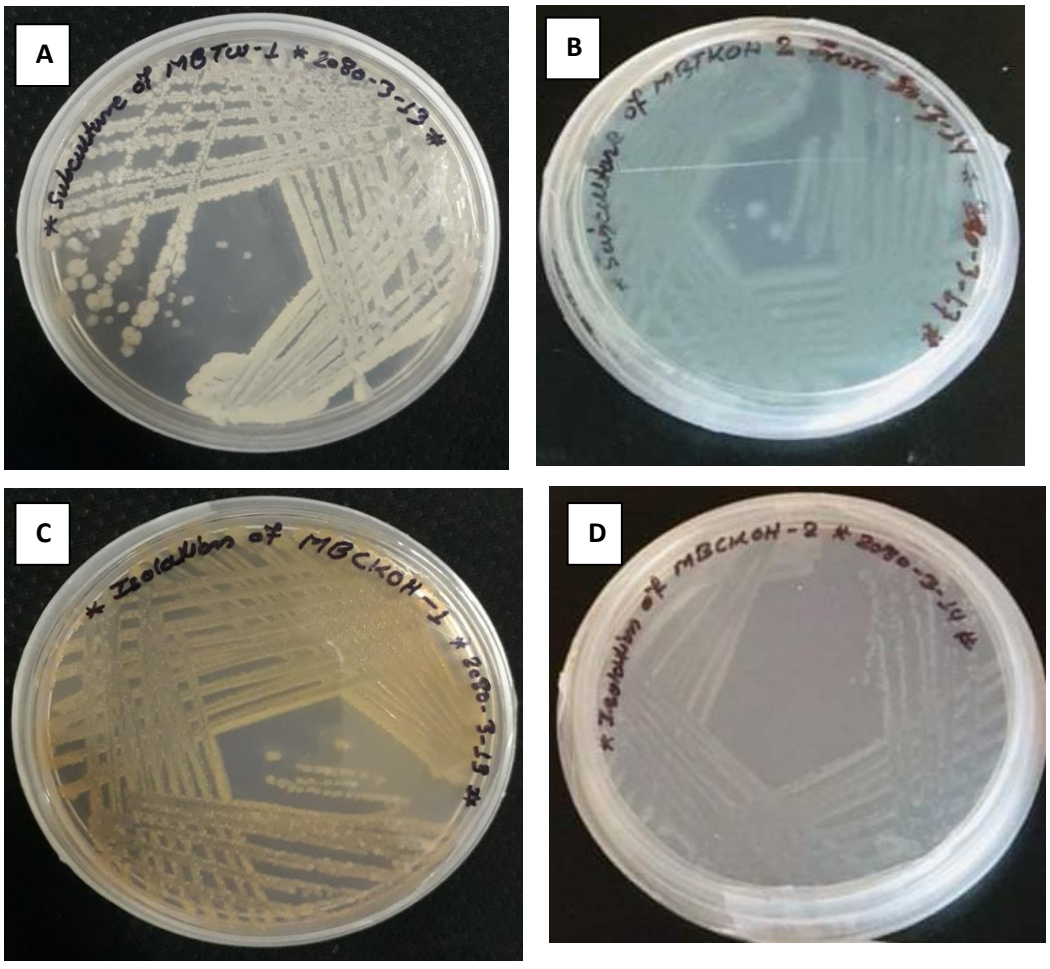

Figure 12: Figures (A), (B), (C), and (D) represent the different isolated colony growth of SK1, SK2, SK3, and SK4 on DMSZ agar media after 24 hours of incubation.

4.8.1 gDNA extraction and PCR amplification of SK1, SK2, SK3, and SK4 isolates.

The CTAB method was utilized to extract the genomic DNA from the four isolates, and a PCR reaction was conducted to target the 16S rRNA region of the bacteria. After amplification gel electrophoresis was performed on 1% agarose gel with 100 bp ladder from Sigma-Aldrich for 60 minutes in 90V. PCR products size of 516bp was visualized and then the same PCR product was sent for sequencing to Center for Molecular Dynamics Nepal (CMDN).

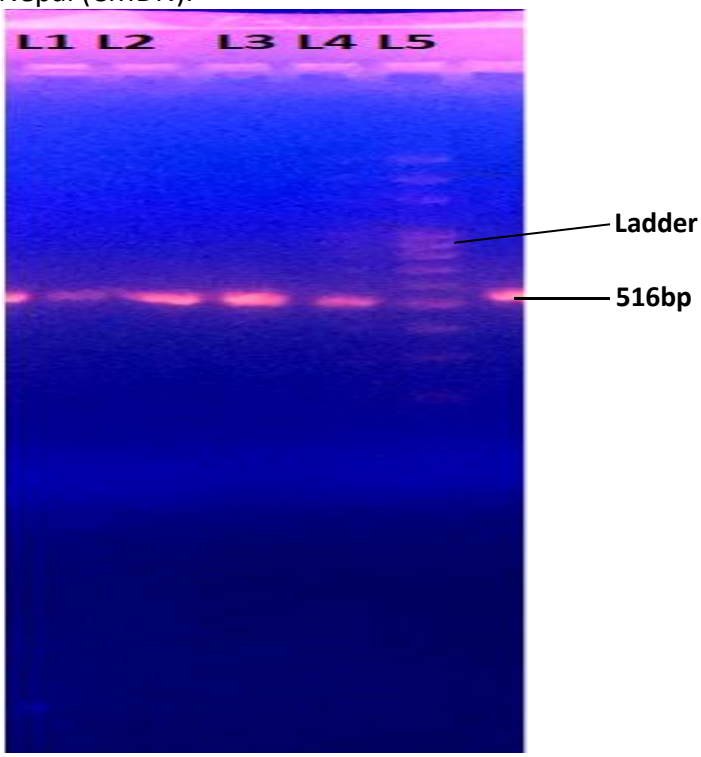

Figure 13: 1% gel electrophoresis of PCR product where L5 contain 100bp ladder and L1, L2, L3 and L4 contain products of Sk1, SK2, SK3 and SK4 respectively.

