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Analysis of Psychrophilic Alternatives for Production of Biogas at Low Temperature Range

by

Ritavrat Joshi

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The undersigned certify that they have read, and recommended to the Institute of Engineering for acceptance, a thesis entitled " **Analysis of Psychrophilic Alternatives for Production of Biogas at Low Temperature Range** " submitted by Ritavrat Joshi in partial fulfillment of the requirements for the degree of Master in Renewable Energy Engineering.

_____

Supervisor, Dr. Ajay Kumar Jha
Program Coordinator
Masters of Science in Renewable Energy Engineering
Department of Mechanical Engineering

_____

External Examiner, Dr. Jit Narayan Shah
Assistant Dean
IOF, Tribhuvan University

_____

Committee Chairperson, Dr. Nawraj Bhattarai
Head
Department of Mechanical Engineering

Date: 21/09/2076

ABSTRACT

The use of biogas technology in Nepal has benefited the country in improving health, environment, and economy and energy conservation. However, the mesophilic temperature ranges in which it has succeeded offer a limitation for scaling up in colder regions of Nepal. Even in warmer areas, winter results in dramatic drops in gas formation in the digesters. This thesis has focused on experimental study of biogas production potential with psychrophilic inoculums.

Various research works throughout the world have suggested that use of psychrophilic methanogens boost the biogas production potential even in low temperatures. Psychrophiles or cryophiles are extremophilic organisms that are capable of growth and reproduction in cold temperatures, ranging from −20 °C to +10 °C. Many such organisms are bacteria, but psychrophiles also include eukaryotes such as lichens, snow algae, fungi, and wingless midges. It is suggestive in relevant research works that such bacteria are found in permafrost (frozen ground) soils and bottom of high altitude lakes.

The samples were collected based on location selection from NAST's research on psychrophiles. Catalase test was considered the major deciding factor for characterization. Further tests were carried out for it and 14 different prototype biogas digesters were made. The digester was also tested adding only the organic wastes for comparative study. Lab tests at RIBB suggested samples from Syangboche to be the best option. The prototype digesters were kept at 5, 10, 15 and 25 degrees Celsius. The pressure reading and temperature of the samples were noted. After 60 days, the gas pressure and the gas composition were tested and for 3 sets of temperature ranges, a total of 5 months' time was used for gas analysis. The results showed increased biogas production while using the sample compared to the digester without the sample at all controlled temperatures. Moreover, even though biogas formation did occur in low temperature ranges with methane content greater than 50%, it was still lower than gas formation in the mesophilic range. Cost benefit analysis for a case study in Chitwan gave a B/C ratio of 1.62, NPV of Rs. 128,113, IRR of 54% and a payback period of 2.75 years for a digester of $6m^3$.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

^{0}C/ Deg. C	Degree Celsius
BSP	Biogas Support Program
AEPC	Alternative Energy Promotion Centre
SNV	Netherlands Development Organization
DNA	Deoxyribonucleic Acid
m^3	Cubic meter
LPG	Liquefied Petroleum Gas
Ltr	Liter

CHAPTER ONE: INTRODUCTION

1.1 Background

Climate change has adverse impacts on forestry due to several climate change induced disasters such as fire, drought, ice-storms and floods, which also results in loss of fertile soil. This effect is more prominent in higher altitude regions of Nepal where the vegetation is already scarce and the timber producing trees are ever more threatened. (Ngamindra Dahal, 2009). During the past two decades, summer temperatures have frequently exceeded the critical threshold of the most valuable timber species of the Himalayan forest. Therefore, the availability of firewood has reduced drastically which has proved to be a major problem for conducting daily household activities. (Gupta, 2010). Moreover, production of biogas in winter and colder temperature range is the challenge.

Biogas has been efficiently produced using mesophilic bacteria, which grow best in moderate temperatures (20-35°C) (Chand, 2012). But these bacteria exhibit significantly reduced biogas production potential for high altitude regions where temperatures fall as lows as 15 degree (Anthony, 2011).To solve this problem, focus has shifted to another group of bacteria termed as psychrophiles, which grow best at temperatures between -20°C to 15°C. Evidences of existence of probable methanogenic psychrophiles have been found in permafrost sediments of frozen lakes (in Alaska and Nepal) (Alaska Centre for Research and Power, 2009-September 2011). These bacteria are capable of production of biogas even at temperature as low as 0^{o} C. However, research based data is not available about methane producing capability of psychrophiles in permafrost sediments in Nepal using conventional feed readily available in rural households.

1.2 Problem Statement

Forest disturbance, dispersion and shifting, bio diversity change, forest disease and pest infestations, abiotic disturbances are major adverse effect of climate change on forestry. (Gupta, 2010). This is a major cause of less availability of firewood in high altitude regions. Sufficient data regarding the methane production potential of mesophilic methanogens has been widely documented (Ramanujam, 2011). Methanogenesis from psychrophiles found in Alaskan and Tibetan thermokarst lakes using food waste as well

as methanol has also been documented (Alaska Centre for Research and Power, 2009-September 2011) (Zhang, 2008). However, in the context of Nepal, no detailed research has been done verifying their existence in the permafrost sediments of high altitude lakes. Moreover, absence of reliable data regarding their methane production ability using common household wastes, has constrained further research to formulate biogas digester design.

In the cold-regions and higher altitudes, mesophiles have been shown to generate biogas in insignificant quantities. Few biogas plants currently in operation in cold climates use different heating techniques to suit the production of biogas by mesophiles (Buysman, 2009). The plants are either inefficient or costly. So, biogas which has been efficiently addressing the impact of climate change (diminishing fuelwood) in warmer regions has been unable to have even minor significance in the colder regions. (Buysman, 2009)

1.3 Rationale of Work

Further research is required to develop a cheap yet efficient alternative to the scarce traditional energy source for cooking purpose. Several organizations in Nepal like BSP-Nepal, AEPC, and SNV have focused on installation of biogas plants in the recent years (BSP Nepal). For instance, BSP-Nepal adds nearly 20,000 biogas plants every year and aims to have a total of 500,000 plants in Nepal. These organizations as well as several academic institutions have also been conducting research to find alternatives to the existing biogas systems for cold climates. Development of guidelines for production of biogas using psychrophiles will aid the organizations to implement it in the probable sites. This thesis was supported by Energy, Environment Research and Development Centre (EERDC).

1.4 Objectives

1.4.1 Main Objective

The main objective of this thesis is to deliver methods for reducing the dependence on firewood as primary means of energy source by identifying and quantifying methanogenesis potential of psychrophiles present in wetlands of Nepal.

1.4.2 Specific Objectives

- To carry out comparative volumetric analysis of bio gas production from household waste between psychrophiles and mesophiles under low, optimal and high temperature range.
- To provide suggestive parameters for digester design modification for using psychrophiles as the solution for gas production at low temperatures.
- To carry out the financial analysis of the proposed digester.

1.5 Hypothesis Setting

For the accomplishment of these objectives, the following hypotheses will be tested:

- Efficiency of biogas production by psychrophiles will be higher at moderate (25-35°C) than mesophiles
- Tanks inoculated with psychrophiles from permafrost lakes will produce more biogas than tanks inoculated with mesophiles, in a cold temperature range.

1.6 Scope of Work

The primary beneficiaries of this research are the people residing in hilly regions where the temperature does not exceed 15-20 °C. Through the findings of this research, the people living in those regions can get the benefit of reduced dependence on firewood, which, in particular, alleviates women's household drudgery and stabilizes their health conditions. Additional benefits are availability of clean renewable energy source and an alternative to chemical fertilizers along with efficient management of household wastes.

1.7 Limitations

The limitations of this study are as follows:

- Short period of study and hence annual variation cannot be studied in gas yield
- Genetic/ DNA tests could not be carried out due to budget limitations and only general sets of morphological tests could be carried out.
- Intermediate products of the overall production cycle have not been studied.

CHAPTER TWO: LITERATURE REVIEW

2.1 Psychrophilic Bacteria

The mesophilic bacteria responsible for methanogenesis are naturally found in the digestive system of ruminant livestock. However, the psychrophilic bacteria do not naturally exist in the kitchen waste or human/cattle waste. So for the use of psychrophiles to generate biogas, it has to be added in to a digester from different sources such as bottom of lakes and swamps (Alaska Centre for Research and Power, 2009-September 2011).

Special challenges to microorganisms in cold ecosystems include reduced enzymatic reaction rates, limited bioavailability of nutrients, and often extremes in pH and salinity. Depending on the local conditions, water activity (the amount of water available to microorganisms) may also be limiting. To thrive successfully in low temperature environments, psychrophiles have evolved a complex range of structural and functional adaptations. Consequently, there is evidence of a wide range of metabolic activities, even at sub-zero temperatures, in cold ecosystems (Miteva, 2011).

Psychrophilic and psychrotrophic microorganisms have the ability to grow at 0°C with an optimum working temperature of 15^{o}C. Psychrotrophic microorganisms have a maximum temperature for growth above 20°C and are widespread in natural environments and in foods. Psychrophilic microorganisms have a maximum temperature for growth at 20°C or below and are restricted to permanently cold habitats. This ability to grow at low temperature may be correlated with a lower temperature characteristic than that of the mesophiles, an increasing proportion of unsaturated fatty acids in the lipid phase of the cell membrane, which makes it more fluid, and a protein conformation functional at low temperature. The relatively low maximum temperature of growth for these microorganisms is often considered to be due to the thermolability of one or more essential cellular components, particularly enzymes, while some degradative activities are enhanced, resulting in an exhaustion of cell energy, a leakage of intracellular substances or complete lysis. Psychrotrophic microorganisms are well-known for their degradative activities in foods. Some of it are even pathogenic or toxinogenic for man, animals or plants. However, in natural microbial ecosystems psychrotrophic and psychrophilic microorganisms can play a large role in the

biodegradation of organic matter during cold seasons. Psychrophiles are extremophiles that are capable of growth and reproduction at low temperatures. The environments they inhabit are ubiquitous on Earth, as a large fraction of our planet's surface is at temperatures lower than 15°C. They are present in the Arctic and Antarctica, glaciers, alpine regions, and deep sea. Psychrophiles utilize a wide variety of metabolic pathways, including photosynthesis, chemoautotrophy, and heterotrophy, and form robust, diverse communities. Psychrophiles are characterized by lipid cell membranes chemically resistant to hardening in response to the cold. In some cases, they create antifreeze protein to maintain their internal space in liquid form even at temperatures below the freezing point of water.