4.8.2 Sequencing of PCR products

Following amplification, the PCR product was sent to the CMDN for sequencing. Sanger's technique was used to sequence the products.

4.8.3 Construction of phylogenetic tree

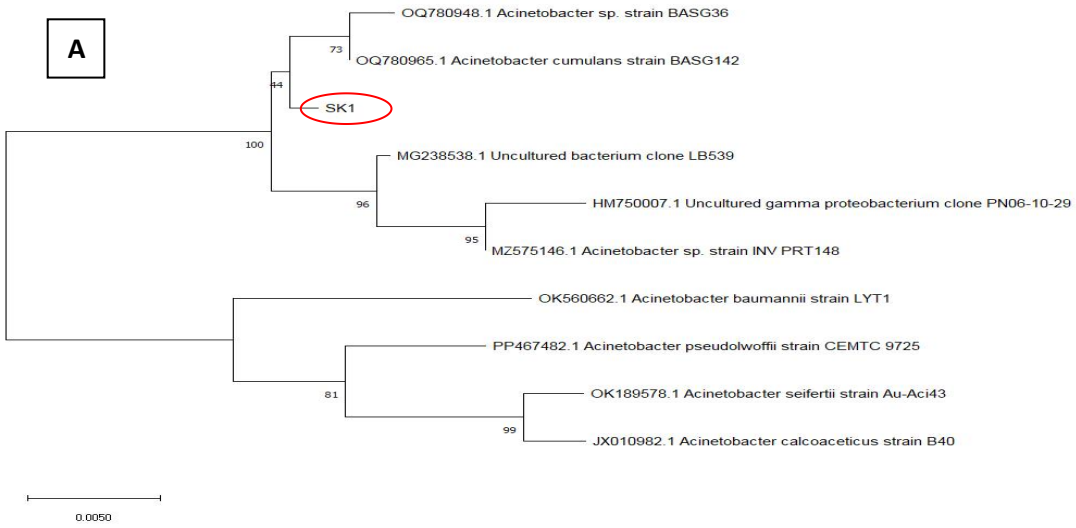

Figure 14: Phylogenetic tree of SK1 isolate of L1

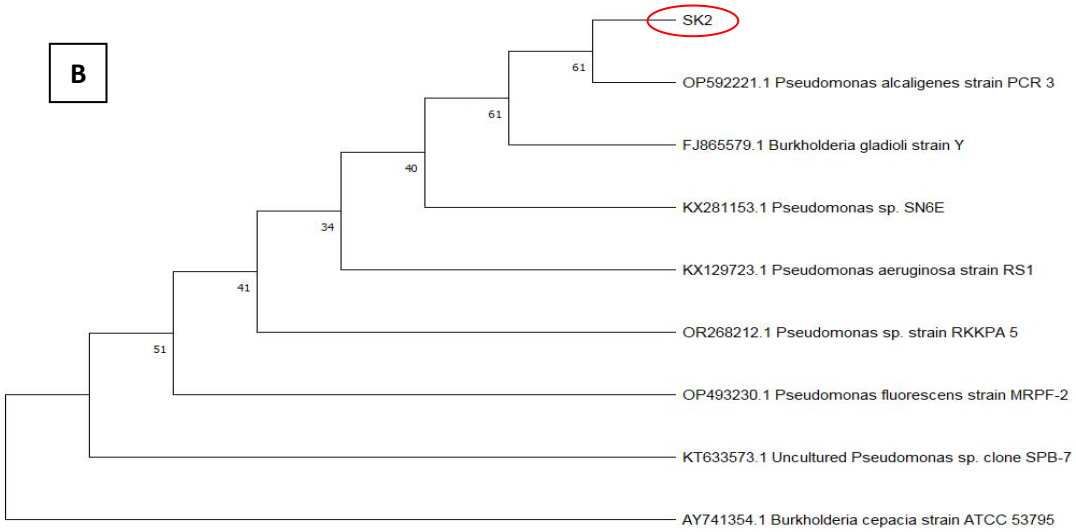

Figure 15: Phylogenetic tree of SK2 isolate of L2

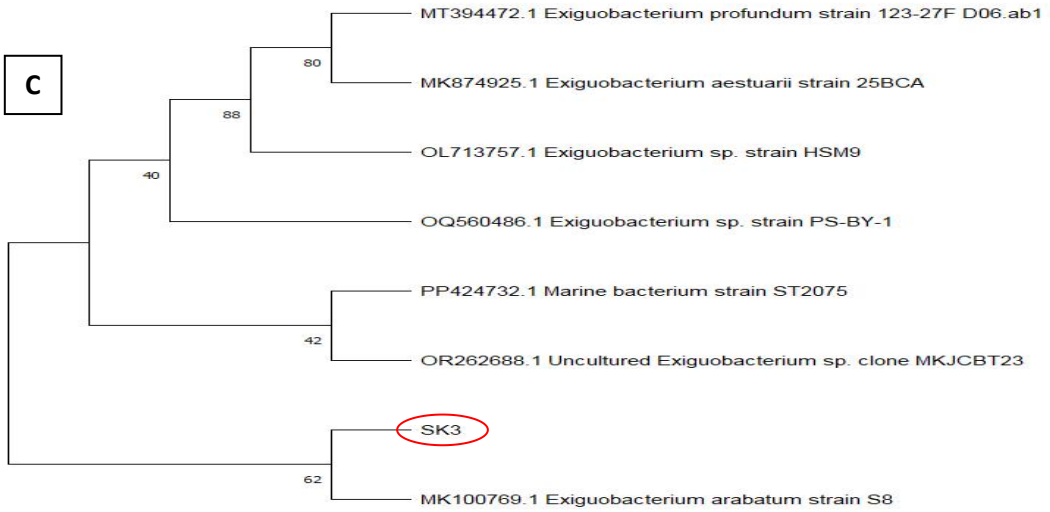

Figure 16: Phylogenetic tree of SK3 isolate of L3

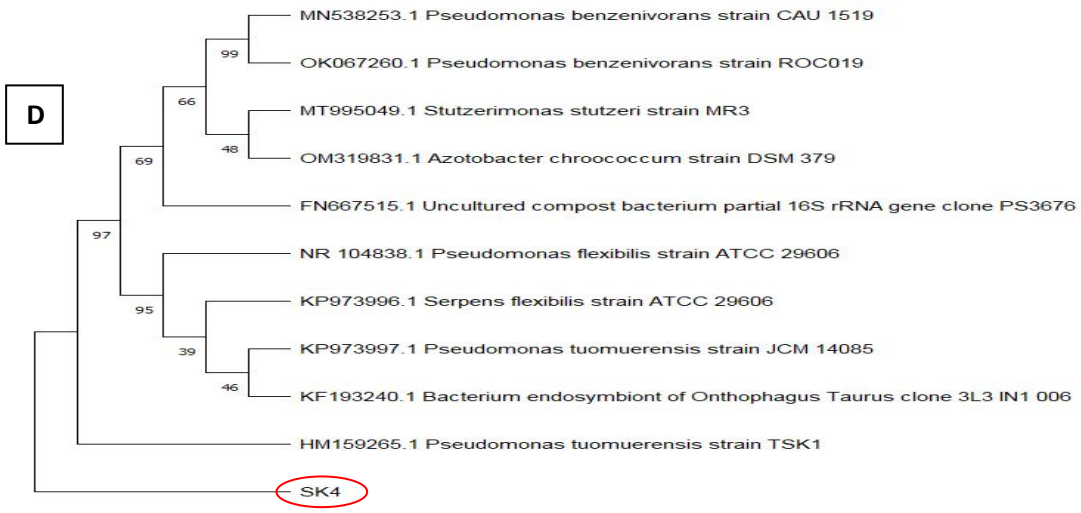

Figure 17: Phylogenetic tree of SK4 isolate of L4

Cladograms A, B, C, and D of isolates L1, L2, L3, and L4 depict a phylogenetic neighbor-joining tree based on 16S rRNA sequences and the tree illustrates the evolutionary relationships among species of the genera *Acinetobacter, Pseudomonas, Exiguobacterium, and Pseudomonas* respectively.

The resulting rRNA sequence was subjected to nucleotide BLAST's sequence homology analysis. The sequence of the SK1 isolate L1 showed a high degree of similarity (99.80%) to *Acinetobacter* spp. Similarly, the SK2 isolate L2 exhibited a 99.80% similarity to *Pseudomonas aeruginosa*. In contrast, the SK3 isolate L3 displayed a 100% similarity to *Exiguobacterium profundum*. However, the SK4 isolate L4's sequence had an 84.99% similarity to *Pseudomonas flexibilis*. Blast sequence homology analyses of the obtained rRNA sequences of all four isolates are documented in Appendix III.

The 16S rRNA sequences obtained were aligned using the MUSCLE algorithm in MEGA11 software, and a phylogenetic analysis was conducted on the data. The cladograms resulting from the analysis are shown in Figure 14, 15, 16, and 17. Based on the analysis, isolates L1, L2,

L3, and L4 were identified as *Acinetobacter cumulans*, *Pseudomonas aeruginosa*, *Exiguobacterium profundum*, and *Pseudomonas flexibilis*, respectively.

Acinetobacter cumulans is a Gram-negative, rod-shaped bacterium known for its diverse metabolic capabilities, such as anaerobic respiration and fermentation. In a study by Yang et al., (2022), *Acinetobacter cumulans* was found to have a relative abundance ranging from 0.01% to 1.84% in the microbial community, indicating its potential involvement in methane production. However, the precise role of Acinetobacter cumulans in this process is not defined.

Pseudomonas spp. plays a crucial role in AD by hydrolyzing carbohydrates, regardless of variations in substrate input and process parameters. This makes *Pseudomonas* spp. essential for the stability and efficiency of anaerobic digestion food webs, leading to diverse downstream metabolic processes for methanogenic pathways (Buettner et al., 2019). *P. aeruginosa* a common species in the rumen, is involved in ureolytic processes (Pang et al., 2020). It is known for its ability to form biofilms during anaerobic respiration, which can influence the microbial community structure in AD systems and impact the production of bioproducts such as methane (Crespo et al., 2016).

Some *Exiguobacterium* spp., such as *E. profundum*, is facultatively anaerobic and produces lactate as the main fermentation product. Lactate, a product of acidic fermentation, is a crucial intermediate in the anaerobic digestion of organic matter (Detman et al., 2018). Numerous studies have demonstrated that lactate is efficiently utilized by methane-producing microbial communities. Analyses of the acidogenic fraction undergoing methanogenesis and the resulting end products in two-stage biogas digesters have consistently shown that lactate is the most effectively utilized component, regardless of its initial concentration (Chojnacka et al., 2015).

Pseudomonas flexibilis is a versatile Gram-negative bacterium with diverse metabolic abilities, including anaerobic respiration and fermentation. While not extensively studied in anaerobic digestion for methane production, a study using high-throughput sequencing identified P. flexibilis in the microbial community at a relative abundance of 0.01-1.84%, indicating its potential involvement in methane production. *Pseudomonas flexibilis* is predominantly responsible for the reduction of N_2O to N_2 during the maturation phase of denitrification (Zhong et al., 2020).