- More than three-quarters of the Earth's surface is cold — oceans with a constant temperature of 4–5°C below a depth of 1,000m cover approximately 70% of the Earth's surface. The microorganisms that occupy these regions are known as psychrophiles. To maintain essential chemical reactions at these temperatures, psychrophilic enzymes are cold active and heat labile.
- Psychrophilic enzymes maintain high activity at low temperatures mainly by decreasing the temperature dependence of the reaction that is catalysed. This is achieved by improving the mobility or flexibility of the active site. As a consequence, substrate binding is generally less efficient, but specific mutations can compensate for this adaptive drift, especially when substrate binding (K_m) has a regulatory function.
- The catalytic centre of cold-active enzymes is identical to that of mesophilic enzymes, to maintain specificity, but local interactions might help to improve catalysis at low temperatures, such as better accessibility to the active site or favorable electrostatic interactions with the substrate. Generally, adaptive mutations favoring active-site flexibility are located outside the catalytic centre. All known interaction types that stabilize a protein are reduced in number and strength, but each enzyme family uses one or a combination of the altered interactions to gain in molecular mobility.
- At least in the case of the best-studied psychrophilic enzyme (chitobiase), the relationships between stability and activity at low temperatures have been shown by site-directed mutagenesis. Stabilizing the psychrophilic enzyme, by engineering the weak interactions found in the mesophilic enzyme, decreases activity and improves substrate binding of the mutants.

- The stability curves of psychrophilic enzymes reveal several unsuspected properties. They are optimally stable at room temperature, which reflects the dominant effect of hydrophobic forces in protein folding. However, they are cold labile and more prone to cold denaturation than mesophilic proteins, which is a phenomenon that might set a biophysical lower limit to life at low temperatures. In addition, the thermodynamic contributions to their stability are the opposite to that of mesophilic proteins, for example the stability of cold-active enzymes is entropy-driven at low temperatures.
- Directed evolution experiments show that several molecular adjustments can lead to cold activity. However, in cold environments, the simplest strategy seems to be to lose stability, in the absence of selection for stable proteins, to gain in flexibility and activity, under a strong selective pressure for cold-active enzymes.

Approximately 80% of our planet's biosphere is permanently cold, that is, at temperatures below 5°C. This includes much of the world's oceans—which cover 70% of the Earth's surface—the polar regions, which encompass Antarctica and parts of North America and Europe that are within the Arctic circle, montane regions (Alps, Himalayas and Rocky Mountains), the mesosphere and stratosphere, and to a lesser extent, man-made habitats such as fridges and freezers. Taken together, this makes low temperature the most wide-spread "extreme" environment and, as such, psychrophiles represent the most abundant, diverse and widely distributed extremophiles on Earth. The diversity of psychrophiles that is associated with various aquatic and terrestrial cold environments has recently been comprehensively reviewed. Organisms that inhabit cold environments have been subdivided into psychrophiles sensu stricto, which grow optimally at less than 15°C (upper limit of 20°C), and psychrotolerant organisms, which survive at temperatures below 0°C but grow optimally at 20–25°C. Psychrophiles sensu stricto predominate in marine ecosystems, where the abyssal oceanic waters are permanently cold (< 5°C), whereas cold-adapted microorganisms isolated from terrestrial environments—which are much more prone to extreme temperature fluctuations are mostly considered as psychrotolerant. As such, there is some bias in research on cold survival and adaptation mechanisms in psychrophilic/psychrotolerant microorganisms, as evidenced by the observed preponderance of genome and metagenome sequences derived from marine

environments, and transcriptome profiling of psychrotolerant microorganisms across wider and experimentally viable temperature spectra. The lack of adequate temperature data also makes it difficult in many cases to assign studied strains as psychrotolerant or psychrophilic. Therefore, we will employ the generic term psychrophiles to encompass both groups, making reference to the psychrophilic/psychrotolerant nature of the strains studied where possible. The lower temperature limit for psychrophiles is not clearly defined, although a limit of 12°C for reproduction and 20°C for metabolic function has been proposed. Photosynthesis in the Antarctic lichen Umbilicaria aprina has been reported to occur at 17°C, and the yeast Rhodotolura glutinis can cause frozen food spoilage at 18°C. Continued cellular functioning has recently been proposed to exist at temperatures below 20°C. Cold temperatures place severe physicochemical constraints on cellular function by negatively influencing cell integrity, water viscosity, solute diffusion rates, membrane fluidity, enzyme kinetics and macromolecular interactions. The ability of an organism to survive and grow in cold conditions is therefore dependent on a number of adaptive strategies in order to maintain vital cellular functions at cold temperatures. As such, psychrophiles have evolved mechanisms to successfully counteract additional stress factors associated with cold environments, such as desiccation, radiation, excessive UV, high or low pH, high osmotic pressure and low nutrient availability.

Psychrophilic microorganisms have successfully colonized all permanently cold environments from the deep sea to mountain and polar regions. Some of these organisms, depending on their optimal growth temperature, are also known by the terms psychrotolerant or psychrotroph (Morita, 1975). Nevertheless, we believe that there is a continuum in temperature adaptation for life with wide or narrow growth temperature ranges depending on the microorganism, and we will use the general term psychrophiles in this review to designate all microorganisms growing well at temperatures around the freezing point of water. This unique property implies that psychrophiles have successfully overcome two main challenges: first, low temperature, because any decrease in temperature exponentially affects the rate of biochemical reactions; and second, the viscosity of aqueous environments, which increases by a factor higher than two between 37 °C and 0 °C. Remarkable adaptations have been observed with some organisms such as *Moritella profunda*, which is a psychropiezophilic organism, a microorganism adapted to cold and living in the deep sea that shows maximal growth rates at 2 °C and a maximum growth temperature of only 12 °C (Xu *et al*, 2003). This

indicates that at temperatures as low as 2 °C, some enzymes or supramolecular structures already show an altered conformation that negatively affects the metabolic flux.

Obvious targets of the deleterious effects of low temperatures are cytoplasmic membranes and enzymes that tend to rigidify when the temperature drops. This affects membrane permeability, and hence the transport of nutrients and waste products, and catalysis, because enzymes require a certain flexibility to function (Goodchild *et al*, 2004b; Ratkowsky *et al*, 2005). Impaired protein folding and protein cold-denaturation can also cause problems at low temperatures, in particular for bacterial strains that sustain biological activities at temperatures as low as −20 °C and resist freezing. Cold-shock proteins have also been described. What are their roles in this adaptation? Key biological activities that involve nucleic acids such as DNA replication, transcription and translation can also suffer from exposure to low temperatures through the formation of secondary structures or super-coiled structures; how do psychrophiles cope with these phenomena? Finally, several genome sequences of psychrophilic microorganisms have been determined, and partial annotation of these has revealed unpredicted cold adaptations, the number of which will obviously expand after completion of the analysis and genome sequencing of other psychrophiles.

Decreasing temperatures have an adverse effect on the physical properties and functions of membranes, typically leading to a reduction in membrane fluidity, the onset of a gel-phase transition and, ultimately, a loss of function. The lipid composition governs the physical properties of membranes and hence it is not surprising that this varies with the thermal habitat of the microorganism. In general, lower growth temperatures produce a higher content of unsaturated, polyunsaturated and methyl-branched fatty acids, and/or a shorter acyl-chain length, with studies reporting a high proportion of *cis*-unsaturated double-bonds and antesio-branched fatty acids (Chintalapati *et al*, 2004; Russell, 1997). This altered composition is thought to have a key role in increasing membrane fluidity by introducing steric constraints that change the packing order or reduce the number of interactions in the membrane. Further adaptations that have been suggested to increase membrane fluidity include an increased content of large lipid head groups, proteins and non-polar carotenoid pigments (Chintalapati *et al*, 2004). However, these adaptive strategies do not seem to be widespread, and studies show more compact lipid head groups (Arthur & Watson, 1976) and decreased non-polar carotenoid pigment synthesis (Fong *et al*, 2001) in some psychrophiles.

The exposure of mesophilic organisms to sudden temperature changes, both upshifts and downshifts, induces the transient overexpression of several proteins—known respectively as heat-shock proteins (Hsps) or cold-shock proteins (Csps)—that are involved in various cellular processes such as transcription, translation, protein folding and the regulation of membrane fluidity (Phadtare, 2004). Although studies of these responses in psychrophilic microorganisms are still in their infancy, similarities with the Csps and Hsps that are induced in mesophiles have been observed. In particular, increased levels of nucleic-acid-binding proteins (for example, CspA-related proteins; Inouye & Phadtare, 2004) and chaperones, such as GroEL (Tosco *et al*, 2003) and DnaK (Yoshimune *et al*, 2005), have been frequently reported. However, distinctions do exist between the mesophilic and psychrophilic cold-shock response, including the lack of repression of housekeeping protein synthesis and the presence of cold-acclimation proteins (Caps) in psychrophiles. Many of the Csps observed in mesophiles act as Caps in psychrophiles, being constitutively rather than transiently expressed at low temperatures. Furthermore, this differential regulation of expression indicates that a temperature sensor system exists in psychrophiles, and thermosensors at the cell membrane level—that sense changes in fluidity—have been reported (Ray *et al*, 1994).

The most important selective pressure of low temperatures is exerted towards chemical reaction rates, most of which exponentially drop with decreasing temperature according to:

$$k_{cat} = K(k_B T/h) \exp(\Delta G^{\#}/RT)$$

in which k_{cat} is the reaction rate, κ is the transmission coefficient, k_B is the Boltzmann constant, T is the absolute temperature in Kelvin, h is the Planck constant, R is the gas constant and $\Delta G^{\#}$ is the activation energy. κ is generally considered to be equal or close to one; however, this transmission coefficient significantly varies with viscosity, resulting in a further decrease of k_{cat} (Siddiqui *et al*, 2004). Despite this, psychrophiles produce cold-adapted enzymes that have high specific activities at low temperatures, often up to an order of magnitude higher than those observed for their mesophilic counterparts (Feller & Gerday, 2003; Georlette *et al*, 2004; Russell, 2000).

The commonly accepted hypothesis for this cold adaptation is the activity–stability–flexibility relationship, which suggests that psychrophilic enzymes increase the flexibility of their structure to compensate for the ‘freezing effect' of cold habitats (Johns & Somero, 2004). This increased flexibility might concern the entire protein or

might be restricted to parts of the structure, especially those implicated in catalysis, and is probably also responsible for the generally observed low stability of cold-adapted proteins (Collins *et al*, 2003; D'Amico *et al*, 2003). Conversely, it has been shown that activity and stability are apparently not always inversely linked (Wintrode *et al*, 2001). However, in this study, multi-substrate enzymes and small-size synthetic substrates were mainly used, which might produce different results to those obtained with natural, large substrates; that is, the specificity of the enzyme might simply be shifted towards the substrate used. Crystallographic structures of psychrophilic proteins indicate that these do not have unusual conformations but instead share a high similarity with their meso and thermophilic homologues. To enhance flexibility, many structural modifications that lead to attenuation in strength and/or number of stabilizing factors viz enthalpic or entropic—have been observed. Common trends include: the reduction of the number of ion pairs, hydrogen bonds and hydrophobic interactions; decreased intersubunit interactions; increased interaction with the solvent; a reduced apolar fraction in the core; higher accessibility to the active site; increased exposure of apolar residues to the solvent; decreased cofactor binding; clustering of glycine residues; and a lower proline and arginine content (Violot *et al*, 2005).