SUMMARY

The increasing demand for renewable and eco-friendly energy for future generations is challenging to meet with current energy plants. In countries like Nepal, where many people depend on traditional biomass fuels for energy, biogas offers a cleaner and more sustainable alternative. Enhanced biogas production from agricultural waste, cow dung, and wastewater treatment can play a crucial role, especially in countries like Nepal. The technique, which may be conducted on both small and large scales, can use a variety of substrate sources, including crops, grasses, leaves, manure, fruit and vegetable waste, and algae. Biogas production from these sources is eco-friendly and considered a clean energy source, contributing to sustainable development globally. The renewable energy market, particularly biogas production, is rapidly growing as it offers a cost-effective alternative to fossil fuels. Microbial Electrochemical Cell (MEC) technology is a new method used for producing bioethanol, hydrogen gas, and methane from lignocellulosic biomass and organic waste, showing great potential. With further technological advancements and cost reductions, bioenergy production is expected to become more efficient and economically viable in the coming years.

The present work focused on optimizing biogas production from cow dung at different temperatures (28°C and 18°C) using low energy input. The study analyzed the chemical parameters of the substrate, including pH, moisture content, soluble sugars, and various chemical components. Biogas production setups were established in digesters of different sizes (1 L, 3.5 L, and 17 L) at different temperatures (15°C, 28°C, and room temperature) with varying voltage inputs (1V, 1.5V, and 2V). Also, various types of electrodes, including carbon felt and steel mesh electrodes, were used to enhance biogas production. The progress of substrate digestion was monitored by analyzing COD and reducing sugar levels, while gas production was quantified by measuring water and KOH displacement. Bacterial colonies in the digesters were isolated and characterized molecularly.

The research findings indicate that biogas production was enhanced with a voltage supply of 1.5 V. Biogas production increased at 18°C, 28°C, and room temperature compared to the control setup. A reduction in COD and reducing sugar was observed during the biogas production process, indicating substrate digestion in anaerobic conditions. Methane gas content analysis showed that methane accounted for 61-72% of the total biogas, consistent with existing literature. In addition, a complete characterization of manure has been carried out in terms of organic matter, its biodegradability, nutrients, and heavy metals (Fe, Cu, Zn, Cd and Pb). Methanogen, the predominant organism, was not isolated in this research due to inadequate maintenance of anaerobic conditions. Four facultative microorganisms were isolated, belonging to the Acinetobacter, Pseudomonas, and Exiguobacterium families. These microorganisms play a crucial role in electron transfer as biocatalysts and biogas production.

This research suggests that biogas production can be increased by supplying a small voltage to the anaerobic digester, even at cold temperatures.

CONCLUSION

This research utilized a laboratory and pilot-scale MEC system to increase biogas production, demonstrating feasibility even at low temperatures. MECs show promise in enhancing biogas production by combining electrochemical principles with microbial metabolism. MECs optimize the conversion of organic matter into methane-rich biogas. Applying an external voltage to MECs accelerates electron transfer processes, boosting biogas production efficiency and rate. MECs also have the potential to improve biogas quality by increasing methane content and reducing impurities.

Four isolates were identified as *Acinetobacter cumulans, Pseudomonas aeruginosa, Exiguobacterium profundum,* and *Pseudomonas flexibilis*. The use of a carbon felt electrode in MEC led to significantly higher volumetric production rates, resulting in 2.7 and 80 times more biogas production compared to the control when supplying 1.5 volts of electric potential at 28°C and 18°C, respectively. The steel mesh electrode produced more biogas at 18°C compared to the control, but biogas production was limited due to corrosion of the electrode. The 3.5L MEC at 28°C exhibited COD and reduced sugar removal efficiencies of 52.23% and 41.40%, making it suitable for waste/manure treatment. In comparison, the MEC at 18°C had lower efficiencies of 18.70% for COD and 17.71% for reduced sugar.

Recent studies have highlighted the importance of electromethanogenesis in producing valuable products from waste. This research was inspired by the global trend of improving waste management and converting waste into energy. While Nepal has started using waste for biogas production, there has been limited exploration of using MECs on a larger scale. The findings from lab-scale experiments can provide a foundation for future pilot and large-scale MEC applications. Further research is needed to validate these findings and explore the potential of MEC technology. Despite some initial challenges, it is evident that methane production rates gradually increased over time using MEC, indicating efficient substrate conversion.

RECOMMENDATIONS

- Investigate the durability and cost reduction of electrodes for large-scale production.
- Validate this strategy by repeated efforts.
- Optimization of substrates and its dilution can be choosen for further enhancement in biogas production.
- Optimization of voltage should be done at low temperature i.e below 15°C.
- Isolate a pure culture of hydrogenotropic methanogen and explore its electromethanogenesis.

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APPENDECES

APPENDIX I

A: Reagents

i: DMSZ 825 media

Table 15: Components of DMSZ825 media

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

Dissolve ingredients (except bicarbonate, vitamins, cysteine and sulfide), sparge medium with 80% H_2 and 20% CO_2 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% H_2 and 20%

CO_2 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% N_2 gas. Vitamins are prepared under 100% N_2 gas atmosphere and sterilized by filtration. Adjust pH of complete medium to 6.8 – 7.0, if necessary. For incubation use sterile 80% H_2 and 20% CO_2 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 16: Components of Trace Element solution

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

Distilled water 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 17: 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	0.10 mg
9	p-Aminobenzoic acid	5.00 mg
10	Lipoic acid	5.00 mg
11	Distilled water	1000.00 ml

iv. PCR components

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

Table 18: PCR components

S.N	Components	Volume (µl)
1	Master mix (2X) (Polymerase, 2X reaction buffer, and dNTP mix	12.5
2	Forward primer	2.5
3	Reverse Primer	2.5
4	Nuclease free water	5
5	Template	2.5

B. Determination of 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. **Potassium Phthalate Stock Solution (1000mg/L)**

In a 100ml volumetric flask, 0.085gm Potassium phthalate was dissolved and volume was maintained.

2. **Preparation of Digestion Solution**

For the preparation of 100ml digestion solution, to 50ml of deionized water, 1.02gm $K_2Cr_2O_7$ was dissolved in 16.7ml 20% H_2SO_4, and 3.33gm Mercuric Sulphate was added and mixed. Then the volume was maintained at 100ml.

3. **Preparation of Catalyst Solution**

For a final volume of 100ml, 0.9mg of Silver Sulphate ($AgSO_4$) was mixed with 100ml of Conc. H_2SO_4.

D. Determination of reducing sugars

For the final volume of 100ml, 30gm of Sodium Potassium Tartrate was mixed in 50ml deionized water (Solution I). To 20ml deionized water, 1.6gm Sodium Hydroxide and 1gm DNS were added and mixed (Solution II). Solution I and II were then mixed and the volume was maintained to 100ml with deionized water.

E. Determination of phosphorus by colorimetric method

1. Stock Solution, PO_4 concentration 1000 mg/L Merck nr 1.19898.

2. Stock Solution, PO_4 concentration 1000 mg/L - Dissolve 1.432 g potassium dihydrogen phosphate, KH_2PO_4 (see remark 2), in about 900 mL water in a volumetric flask of 1000 mL. Make up to 1000 mL with water.

3. Ammonium Molybdate Solution - Dissolve 40 g ammonium molybdate tetrahydrate, $(NH_4)6Mo_7O_{24}.4H_2O$, in ultra pure water and make up to 1000 mL. This solution should be stored in a bottle made of hard glass.

4. Potassium Antimonyl Tartarate Solution - Dissolve 0.274 g potassium antimonyl tartrate, $KSbOC_4H_4O_6.5H_2O$, in ultra pure water and make up to 100 mL with ultra pure water.

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 1000mL 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

F. 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 minutes (J. Liu et al. 2017b).

G. Formula for calculation

$$TSS = \frac{Weight\ of\ dry\ solid}{Weight\ of\ wet\ solid} \times 100\%$$

$$VSS = \frac{Wt.\ of\ dry\ solids - Wt.of\ ash}{Wt.of\ dry\ solids} \times 100\%$$

APPENDIX II

Standard graph of Chemical oxygen demand soluble reducing sugar and phosphorus

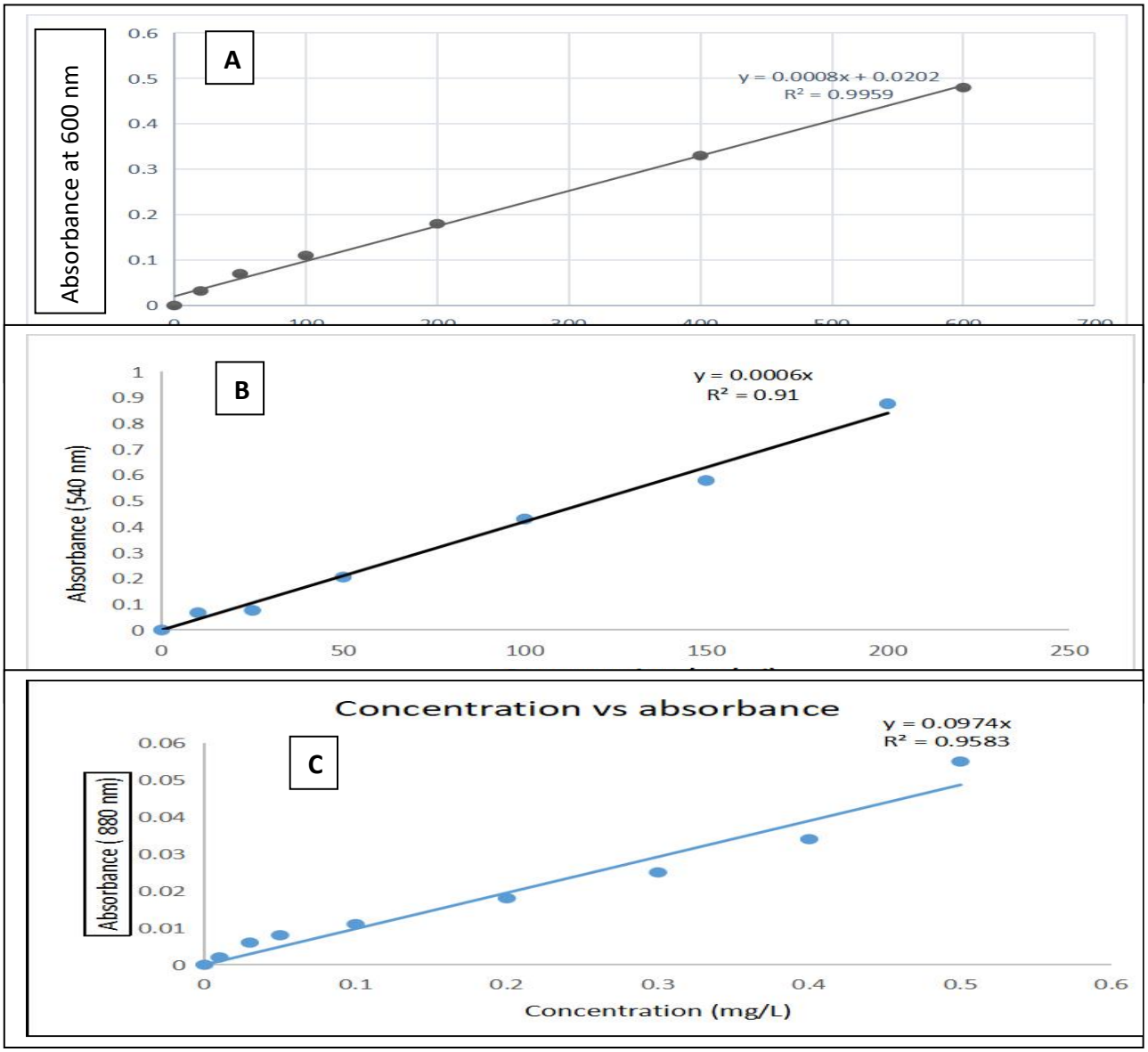
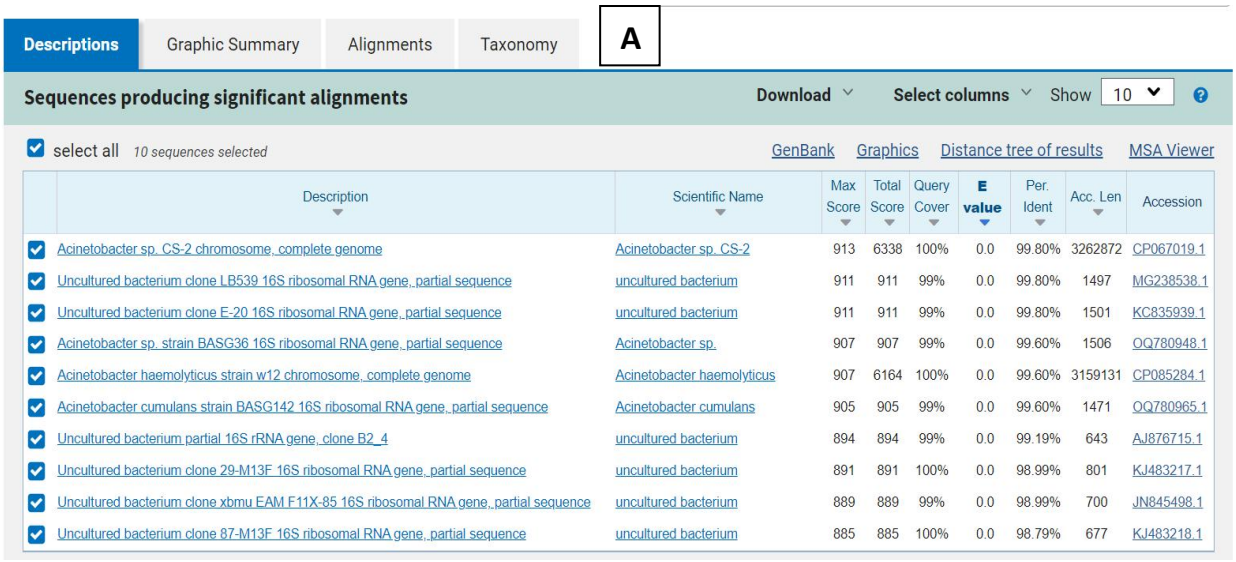