Comparison of the thermodynamic parameters of activation of psychrophilic enzymes with those of their mesophilic homologues (Lonhienne *et al*, 2000), indicates that the high k_{cat} of these at low temperatures is due to a decrease of the activation enthalpy $\Delta H^{\#}$—a decrease in the number of enthalpy-driven interactions that have to be broken during catalysis. This decrease is partially compensated by a less favorable activation entropy $\Delta S^{\#}$. As a result, and as supported by the negative values of $\Delta(\Delta S^{\#})_{psychro–meso}$, the ground-state enzyme–substrate complex shows a broader distribution of conformational states. Consequently, a further effect of the enhanced flexibility should be a looser binding of the substrate, which is observed through high K_M values for many psychrophilic enzymes that interact with large substrates (Collins *et al*, 2002). These catalysts therefore increase k_{cat} at the expense of K_M, whereas in some intracellular enzymes this adaptive drift of K_M is counteracted by the retention of rigid structural domains (Bentahir *et al*, 2000). Another effect of low temperatures on proteins is cold denaturation, a phenomenon that is thought to occur from destabilizing hydration (Makhatadze & Privalov, 1995).

Psychrophilic microorganisms have successfully confronted the two main physical challenges to which they are exposed: low thermal energy and high viscosity, both of

which slow metabolic flux. Proteins are the main targets of these adaptations as they control the equilibrium between substrates and products, influx of nutrients, outflow of waste products, macromolecular assemblies, nucleic-acid dynamics and appropriate folding. Their adaptation seems to rely on a higher flexibility of key parts of the molecular structure or of the whole edifice through a decreased stability that partly compensates the freezing effect of low temperatures on the three-dimensional structure. As shown by genomics and proteomics, cold-shock proteins are also highly expressed and can have crucial roles in protein folding, control of nucleic-acid secondary structure, and transcription and translation. Approaches such as genome sequencing will undoubtedly shed new light on other characteristics of these fascinating organisms. These microbes might thrive inside cold digesters led to McFadden's experiments with digesters containing psychrophile-rich mud from a frozen lake in Alaska. Using a 1000-litre digester, the pair were able to produce 200 to 300 litres of methane per day. Similar-sized digesters in warmer regions can produce 1000 litres of biogas a day – enough to power a fridge for 16 to 20 hours. A new study by Concordia University's Rajinikanth Rajagopal, David Bellavance and Mohammad Saifur Rahaman demonstrated the viability of using anaerobic digestion in a low temperature environment (5 ° C to 25 ° C). The results of the research published in the journal Process Safety and Environmental Protection have shown that it is possible to convert solid food waste into renewable energy and organic fertilizer.

According to research, the level of global municipal waste will reach 2.2 billion tons by 2025. The greenhouse gases produced by this waste should constitute a serious climate threat to the planet. The researchers behind the new study claim that this gas can be captured through anaerobic digestion techniques designed and transformed into renewable energy. The low temperature bioreactor is therefore an effective solution.

2.2 Psychrophiles and Biogas

Biogas is used as a fuel, making its energy content an important characteristic. The heat of combustion, which is the total energy released as heat from complete combustion, is related to its methane content and can be estimated by equation 1 (Smith, et al., 2005).

$$Hc,ogas = HcCH_4 \cdot pCH_4 \quad (1)$$

Where, H$c,ogas$ is the heat of combustion of the biogas [kJ/mol]

$HcCH_4$ is the heat of combustion of methane [kJ/mol]

pCH_4 is the portion of methane present in the biogas, %

Methane usually makes up 50-75% of the biogas. Other components include carbon dioxide (25-45%), water vapor (2-7%), oxygen (< 2%), nitrogen (< 2%), ammonia (< 1%), hydrogen (< 1%) and hydrogen sulfide (< 1%) (Al Seadi, et al., 2008). Mesophiles have a working temperature range of 20-40 ^{o}C with 35-37 ^{o}C being the optimal range whereas psychrophiles have the optimal range of 15-20 ^{o}C.

Denali commission research has successfully identified the potential of psychrophilic methanogens to produce biogas. The research was mainly based on the comparative biogas production potentials of mesophilic and psychrophilic methanogens at warm and cold temperatures. The feed supplied for the purpose was food waste from kitchen. Digesters containing psychrophiles produced significantly more biogas than those containing mesophiles only, both at 15°C and 25°C. Interestingly, it was found that psychrophilic-digesters kept at 25°C produced more biogas as compared to digesters at 15°C (Alaska Centre for Research and Power, 2009-September 2011). Another study showed that 0.66 to 0.92 m^3 m^{-2} per day of biogas (70% methane) was collected at a 10-11ºC lagoon in California. (Chandler et al, 1983).

The methanogenesis capability of the psychrophilic methanogens is also supported by the research by Zhang et al. (2008). However, the research uses methanol as feed. So, research based on the use of feed which is commonly available in households, is of utmost importance to quantify the methane production depending upon various varying parameters. The result from the characterizations and tests will be the starting point for the design of digester for household biogas production (Dhadse, 2012). Psychrophilic methanogens have been isolated from sediment samples from Baldegger Lake and Soppen lake in Switzerland using an anaerobic mineral medium supplemented with microelements and vitamins (Nozhevnikov, 2003). A psychrophilic hydrogen utilizing methanogen, *Methanogenium frigidium*, has also been isolated using MSH medium from Ace Lake, Antarctica (Franzmann, 1997).

2.3 Psychrophiles in Nepal

Isolation of psychrophilic methanogens from wetlands in high altitude regions of Nepal has not been done. This research is also highly urgent to determine whether the lakes in the high altitudes of Nepal emit methane or not, as several lakes in Alaska have been shown to exhibit continuous methane bubbling (Walter, 2007).

Several other technologies are being used and researched about to make the biogas in cold climate regions efficient. However, the biogas digesters use conventional mesophilic bacteria. Furthermore, these researches are mainly based on increasing the temperature of the digester by using various added systems such as solar heater, heat composting, multifunctional green house and other approaches (Buysman, 2009). Moreover, it is to be noted that alpine characteristic of Nepal varies in terms of higher altitude and even lesser vegetation.

Research regarding supporting the conventional biogas digester has also been done. The research focuses on finding out appropriate ratio of mixtures and other design parameters when the digester is to be heated to raise the methane production. The heating methodology used is the combination of solar heating and biogas heating. The biogas heating is prevalent when solar heating is not available (Rennuit, 2013). However, this kind of system requires high amount of feed which may not be available in high altitude rural regions of places as Nepal. Hence, such technology has been proved to be inefficient compared to biogas in warmer climates and costly as well.

2.4 Climate Change and Psychrophiles

Forest disturbance, dispersion and shifting, bio diversity change, forest disease and pest infestations, abiotic disturbances are major adverse effect of climate change on forestry. (Gupta, 2010). Increase in temperature and decrease in precipitation causes vegetation shift and decrease in vegetation (ACAP, 2012). Providing an alternative to the already scarce firewood would lead to conservation of forests and in addition would reduce the expenditure in comparatively costlier forms of energy like LPG gas and fossil fuels. Furthermore, soil fertility is naturally poor in upper-hilly regions and this problem is further amplified by the decrease in precipitation as a result of climate change. The end products of biogas digestion can be utilized as a pathogen free, nutrient-rich fertilizer which can significantly enhance soil fertility and also promote healthy sanitary practices (Wright) (Lukehurst, 2010). Several organizations in Nepal like BSP-Nepal, AEPC, and SNV have focused on installation of biogas plants in the recent years. For instance, BSP-Nepal adds nearly 20,000 biogas plants every year and aims to have a total of 500,000 plants in Nepal. These organizations as well as several academic institutions have also been conducting research to find alternatives to the existing biogas systems for cold climates. Development of guidelines for production of biogas using psychrophiles will aid the organizations to implement it in the probable sites.

2.5 Morphology of Psychrophiles

The morphology of psychrophiles consists of them being Gram negative, citrate utilizing, oxygenic photosynthetic bacteria that are prokaryotic in nature. Catalase test must be negative.

2.6 Isolation of Bacteria

Serial Dilution is a method commonly used in microbiology to dilute the source sample so as to minimize the diversity of microorganisms in the sample and facilitate easier isolation of the desired organism. This process involves the following steps:

- Transfer 1gm sample to a test tube containing 9ml distilled water. This solution is now said to be of the dilution order 10^{-1}
- Then, transfer 1ml solution from the first test tube to a second tube containing 9ml distilled water. This solution is of the dilution order 10^{-2}
- Repeat the process until the desired dilution order is reached.

2.7 Biological Characterization

The following tests can be carried out for the morphology and biochemical characterization of the collected samples. It is to be noted that oxidase and catalase tests are a part of reference to the Bergey's Manual of Systematic Bacteriology for determination of genera. Moreover, Sugar Fermentation Test and IMViC tests could not be carried out due to budget and time constraints.

- **Gram's staining**

  Gram staining or Gram stain, also called Gram's method, is a method of staining used to differentiate bacterial species into two large groups (gram-positive and gram-negative).

- **Catalase test**

  The catalase test is also one of the main three tests used by microbiologists to identify species of bacteria. The presence of catalase enzyme in the test isolate is detected using hydrogen peroxide. If the bacteria possess catalase (i.e., are catalase-positive), when a small amount of bacterial isolate is added to hydrogen peroxide, bubbles of oxygen are observed.

  1. If the mixture produces bubbles or froth, the organism is said to be 'catalase positive. Staphylococci and Micrococcus are catalase-positive. Other catalase-

positive organisms include *Listeria, Corynebacterium diphtheriae, Burkholderia cepacia, Nocardia, the family Enterobacteriaceae (Citrobacter, E.coli, Enterobacter, Klebsiella, Shigella, Yersinia, Proteus, Salmonella, Serratia), Pseudomonas, Mycobacteriumtuberculosis, Aspergillus, Cryptococcus and Rhodococcus equi.*

2. If not, the organism is 'catalase-negative'. *Streptococcus* and *Enterococcus* spp. are catalase-negative.

- **Oxidase test**

The oxidase test is used to identify bacteria that produce cytochrome c oxidase. All bacteria that are oxidase positive are aerobic, and can use oxygen as a terminal electron acceptor in respiration

1. OX+
    - OX+ normally means the bacterium contains cytochrome c oxidase and can therefore use oxygen for energy production by converting O_2 to H_2O_2 or H_2O with an electron transfer chain.
    - The Pseudomonadaceae are typically OX+

    The Gram-negative diplococci Neisseria and Moraxella are oxidase-positive.
    - Many Gram-negative, spiral curved rods are also oxidase-positive, which includes Helicobacter pylori, Vibrio cholerae, and Campylobacter jejuni.
    - Also Legionella pneumophila is oxidase-positive.

2. OX-

    OX- normally means the bacterium does not contain cytochrome c oxidase and, therefore, either cannot use oxygen for energy production with an electron transfer chain or employs a different cytochrome for transferring electrons to oxygen. Enterobacteriaceae are typically OX-.

- **Citrate Utilization test**

The citrate test detects the ability of an organism to use citrate as the sole source of carbon and energy. Bacteria that grow in the medium turn the medium alkaline. This is indicated by the change of color of bromothymol blue indicator from green to blue.

1. **Positive Reaction:** Growth with color change from green to intense blue along the slant.

Examples: *Salmonella, Edwardsiella, Citrobacter, Klebsiella, Enterobacter, Serratia, Providencia,* etc.

2. **Negative Reaction:** No growth and No color change; Slant remains green.
   Examples: *Escherichia, Shigella, Morganella, Yersinia* etc.

CHAPTER THREE: METHODOLOGY

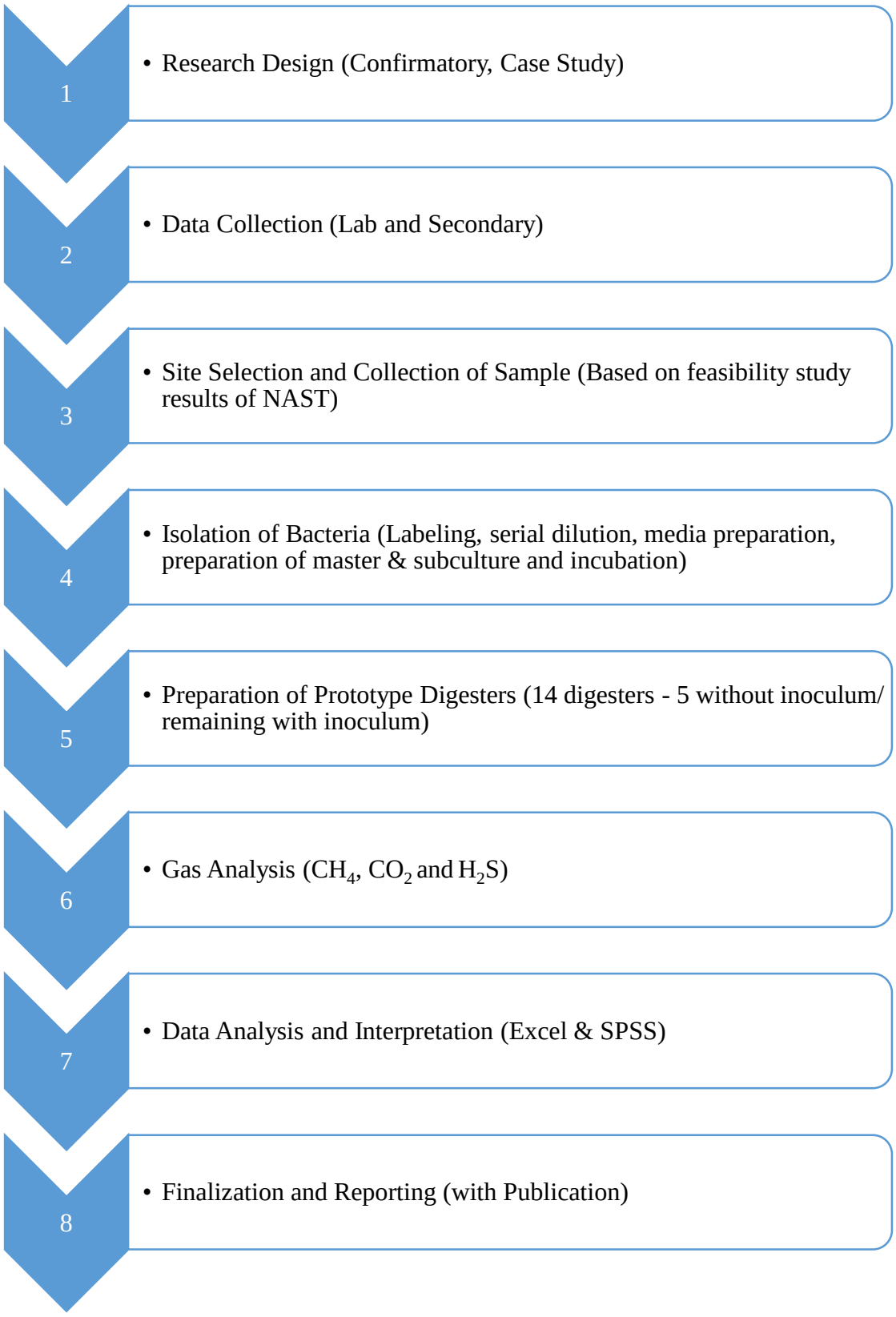

Figure 3.1 Methodology Hierarchy

3.1. Research Design

The proposed research follows a confirmatory approach and a case study design. Since the research objective and approach requires both field level work as well as theoretical review in areas with little known work, the research was exploratory in nature.

3.2. Data Collection

The primary data source for the verification of psychrophilic bacteria was the confirmatory lab tests to be carried out whereas secondary data was attained through research papers and articles as well as other relevant ongoing projects. Permafrost was the best sample type for this purpose and keeping this in mind, organic soil was taken from such areas (1-2m deep). This constituted of slimy and sludgy soil as well.

3.3 Site Selection and Collection of Sample

The possible sampling sites were identified as wetlands of Manang (Gangapurna, Te Manang and Pisang area), Solukhumbu and Gosaikunda in Rasuwa district) as shown in Table 3.1. The primary objective was isolation and characterization of Psychrophiles from samples collected at a depth of about 1-2 meters below the water level. A total of 12 samples were stored in falcon tubes, sealed with parafilm and stored in ice boxes maintained at <10 deg. C for transport. However, samples from Solukhumbu were taken as the primary ones based on NAST's research on "Development of Design Guidelines of Digester for Biogas Generation at High Altitude" funded by ADB on 2016. Higher number of samples taken show the incidence of relatively high amount of organic matter available. Syangboche area's sample was taken from decomposing waste.

Table 3.1: Sample collection and site details

S.No.	Location	No. of samples	Nature of samples	Altitude (m)	Soil temp. (deg. C)
1.	Natural Pond, Te Manang	1	Lake Sediment	3572	-1.3
2.	Gangapurna, Manang	3	Lake Sediment	3570	0.9

S.No.	Location	No. of samples	Nature of samples	Altitude (m)	Soil temp. (deg. C)
3.	Natural Pond, Pisang	1	Lake Sediment	3314	1
4.	Natural Pond, Pisang	1	Lake Sediment	3348	1.5
5.	Gosainkunda, Rasuwa	2	Lake Sediment	4380	-1.5
6.	Natural Pond, Lauribinayak, Rasuwa	1	Wetland Sediment	3846	0.2
7.	Gokyo Lake, Solukhumbu	2	Lake Sediment	4788	-2.1
8.	Syangboche, Solukhumbu	1	Fecal waste	3846	1.7

3.4 Isolation of Bacteria

The isolation of bacteria from the collected samples involved the following steps:

- **Labeling of samples:**
  For ease during isolation, the soil samples collected from Gangapurna lake were named with their respective abbreviations as follows:
  - Te Manang Sample: T
  - Gangapurna Sample: G1, G2, G3 (collected from different parts of the same lake)
  - Pisang samples : P1, P2
  - Gosaikunda sample: GO1, GO2
  - Lauribinayak sample: L
  - Gokyo Lake sample: S1 (greener part), S2 (barren part)

- Syangboche sample: S3

- **Serial dilution:**

  The soil samples were serially diluted to concentrations ranging from 10^{-1} to 10^{-5}. Dilutions 10^{-3}, 10^{-4} and 10^{-5} were used for inoculation of samples G3 and T whereas10-^{3} and 10^{-4} was used for inoculation of the rest of the samples.

- **Media Preparation:**

  MSH media was used for the isolation of desired bacteria. Stock solutions were prepared and kept for use.

- **Preparation of Master and Sub Culture**

  Master plates were prepared from the soil samples using the spread plate technique. In total, 24 plates were inoculated. For samples G3 and T, two plates each were prepared

  for the dilutions 10^{-3}, 10^{-4} and 10^{-5} and these were the first samples to be inoculated. Later, one plate each for dilutions 10^{-3} and 10^{-5} were prepared for samples G1, G2, G4, P and DP. Subculture was done through quadrant streaking.

- **Incubation:**

  After inoculation, plates were placed inside an airtight chamber (box). This was censured by lighting a candle and the lid was closed. The candle extinguished after about 3 minutes and the chamber was now assumed airtight. The box was placed in a refrigerator maintaining 0 to 5 deg. C temperature.

3.5 Preparation of Prototype Digesters

In total, 14 prototype digesters were prepared from jars with different samples and were operated at three different temperatures (10°C, 15^{o}C, 25^{o}C and one at room temperature) with retention period of 60 days for each. Gokyo Lake samples were used for two different sets of digesters operated at temperatures 10, 15 and 25°C. Digesters with different feedstock composition were set up.

Table 3.2 Prototype digesters with Mesophilic conditions

Composition Jar	Kitchen waste(kg)	Cow dung (kg)	Sample (kg)	Water (kg)	Temperature
Jar 0	1.5	1.5	-	2	15^{o}C
Jar 1	1.5	1.5	-	2	Room Temp.
Jar 2	1.5	1.5	-	2	10°C
Jar 3	1.5	1.5	-	2	25^{o}C
Jar 4	1.5	1.5	-	2	5°C

Table 3.2 above shows the setup of simple mesophilic conditions for 4 prototype digesters without the addition of samples at different temperatures.

Table 3.3 Prototype digesters with S1 and S2 inoculums

Composition Jar	Kitchen waste(kg)	Cow dung (kg)	Sample (kg)	Water (kg)	Temperature (T1 for 60 days)	Temperature (T2 for 60 days)	Temperature (T3 for 60 days)
Jar 5	1.5	1.5	1(S1)	2	25°C	15^{o}C	10°C
Jar 6	2	-	1(S1)	2	25°C	15^{o}C	10°C
Jar 7	2	-	1(S1)	2	25°C	15^{o}C	10°C
Jar 8	1.5	1.5	1(S2)	2	25°C	15^{o}C	10°C
Jar 9	2	-	1(S2)	2	25°C	15^{o}C	10°C
Jar 10	1.5	1.5	1(S2)	2	25°C	15^{o}C	10°C

Table 3.3 shows the proportion of kitchen waste and cow dung mixed to form a slurry with water and inoculation was done with S1 and S2 colonies.