Figure 18: Standard graph, (A) Chemical oxygen demand, (B) Soluble reducing sugar and (C) Phosphorus

APPENDIX III

Figure 19: BLAST sequence homology analysis of obtained rRNA sequence of isolate, (A) SK1, (S) SK2, (C) SK3 and (D) SK4



Descriptions | Graphic Summary | Alignments | Taxonomy

A

Sequences producing significant alignments

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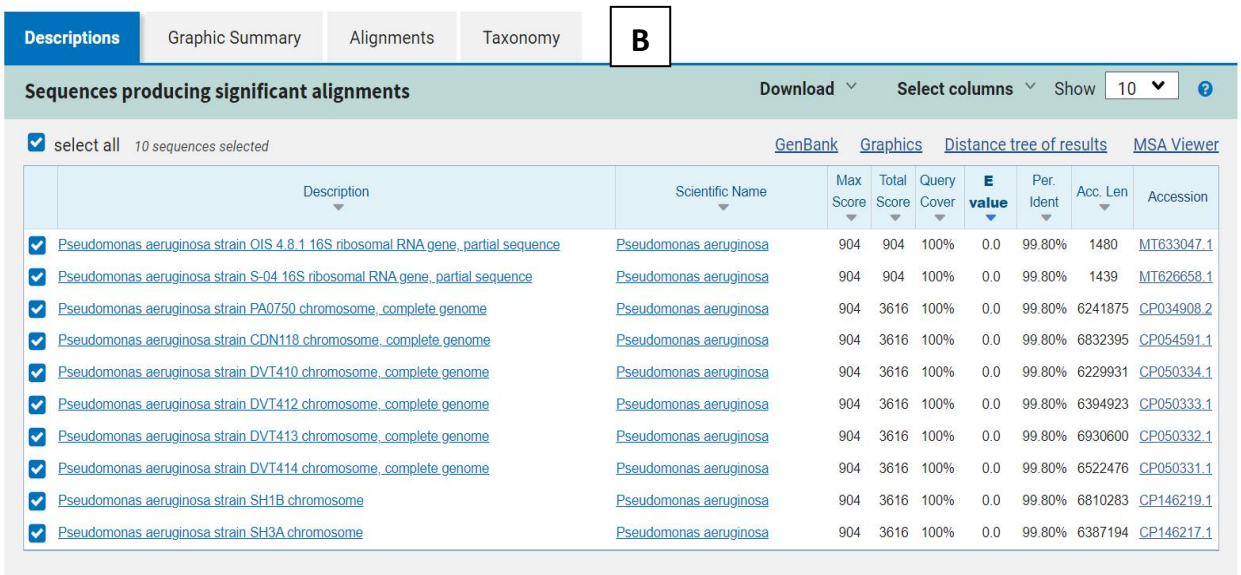
Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Acinetobacter sp. CS-2 chromosome, complete genome	Acinetobacter sp. CS-2	913	6338	100%	0.0	99.80%	3262872	CP067019.1
Uncultured bacterium clone LB539 16S ribosomal RNA gene, partial sequence	uncultured bacterium	911	911	99%	0.0	99.80%	1497	MG238538.1
Uncultured bacterium clone E-20 16S ribosomal RNA gene, partial sequence	uncultured bacterium	911	911	99%	0.0	99.80%	1501	KC835939.1
Acinetobacter sp. strain BASG36 16S ribosomal RNA gene, partial sequence	Acinetobacter sp.	907	907	99%	0.0	99.60%	1506	OQ780948.1
Acinetobacter haemolyticus strain w12 chromosome, complete genome	Acinetobacter haemolyticus	907	6164	100%	0.0	99.60%	3159131	CP085284.1
Acinetobacter cumulans strain BASG142 16S ribosomal RNA gene, partial sequence	Acinetobacter cumulans	905	905	99%	0.0	99.60%	1471	OQ780965.1
Uncultured bacterium partial 16S rRNA gene, clone B2_4	uncultured bacterium	894	894	99%	0.0	99.19%	643	AJ876715.1
Uncultured bacterium clone 29-M13F 16S ribosomal RNA gene, partial sequence	uncultured bacterium	891	891	100%	0.0	98.99%	801	KJ483217.1
Uncultured bacterium clone xbmu EAM F11X-85 16S ribosomal RNA gene, partial sequence	uncultured bacterium	889	889	99%	0.0	98.99%	700	JN845498.1
Uncultured bacterium clone 87-M13F 16S ribosomal RNA gene, partial sequence	uncultured bacterium	885	885	100%	0.0	98.79%	677	KJ483218.1