Table 3.4 Prototype digesters with S3 inoculums

Composition Jar	Kitchen waste(kg)	Cow dung(kg)	Sample (kg)	Water (kg)	Temperature (T1 for 60 days)	Temperature (T2 for 60 days)	Temperature (T3 for 60 days)
Jar 11	1.5	1.5	1(S3)	2	25°C	15°C	10°C
Jar 12	2		1(S3)	2	25°C	15°C	10°C
Jar 13	1.5	1.5	1(S3)	2	25°C	15°C	10°C

Tables 3.3 and 3.4 show the setup for samples S1, S2 and S3 at 25, 15 and 10 deg. Celsius for gas production and analysis. S1 represents samples from greener part of Gokyo, S2 was samples from barren area whereas S3 represents samples from Syangboche.

3.6 Gas Analysis

The following equipments were used for pressure measurement

- Digital Multimeter: Fluke DMM 117
- Digital Pressure Module: Fluke-Module PV 350

The composition of the collected gas was determined using a digital gas analyzer: German gas analyzer Gasboard – 3200P, which detected gases Methane (CH_4), Carbondioxide (CO_2) and Hydrogen sulphide (H_2S). A burning flame from the gas outlet when exposed to a flame indicated positive result.

3.7 Data Analysis and Interpretation

The collected data was analyzed for volumetrics, production and feedstock using software tools as per the requirement of analysis and design which may include MS-Excel, SPSS, etc.

3.8 Finalization and Reporting

The research paper for the thesis was published in SAS Publications November 2019 edition and the reporting has been carried out as per the norms of Pulchowk campus.

CHAPTER FOUR: RESULTS AND DISCUSSION

4.1. Identification of Temperature Conditions of Manang and Solukhumbu

The average high temperature was 21°C in Manang and 17°C in Solukhumbu and the average low temperature was 0°C-1°C. The temperature of the soil at sample collection sites ranged from -2°C to 3°C.

4.2. Colony Morphology of Samples

After about 15 days of incubation, colonies started to appear on most of the master plates, except some. A common observation among all the colonies was that almost all of them were brightly colored, circular in shape and exhibited liquid-like colonies rather than solid ones. Distinct colonies from all the above master plates were sub-cultured and growth was observed in all of the plates. Again, the sub-cultured plates displayed continuous liquid-like morphology. Out of the obtained plates, 10 plates were selected for further biochemical tests and were named using numbers after the first letter M (eg. M1, M2, M3, M4 etc).

Table 4.1 Colony morphology of samples

Samples	**Colony Morphology**
G3 (10^{-3}) a	Pinkish Red (dense, watery)
G3 (10^{-4}) a	No growth observed
G3 (10^{-4}) b	No growth observed
G3 (10^{-5}) a	No growth observed
G3 (10^{-5}) b	Yellow (dense, watery)
T (10^{-3}) a	No growth observed
T (10^{-3}) b	White + Yellow + Orange (all thin, watery)
T (10^{-4}) a	Yellow (thin, watery)
T (10^{-4}) b	No growth observed

Samples	**Colony Morphology**
T (10^{-5}) a	Slimy creamy+Orange (dense, watery)
T (10^{-5}) b	No growth observed
G1 (10^{-3})	No growth observed
G1(10^{-4})	No growth observed
G2(10^{-3})	No growth observed
G2(10^{-4})	No growth observed
G4(10^{-3})	Yellow + White (both thin, watery)
G4(10^{-4})	Orange (thin watery)
P(10^{-3})	Yellowish orange (dense, watery)
P(10^{-4})	Yellowish orange (dense, watery)
DP (10^{-3})	No growth observed
DP (10^{-4})	No growth observed
S1(10^{-3})	Slimy creamy + orange
S1(10^{-4})	Slimy creamy + orange
S2(10^{-3})	Slimy creamy + orange
S2(10^{-4})	Yellowish orange (dense, watery)
S3(10^{-3})	Slimy creamy + orange
S3(10^{-4})	Slimy creamy + orange

As seen in the table above, the colonies without any growth were discarded as it would be of no interest to the cause of this research.

4.3 Characterization of Bacterial Samples

Four biochemical tests were performed followed by a fluorescence test for characterization of the bacterial samples obtained. The results for these tests are shown in Table 4.2.

Table 4.2: Biochemical test results

Colonies	**Colony Name**	**Gram Staining Test**	**Catalase Test**	**Oxidase Test**	**Citrate Utilization Test**
G3(10-3)a (pinkish red)	M1	+ve (spherical)	+ve	+ve	-ve
T(10-5) a (orange)	M2	-ve (spherical)	+ve	-ve	-ve
T (10-4)a (yellow)	M3	+ve (spherical)	+ve	-ve	+ve
T (10-3)b (whitish yellow)	M4	+ve (rods)	+ve	-ve	+ve
T(10-3)b (orange)	M5	+ve (spherical)	+ve	-ve	-ve
G3 (10-5)b (yellow)	M6	+ve (spherical)	+ve	-ve	+ve
P(10-4) (yellowish orange)	M7	+ve (spherical)	+ve	+ve	-ve
G4(10-4) (orange)	M8	+ve (spherical)	+ve	+ve	-ve
G4 (10-4) (yellow)	M9	+ve (spherical)	+ve	+ve	-ve
G4(10-3) (white)	M10	+ve (spherical)	-ve	-ve	+ve
S1(10^{-3}) (Slimy creamy + orange)	M11	-ve (rods)	-ve	+ve	+ve

Colonies	Colony Name	Gram Staining Test	Catalase Test	Oxidase Test	Citrate Utilization Test
S1(10^{-4}) (Slimy creamy + orange)	M12	-ve (rods)	-ve	+ve	+ve
S2(10^{-3}) (Slimy creamy + orange)	M13	-ve (rods)	-ve	+ve	+ve
S2(10^{-4}) (Yellowish orange)	M14	+ve (spherical)	-ve	+ve	-ve
S3(10^{-3}) (Slimy creamy + orange)	M15	-ve (rods)	-ve	+ve	+ve
S3(10^{-4}) (Slimy creamy + orange)	M16	-ve (rods)	-ve	+ve	+ve

Any colony exhibiting positive results to Catalase tests were discarded since it inidicates that the bacteria is of aerobic in nature which is contradictory to the purpose of methanogenesis. Since M11, M12, M13, M15 and M16 demonstrated the closest morphology to that of Psychrophiles, it was decided that from then on, the thesis should be focused on the prototype digester research with samples from Solukhumbu.

4.4. Gas Analysis

The total volume of each digester formed were 20 liters. The volume of the gas in the jar was the total volume after the volume of the feed was deducted. A total of 60 days retention time was given before checking the gas composition. Jars 0-4 were

under mesophilic conditions whereas the remaining was inoculated with samples from Solukhumbu as see in Table 4.3.

As seen in table 4.3, the flame test continued to be positive only till 10 deg. C and was negative at 5 deg. C along with a dramatic drop in the gauge pressure and gas volume.

Table 4.3 Methane production from mesophiles

Jar	Temp (K)	Gauge Pressure (kPA)	Gas Composition (vol %)					Gas volume in Jar (Ltr)	Flame Test
			CH_4	CO_2	H_2S (ppm)	N_2	H_2		
Jar 0	288	202	31	28.2	0.714	38.2	1	15.0	+ve
Jar 1	296	448	64	21	0.798	12.8	1	16.0	+ve
Jar 2	283	192	24	37.1	0.612	37.2	1	15.0	+ve
Jar 3	298	545	63	22	0.768	12.9	1	14.0	+ve
Jar 4	278	171	9	17	0.186	72.3	1	14.8	-ve

Table 4.4. shows the favorable production of biogas at 25 deg. C with proper methane proportion level maintained. Table 4.5 shows that this was maintained even at 15 deg. C for jars with the psychrophilic inoculum.

Table 4.4 Methane production (60 days at T1 25 deg. C)

Jar	Temp (K)	Gauge Pressure (kPA)	Gas Composition (vol %)					Gas volume (Ltr)	Flame Test
			CH_4	CO	H_2S (ppm)	N_2	H_2		
Jar 5	298	426	66	20.8	0.347	11.8	1	14.0	+ve
Jar 6	298	422	61	21	0.008	16.9	1	15.0	-ve
Jar 7	298	412	62	19	0.012	17.2	1	14.0	-ve

Jar	Temp (K)	Gauge Pressure (kPA)	Gas Composition (vol %)					Gas volume (Ltr)	Flame Test
			CH_4	CO	H_2S (ppm)	N_2	H_2		
Jar 8	298	456	65	18	0.044	15.8	1	14.0	-ve
Jar 9	298	474	61	21	0.006	16.5	1	15.0	-ve
Jar 10	298	432	64	20.4	0.346	14.2	1	15.0	+ve
Jar 11	298	458	64	22.6	0.408	11.2	1	15.0	+ve
Jar 12	298	434	63	19.8	0.206	15.3	1	16.0	+ve
Jar 13	298	414	66	19.1	0.169	13.2	1	14.0	+ve

Table 4.5 Methane Production at 15 deg C

Jar	Temp (K)	Gauge Pressure (kPA)	Gas Composition (vol %)					Gas volume (Ltr)	Flame Test
			CH_4	CO_2	H_2S	N_2	H_2		
Jar 5	288	392	58	21	0.242	19.2	1	14.0	+ve
Jar 6	288	348	58	20.1	0.009	20.2	1	15.0	-ve
Jar 7	288	372	61	22.7	0.017	15.2	1	14.0	-ve
Jar 8	288	339	62	20.8	0.049	17	1	14.0	-ve
Jar 9	288	344	58	26.7	0.007	13.5	1	15.0	-ve
Jar 10	288	362	61	26.4	0.398	11.2	1	15.0	+ve
Jar 11	288	329	62	20	0.416	16.1	1	15.0	+ve
Jar 12	288	337	63	17.2	0.217	18.4	1	16.0	+ve

Jar	Temp (K)	Gauge Pressure (kPA)	Gas Composition (vol %)					Gas volume (Ltr)	Flame Test
			CH_4	CO	H_2S (ppm)	N_2	H_2		
Jar 13	288	391	61	19.1	0.169	17.8	1	14.0	+ve

It can be seen in the above tables that with the drop in temperature - gas pressure, methane content and gas volume also decrease in the jars without inoculum. This also yielded negative flame tests as well.