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Sequences producing significant alignments

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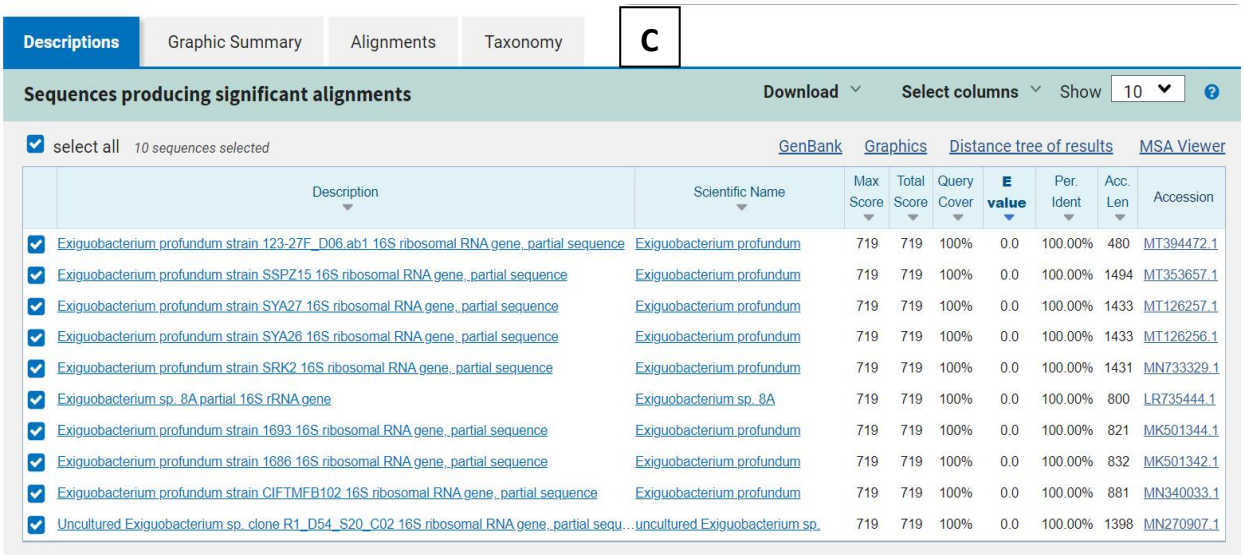
Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Pseudomonas aeruginosa strain OIS 4.8.1 16S ribosomal RNA gene, partial sequence	Pseudomonas aeruginosa	904	904	100%	0.0	99.80%	1480	MT633047.1
Pseudomonas aeruginosa strain S-04 16S ribosomal RNA gene, partial sequence	Pseudomonas aeruginosa	904	904	100%	0.0	99.80%	1439	MT626658.1
Pseudomonas aeruginosa strain PA0750 chromosome, complete genome	Pseudomonas aeruginosa	904	3616	100%	0.0	99.80%	6241875	CP034908.2
Pseudomonas aeruginosa strain CDN118 chromosome, complete genome	Pseudomonas aeruginosa	904	3616	100%	0.0	99.80%	6832395	CP054591.1
Pseudomonas aeruginosa strain DVT410 chromosome, complete genome	Pseudomonas aeruginosa	904	3616	100%	0.0	99.80%	6229931	CP050334.1
Pseudomonas aeruginosa strain DVT412 chromosome, complete genome	Pseudomonas aeruginosa	904	3616	100%	0.0	99.80%	6394923	CP050333.1
Pseudomonas aeruginosa strain DVT413 chromosome, complete genome	Pseudomonas aeruginosa	904	3616	100%	0.0	99.80%	6930600	CP050332.1
Pseudomonas aeruginosa strain DVT414 chromosome, complete genome	Pseudomonas aeruginosa	904	3616	100%	0.0	99.80%	6522476	CP050331.1
Pseudomonas aeruginosa strain SH1B chromosome	Pseudomonas aeruginosa	904	3616	100%	0.0	99.80%	6810283	CP146219.1
Pseudomonas aeruginosa strain SH3A chromosome	Pseudomonas aeruginosa	904	3616	100%	0.0	99.80%	6387194	CP146217.1



Descriptions | Graphic Summary | Alignments | Taxonomy

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Sequences producing significant alignments