Table 4-6: Methane Production at 10 deg C

Jar	Temp (K)	Gauge Pressure (kPA)	Gas Composition (vol %)					Gas volume (Ltr)	Flame Test
			CH_4	CO_2	H_2S	N_2	H_2		
Jar 5	283	224	48	26	0.268	24.7	1	14.0	+ve
Jar 6	283	208	59	22.4	0.008	17.5	1	15.0	-ve
Jar 7	283	212	51	28	0.012	19.9	1	14.0	-ve
Jar 8	283	209	52	25	0.044	21	1	14.0	-ve
Jar 9	283	224	58	28.5	0.006	12.4	1	15.0	-ve
Jar 10	283	232	44	38.7	0.346	15.7	1	15.0	+ve
Jar 11	283	219	59	22	0.408	16.9	1	15.0	+ve
Jar 12	283	241	61	18.4	0.206	19.2	1	16.0	+ve
Jar 13	283	231	55	30.6	0.169	12.8	1	14.0	+ve

It was seen that even in lower temperature ranges of 10 deg. C, jars with the psychrophilic inoculum produced suitable amounts of gas volume. Further observation at even 5 and 0 deg. C showed that Methane production constituted of an average of 52% in the jars with a low CH_4 : CO_2 ratio but a final table could not be developed due

to leakage development in the prototype digesters. As seen in Figures 4.1 and 4.2, it is an obvious fact that gas pressure and methane production dropped with temperature drop under mesophilic bacterial decomposition.

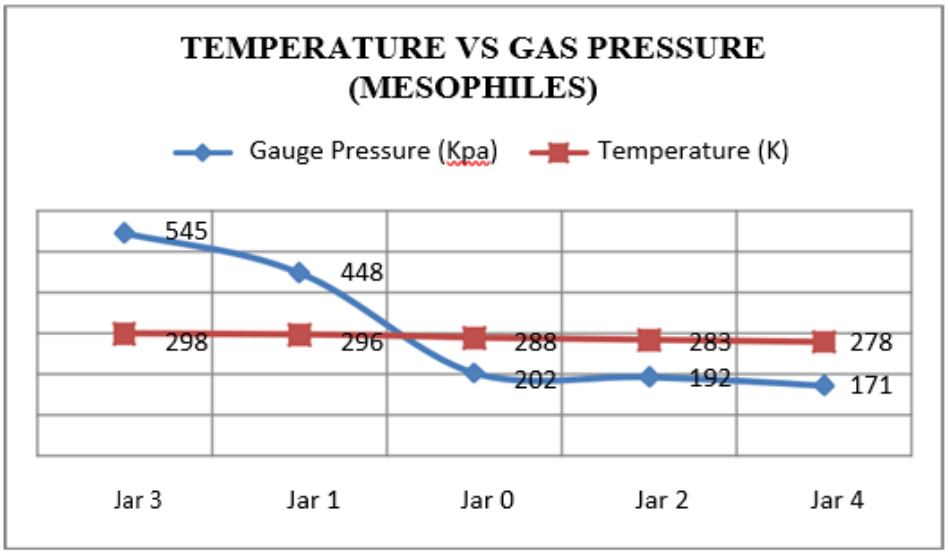

Figure 4.1 Temperature VS Gas Pressure (Mesophiles)

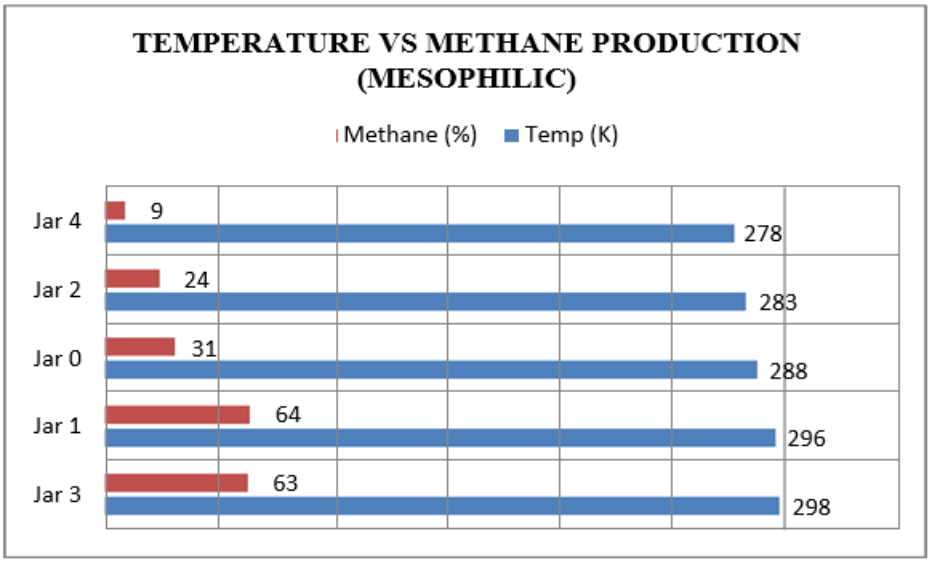

Figure 4.2 Temperature VS Methane Production (Mesophiles)

4.5 Comparative Production of Methane

The main purpose of this was to see the performance of Psychrophiles at high temperature (25 deg. C), optimal temperature (15 deg. C) and lo temperature (10 deg. C). It was seen that collected sampled psychrophiles exhibited either higher or similar

methane production potential at 25^{O}C. Jar 2 here was the prototype digester maintained at 25^{O}C under mesophilic conditions.

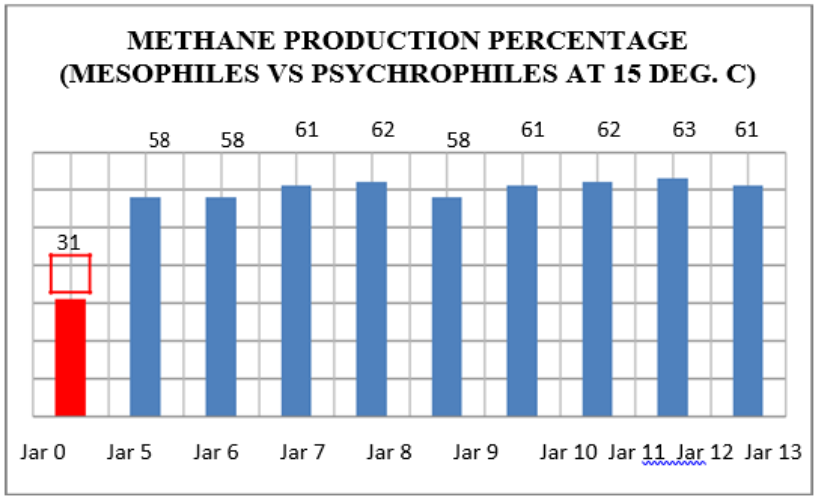

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Figure 4.3: Methane Production Percentage (Mesophiles vs Psychrophiles at 25 deg. C)

As seen in Figure 4.4, at 15 deg. C which is considered the optimal temperature for Psychrophiles, the methane content was in usable and good range throughout the prototype digesters. On the contrary, mesophilic activity dropped in Jar 0 at the same temperature with the methane percentage dropping to 31. A feeble positive flame test was observed.

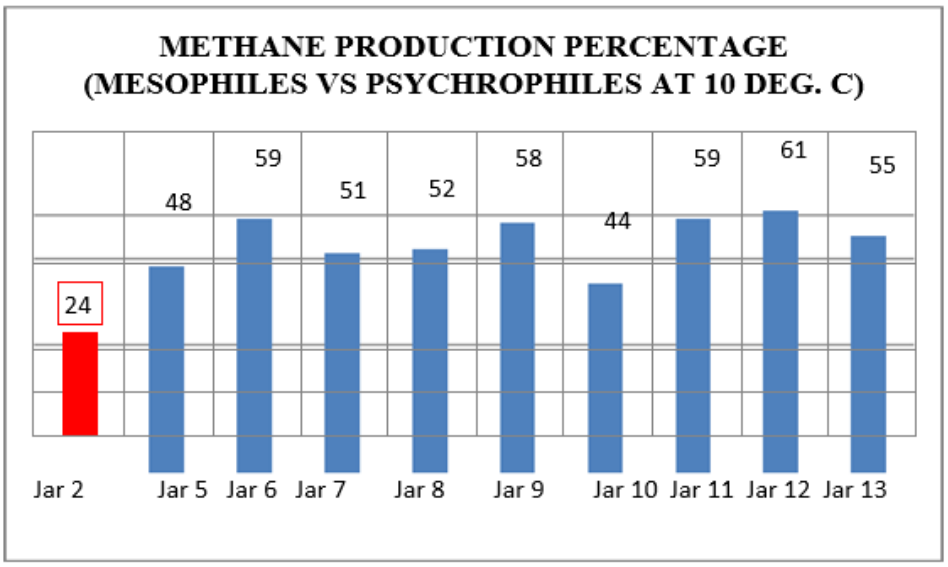

Figure 4.4: Methane Production Percentage (Mesophiles vs Psychrophiles at 15 deg. C)

4.5.1 Assumptions:

Due to the limitation of the gas analyzer, it is assumed that, the composition of biogas derived from various prototype digesters consists of 1% of Hydrogen. The partial pressure of each constituents of the gas was calculated from the Dalton's Law of Partial Pressure and the mass of each constituent was calculated by using ideal gas equation which is

$$PV = mRT$$

Where,

- P is pressure in kPa
- V is volume in m3
- m is mass in kg
- R is gas constant in KJ/kg K
- T in temperature in Kelvin

The values of gas constant of various gases are shown below in Table 4.7:

Table 4.7: Gas Constant Values for Constituent Gases in Biogas

Gas	**Gas Constant (J/kg K)**
Methane	518.3
Carbon di-oxide	188.9
Hydrogen sulphide	244
Nitrogen	297
Hydrogen	412

The gas constant value of the composition mixture was calculated by using the formula

$$R = \frac{R1 * M1 + R2 * M2 + R3 * M3}{M1 + M2 + M3}$$

Where,

- R = overall gas constant
- R1, R2, R3 = Individual gas constant
- M1, M2, M3 = Mass of Individual gas

The table in Annex 2 depicts the partial pressure and gas constant values obtained in the prototype digesters. It is to be noted that apart from the leakages developed, jars with psychrophilic inoculum generally yielded higher and favorable values of gas production.

4.6 Total Solid (TS) value

Total solid contained in a certain amount of materials is usually used as the material unit to indicate the bio-gas producing rate of materials. 1 kg TS of cattle dung produces 0.25 m3 of biogas at 25°C. Again from ideal gas equation for biogas,

Gas constant for biogas = 0.518 kJ/ kg K
For 1 atm pressure, 1 kg TS of cattle dung produces 0.164 kg of biogas.

As Jar 1, Jar 10 and Jar 13 were kept in room temperature, it can be seen that the amount of biogas produced is 25.9 g, 26.7 g and 22.2 g respectively. The total amount of organic feed in Jar 1 was 3 kg.

So, $TS * 3 * 0.164 = 25.9 * 10^{-3}$

So, TS = 5.26 % for the organic feed which was put in the prototype digesters.

In the prototype digesters, Hydraulic Retention Time was kept to be 55 days.

4.7 Design of Digester of Volume 2 m^3

$V = 2\ m^3$

Cross –section of a digester:

a) Volume of gas collecting chamber: Vc
b) Volume of gas storage chamber = Vgs
c) Volume of fermentation chamber = Vf
d) Volume of hydraulic chamber = Vh
e) Volume of Sludge layer = Vs

Table 4.8 Geometrical Dimension of the Cylindrical Shaped Biogas Digester Body

For Volume	For geometrical dimensions
$V_c \leq 5\%$ V	$D = 1.3078 * V^{1/3}$
$V_s \leq 15\%$ V	$V_1 = 0.0827 D^3$
$V_{gs} + V_f = 80\%$ V	$V_2 = 0.05011 D^3$
$V_{gs} = V_h$	$V_3 = 0.3142 D^3$
$V_{gs} = 0.5 (V_{gs} + V_f + V_s) * K$	$R_1 = 0.725 D$
Where K = gas production rate per m3	$R_2 = 1.0625 D$
digester volume per day	$f_1 = D/5$
	$f_2 = D/8$
	$S_1 = 0.911 D^2$
	$S_2 = 0.8345 D^2$

For V = 2m3

D = 1.65 m

Similarly,

V3 = 3.14 * D2 * H/4

H = 0.66 m

f1 = D/5 = 0.33 m

f2 = D/8 = 0.21 m

R1 = 1.2 m

R2 = 1.75 m

V1 = 0.37 m3

Vc = 0.1 m3

Vgs = 0.5 (Vgs + Vf+ Vs) * K

=0.5*(0.8*2+0.15*2)*0.4

= 0.38 m3

Also Vgs = 50% of gas yield

= 0.5 * TS * gas producing rate per kg TS

=0.5 * TS * (0.011/(3*0.0526))

At 10℃, 1 atm pressure, the biogas production rate when the sample is added,

Production rate = 0.067 kg/kg TS

Production rate (volume) = 267 * 283*0.067/100000 = 0.05 m3

Gas production rate per kg TS = 0.05 m3/ kg TS

TS required = 15.2 kg TS

Considering 49 % of total solid in available discharge

Total discharge required = 15.2/0.49 = 30 kg per day 30 kg per day of organic wastes is required per day

Considering hydraulic retention time of 55 days,

Vgs + Vf = Q . HRT

Q = 1600/55 = 30 kg

So, this is confirmed by the digester volume as well that 30 kg organic feed is required per day for the given dimension of the cylindrical digester of 2 m3 volume.

a) Volume of gas collecting chamber: Vc
b) Volume of gas storage chamber = Vgs
c) Volume of fermentation chamber = Vf
d) Volume of hydraulic chamber = Vh
e) Volume of Sludge layer = Vs

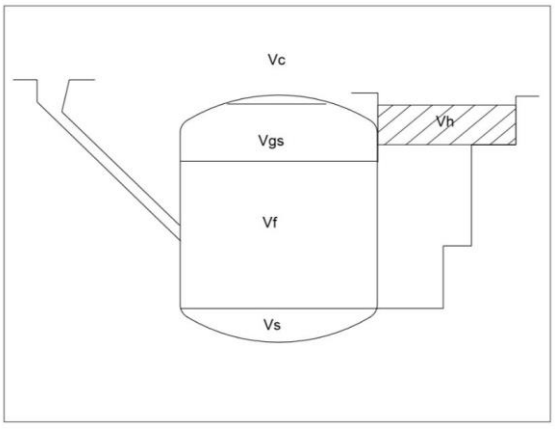

Figure 4.6 Cross Section of Digester

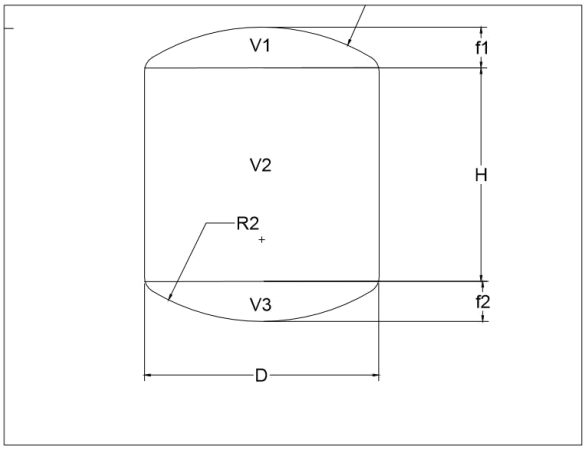

Figure 4.7 Geometrical Dimensions of Digester

4.8 Financial Analysis

The CBA was done taking Chitwan district, the central region of inner Terai of Nepal as a sample district. It was purposively selected for the study as research data was available. The study results focused on the adoption of the biogas in peri-urban area to cope with vulnerable climate change for raising livestock and farming purpose in two VDCs namely, Gitanagar and Patihani purposively; as they are marked as vulnerable to climate change (Dhakal, 2018). The cost and the benefits associated with the biogas plant were quantified and estimated on the basis of the valuation of the time saved, fuel used, and chemical fertilizer. The economic analyses were based on the following assumptions:

- Gas production maintained even in winter
- 10 kg of dung (or equivalent 1:1 ratio of organic waste and dung slurry) is required per m^3 size of biogas plant
- 0.036 m^3 of gas is produced per kg of fresh cattle dung
- m^3 of gas is equivalent to 3.5 kg of firewood
- The cost of firewood is Rs 30.00/kg
- Cost of psychrophilic inoculum incorporated in the plant cost

The use of biogas has significant reduction in the time for firewood collection, mosquito breeding, flies and rodents, foul odor, and smoke in overall. As per the response of the household, the findings showed that the monthly mean firewood use was 7.30 bundles before and 2.81 bundles after. The least quantity of the firewood use was three bundles in a month before and no use at all after biogas installation. The maximum amount of

the firewood use was 20 bundles before and 6 bundles after by the household. As the t-statistics showed, the firewood consumption decreased after biogas plant installation at 1% level of significance.

Table 4.9 Statistics About Firewood Use

Statistics	Before	After
Mean	7.30	2.81
Minimum	3.00	0.00
Maximum	20.00	6.00
Mean difference	-4.49***	

The findings showed that the mean sufficiency of one LPG cylinder was for 2.31 months before and 5.82 month after. The least duration of the LPG use was 1 month before and 3 months after biogas installation. The maximum duration of the LPG use was for 3 months before and 7 months after using the biogas by the household. As the t-statistics showed, the duration of one LPG increased after biogas plant installation at 1% level of significance.

Table 4.10 Statistics About the Sufficiency of One Cylinder of LPG (in month)

Statistics	Before	After
Mean	2.31	5.80
Minimum	1.00	3.00
Maximum	3.00	7.00
Mean difference	3.49***	

The study revealed that the mean time spent for firewood collection was 2.2 and 0.33 hour before and after respectively. Maximum 4 hours were spent by the household for the collection of the firewood before and 1 hour after. It was found that the time spent for firewood collection decreased from 132 hours to only 20 hours for sixty households on an aggregate. The t-statistics showed that the use of biogas has significant role in the reduction of time for collecting the firewood by the household. The level of significance was at 1% with mean difference of 1.87 hours.

Table 4.11 Statistics About the Time Spent for Firewood Collection in a Day (in hour).

Statistics	Before	After
Mean	2.20	0.33
Minimum	1.00	0.00
Maximum	4.00	1.00
Mean difference	-1.87***	

The initial investment to be made for the construction of six cubic meter size was Rs. 57000. Assuming the discount rate 14% (AEPC, 2013), the total discounted cash inflow and out flow were calculated. The payback period was calculated using the formula mentioned in the methodology. From the financial analysis, analysis it was found that the Benefit cost ratio was found to be 1.62 at 14% rate of discount. This means that the investment in the biogas by Re

1 would provide return of 62 paisa. Therefore, the use of biogas plant is more viable as cost associated with it is outweighed by the benefit obtained. The Net Present Value (NPV) was found to be Rs. 128,113.10 which suggests that the construction of the biogas plant is economically viable.

The payback period was found to be 2 years and 9 months. It means that the initial capital investment made is recovered by annual outcome with 2.75 years. The internal rate of return is more than 54.67%, which is greater than the interest rate of bank. The sensitivity analysis also indicated that under the various changed parameter of biogas, the installation is economically viable. Therefore, it is worth considering that investment in the biogas plant construction by a household is rational and the economic return is higher than depositing in bank.

Table 4.12 Summary of the economic analysis of biogas plant installation.

Particulars	Unit	Value
B/C Ratio	-	1.62
NPV	Rs.	128113.10
IRR	%	54.67
Payback Period	Yrs.	2.75

The above finding suggest that the installation of biogas in economically viable. The benefits of the biogas even in winter will be remarkable if it is feasible even in the winter

season and this hypothetical CBA if for the verification of the same. The installation of the biogas is financially viable and the rational decision of any households that reduces the vulnerability due to climate change. The information about the benefits of the biogas and the various appropriate policy, schemes, subsidy, loan, for installation and post installation maintenance of biogas should be provided by the distributors and government to reduce the GHGs from household level.

CHAPTER FIVE: CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

A maximum capacity utilization factor (CUF) of 18.49% was observed in the month of January 2017 and a minimum CUF of 16.5% was observed in the month of July of the same year. The average capacity factor of the power plant was 17.49%. Annual energy generation from the power plant was 13,601.41 kWh. The average performance ratio calculated was 36.98% with a maximum of 67.61% and had a minimum of 25.77% with respect to different reference insolation. The extra demand would be 30,006 kWh from the sample households of 55 out of 115. When this pattern of load distribution was generalized in the population then the total demand would be 64,992kWh per year. However, with an annual supply of 13,601.41 kWh the rising demand of the community would be very difficult to fulfil. In order to fulfill the increased demand, the power plant must run at 82.44%.

Average annual income of the beneficiaries of micro grid was Rs 364,550/- with a modal income of Rs. 237,500/- which has escalated around by 20 folds within 2 years. The net present value of NPR -10,978,605.76 was obtained due to unavoidable replacement cost of batteries, charge controllers, inverters and high initial investment without the consideration of the grant amount. The grant for the project was of value NPR 11,295,000.00 from AEPC, IFAD and UNESCAP. Whereas, with 84.32% utilization of grant, NPV worth of NPR 384,394.22 was obtained for the project. In breakeven analysis, a breakeven point of the project is obtained at 81.87% utilization of the grant. Average unit cost of electricity was found to be Rs 37.08 but it varied from Rs 16.67 to Rs 80.81. Household consuming more electricity had to pay less unit cost of electricity whereas household consuming less electricity had to pay higher unit cost of electricity. The return on investment was 10.61% on SUN's investment, 12.36% on net cash outlay, 20.59% on initial cash outlay on net income and 3.84% on initial investment.