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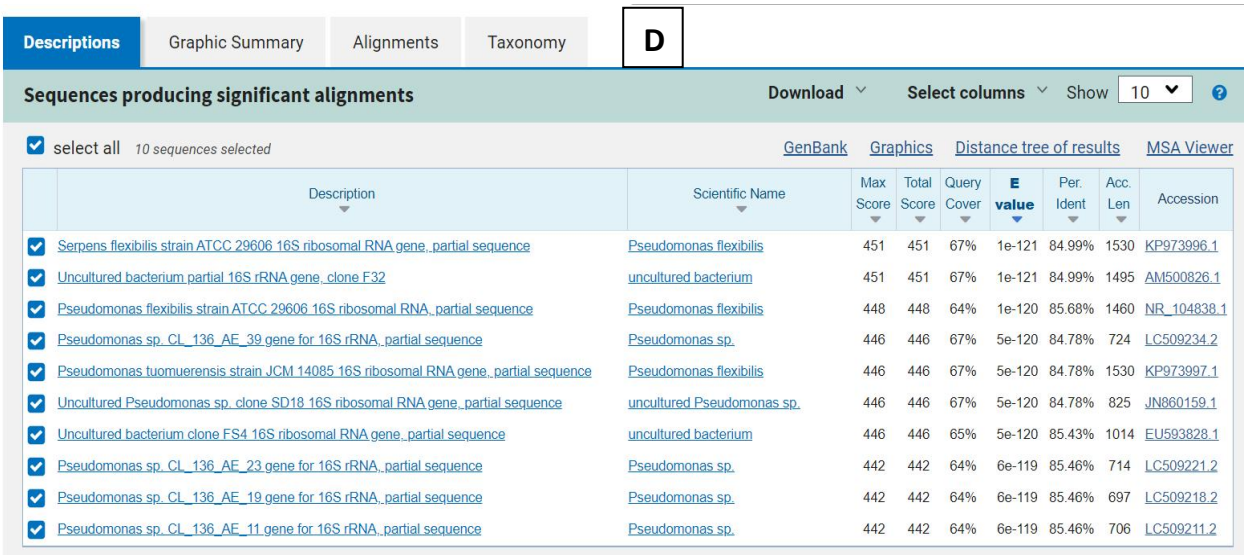
Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Exiguobacterium profundum strain 123-27F_D06.ab1 16S ribosomal RNA gene, partial sequence	Exiguobacterium profundum	719	719	100%	0.0	100.00%	480	MT394472.1
Exiguobacterium profundum strain SSPZ15 16S ribosomal RNA gene, partial sequence	Exiguobacterium profundum	719	719	100%	0.0	100.00%	1494	MT353657.1
Exiguobacterium profundum strain SYA27 16S ribosomal RNA gene, partial sequence	Exiguobacterium profundum	719	719	100%	0.0	100.00%	1433	MT126257.1
Exiguobacterium profundum strain SYA26 16S ribosomal RNA gene, partial sequence	Exiguobacterium profundum	719	719	100%	0.0	100.00%	1433	MT126256.1
Exiguobacterium profundum strain SRK2 16S ribosomal RNA gene, partial sequence	Exiguobacterium profundum	719	719	100%	0.0	100.00%	1431	MN733329.1
Exiguobacterium sp. 8A partial 16S rRNA gene	Exiguobacterium sp. 8A	719	719	100%	0.0	100.00%	800	LR735444.1
Exiguobacterium profundum strain 1693 16S ribosomal RNA gene, partial sequence	Exiguobacterium profundum	719	719	100%	0.0	100.00%	821	MK501344.1
Exiguobacterium profundum strain 1686 16S ribosomal RNA gene, partial sequence	Exiguobacterium profundum	719	719	100%	0.0	100.00%	832	MK501342.1
Exiguobacterium profundum strain CIFTMFB102 16S ribosomal RNA gene, partial sequence	Exiguobacterium profundum	719	719	100%	0.0	100.00%	881	MN340033.1
Uncultured Exiguobacterium sp. clone R1_D54_S20_C02 16S ribosomal RNA gene, partial sequ...	uncultured Exiguobacterium sp.	719	719	100%	0.0	100.00%	1398	MN270907.1



Descriptions | Graphic Summary | Alignments | Taxonomy

D

Sequences producing significant alignments

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GenBank | Graphics | Distance tree of results | MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Serpens flexibilis strain ATCC 29606 16S ribosomal RNA gene, partial sequence	Pseudomonas flexibilis	451	451	67%	1e-121	84.99%	1530	KP973996.1
Uncultured bacterium partial 16S rRNA gene, clone F32	uncultured bacterium	451	451	67%	1e-121	84.99%	1495	AM500826.1
Pseudomonas flexibilis strain ATCC 29606 16S ribosomal RNA, partial sequence	Pseudomonas flexibilis	448	448	64%	1e-120	85.68%	1460	NR_104838.1
Pseudomonas sp. CL_136_AE_39 gene for 16S rRNA, partial sequence	Pseudomonas sp.	446	446	67%	5e-120	84.78%	724	LC509234.2
Pseudomonas tuomuerensis strain JCM 14085 16S ribosomal RNA gene, partial sequence	Pseudomonas flexibilis	446	446	67%	5e-120	84.78%	1530	KP973997.1
Uncultured Pseudomonas sp. clone SD18 16S ribosomal RNA gene, partial sequence	uncultured Pseudomonas sp.	446	446	67%	5e-120	84.78%	825	JN860159.1
Uncultured bacterium clone FS4 16S ribosomal RNA gene, partial sequence	uncultured bacterium	446	446	65%	5e-120	85.43%	1014	EU593828.1
Pseudomonas sp. CL_136_AE_23 gene for 16S rRNA, partial sequence	Pseudomonas sp.	442	442	64%	6e-119	85.46%	714	LC509221.2
Pseudomonas sp. CL_136_AE_19 gene for 16S rRNA, partial sequence	Pseudomonas sp.	442	442	64%	6e-119	85.46%	697	LC509218.2
Pseudomonas sp. CL_136_AE_11 gene for 16S rRNA, partial sequence	Pseudomonas sp.	442	442	64%	6e-119	85.46%	706	LC509211.2

APPENDIX IV

Fig: Sample collection at chovar cow farm

Fig: Crucible mug inside muffle furnace

Fig: Muffle furnace at 500°C

Fig: 1L setup of MEC and control

Fig: 3.5L setup of MEC and control

Fig: C-felt electrode after 15 days of setup

Fig: Corroded steel electrode after 15 days of setup

Fig: Digested sample with H_2SO_4-salicylic acid-H_2O

Fig: 17L MEC setup during gas collection

Fig: Certificate provided by ICC 2023 after poster presentation.