5.2 Recommendations

- It is recommended that genetic/ DNA tests be carried out for concrete identification of Psychrophiles
- Subsidies must be provided for psychrophilic inoculum

REFERENCES

ACAP. (2012, July). Climate change impact on livelihood and natural resources of Upper Mustang. National trust for Nature Conservation.

Ajay Kumar Jha, Jianzheng Li and Tri Ratna Bajracharya (2014). Performance Characteristics of Semi-Dry Anaerobic Digestion of Cow Dung and Excess Sludge Mixture under Psychrophilic Condition

Alaska Centre for Research and Power. (2009-September 2011). *An investigation of psychrophiles for generating heating gas in arctic environments: A Project by Cordova Electric Cooperation.* Alaska: Denali Commission.

Alaska Centre for Research and Power. (2009-September 2011). An investigation of psychrophiles for generating heating gas in arctic environments: A Project by Cordova Electric Cooperation. Alaska: Denali Commission.

Alla N. Nozhevnikov, K. Z. (2003). Evidence for the Existence of Psychrophilic Methanogenic Communities in Anoxic Sediments of Deep Lakes. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY* , 1832-1835.

Alla N. Nozhevnikov, K. Z. (2003). Evidence for the Existence of Psychrophilic Methanogenic Communities in Anoxic Sediments of Deep Lakes.

Amarasekara, R. (1994). Energy for Cooking. Integrated Development Association (IDEA).

Buysman, E. (2009). *Biogas for Developing Countries with Cold Climates: Addressing the challenge of Biogas production in cold regions.*

Buysman, E. (2009). Biogas for Developing Countries with Cold Climates: Addressing the challenge of Biogas production in cold regions.

C. T. Lukehurst, P. F. (2010, June). Utilisation of digestate from biogas plants as biofertiliser. IEA Bioenergy.

Charlotte Rennuit, S. G. (2013). Decision Support for the Construction of Farm-Scale Biogas Digesters in Developing Countries with Cold Seasons. *Energies*

Charlotte Rennuit, S. G. (2013). Decision Support for the Construction of Farm-Scale Biogas Digesters in Developing Countries with Cold Seasons. Energies

Dr Arwa Abdelhay, Dr Abeer Albsoul, Master Fatimeh Hadidi, Master Abeer Abuothman (2014). Optimization and modeling of biogas production from green waste/biowaste co-digestion using leachate and sludge

Gefu Zhu, Jianzheng Li and Ajay Kumar Jha (2014). Anaerobic Treatment of Organic Waste for Methane Production under Psychrophilic Conditions

Gefu Zhua and Ajay Kumar Jha (2012). Psychrophilic dry anaerobic digestion of cow dung for methane production: Effect of inoculums

George Otim, D. O. (n.d.). Design of Biogas Plant for Rural Households in Uganda (Case Study: Apac District). Second International Conference on Advances in Engineering and Technology, (pp. 544-550).

Grassi, D. I. (n.d.). Biomass Gas Generators to supply Low pollution Cooking Fuels. Latin America Thematic Network on Bioenergy.

Guishan Zhang, N. J. (2008). Methanogenesis from Methanol at Low Temperatures by a Novel Psychrophilic Methanogen, " Methanobolus psychrophilus" sp. nov., Prevalent in Zoige Wetland of the Tibetan Plateau. *Appl. Environ. Microbiol* .

Guishan Zhang, N. J. (2008). Methanogenesis from Methanol at Low Temperatures by a Novel Psychrophilic Methanogen, “Methanobolus psychrophilus" sp. nov., Prevalent in Zoige Wetland of the Tibetan Plateau. Appl. Environ. Microbiol.

Gupta, S. P. (2010). *Climate Change and its Impact on Forest Resource Base at Global and Local Level.* HNRSC, Kathmandu University.

J. M. LARKIN AND J. L. STOKES (1967). Taxonomy of Psychrophilic Strains of Bacillus

Jianzheng Li, Qiaoying Ban, Liguo Zhang and Ajay Kumar Jha (2012). Syntrophic Propionate Degradation in Anaerobic Digestion: A Review

K. M Walter, L. C. (2007). Methane bubbling from northern lakes: present and future contributions to the global methane budget. *Phil. Trans. R. Soc. A , 365*, 1657– 1676.

K. M Walter, L. C. (2007). Methane bubbling from northern lakes: present and future contributions to the global methane budget. Phil. Trans. R. Soc. A, 365, 1657–1676.

Katey Walter Anthony, C. P. (2011). *Denali Emerging Energy Technology Grant:"Improving Cold Region Biogas Digester Efficiency".* Alaska: Denali Commission.

Katey Walter Anthony, C. P. (2011). Denali Emerging Energy Technology Grant:"Improving Cold Region Biogas Digester Efficiency". Alaska: Denali Commission.

M.B. Chand, B. U. (2012). Biogas option for mitigating and adaptation of climate change. *Rentech Symposium Compendium , 1*, pp. 5-9.

M.B. Chand, B. U. (2012). Biogas option for mitigating and adaptation of climate change. Rentech Symposium Compendium, 1, pp. 5-9.

Maheta, P. N. (2015, July). Design of Digester for Biogas Plant for Kadi Sarva Vishwa Vidhlya Gandhinagar Gujarat. International Journal of Innovative Research in Science Engineering and Technology, 4(7), 5401-5408.

MIHIC, S. (2004). Biogas Fuel for Internal Combustion Engines. Annals of the Faculty of Engineering Hunedoara.

Miteva, R. M. (2011). Diversity and ecology of psychrophilic microorganisms. *Research in Microbiology, 162,* 346-361.

Miteva, R. M. (2011). Diversity and ecology of psychrophilic microorganisms. Research in Microbiology, 162, 346-361.

Ngamindra Dahal, H. O. (2009). *Impact of Climate Change on Forests and Livelihoods: Issues and Options for Nepal.* Livelihood and Forestry Programme.

Ngamindra Dahal, H. O. (2009). Impact of Climate Change on Forests and Livelihoods: Issues and Options for Nepal. Livelihood and Forestry Programme.

P. D. Franzmann, Y. L. (1997). Methanogenium frigisium sp. Nov., a Psychrophilic, H2 using Methanogen from Ace Lake, Antarctica. International Journal of systematic Bacteriology, 44(4).

P. E. Wright, S. F. (n.d.). Reduction of selected pathogens in anaerobic digestion.

Ramanujam, B. V. (2011). Anaerobic Digestion of Vegetable Wastes for Biogas Production in a Fed-Batch Reactor. *Int. J. Emerg. Sci. , 1(3),* 478-486. 18

Ramanujam, B. V. (2011). Anaerobic Digestion of Vegetable Wastes for Biogas Production in a Fed-Batch Reactor. Int. J. Emerg. Sci., 1(3), 478-486.

S. Dhadse, N. C. (2012). Study of diverse methanogenic and non-methanogenic bacteria used for the enrichment of biogas production. *Int J. LifeSc. Bt & Pharm. Res. , 1(2).*

S. Dhadse, N. C. (2012). Study of diverse methanogenic and non-methanogenic bacteria used for the enrichment of biogas production. Int J. LifeSc. Bt & Pharm. Res., 1(2).

Teodorita Al Seadi, D. R. (2008). http://lemvigbiogas.com.

Viessmann. (n.d.). The Practical Guide to AD. www.adbiogas.co.uk.

Wellinger, A. (1999). *Process design of agricultural digesters.* Nova Energie GmbH Elggerstrasse.

Wellinger, A. (1999). Process design of agricultural digesters. Nova Energie GmbH Elggerstrasse.

Wilkes, A. (2008). Towards mainstreaming climate change in grassland management policies and practices on the Tibetean Plateau. World agroforestry center. Beijing: ICRAF-China.

Yaru S. S., A. I. (2015). Characterization and Determination of Some Thermo-Physical Properties of Cattle Dung Biogas. Applied Science Reports, 34-39.

Zhang, P. R. (2012). Biogas Production Technologies. National Renewable Energy Laboratory.

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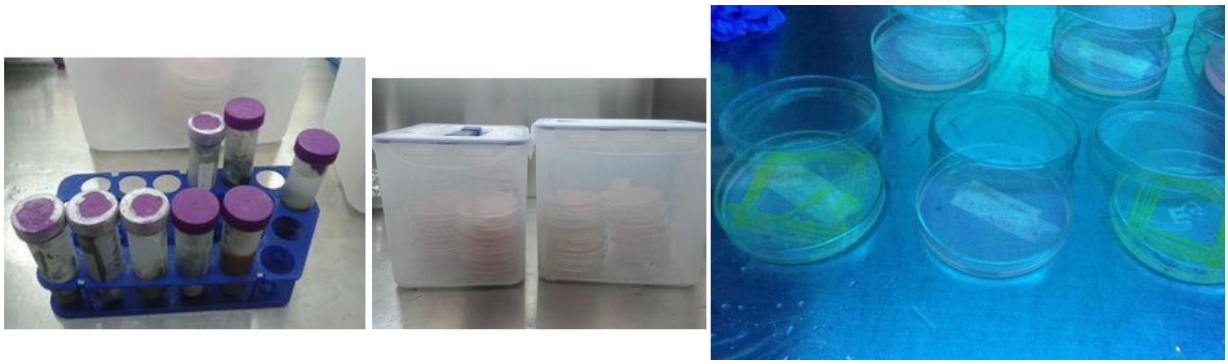
ANNEX I: PHOTOGRAPHS

Prototype Digesters at room temperature and at low temperature inside refrigerator



Collected samples and lab tests

ANNEX II: PARTIAL PRESSURE COLLECTION

	Temperature	Pressure	Gas Composition (vol %)					Volume of gas in Jar	Partial Pressure (kPa)					Mass (g)					Total Mass (g)	Gas Constant (J/kg K)
Jar	(Kelvin)	(KPa)	CH_4	CO_2	H_2S (ppm)	N_2	H_2		CH_4	CO_2	H_2S	N_2	H_2	CH_4	CO_2	H_2S	N_2	H_2		
Jar 1	298	450	53	32	853	13.1	1.0	15.0	238.5	144.0	3.8	59.2	4.5	12.276	12.279	0.00675	1.318	0.0183	25.9	350.7
Jar 2	283	172	28	35	607	35.4	1.0	15.0	48.2	60.2	1.0	60.9	1.7	1.379	5.912	0.00138	3.845	0.0074	11.1	267.1
Jar 3	283	228	48	26	268	24.7	1.0	14.0	109.4	59.3	0.6	56.4	2.3	5.372	4.325	0.00036	2.489	0.0098	12.2	356.2
Jar 4	283	182	8	22	10	69.0	1.0	15.0	14.6	40.0	0.0	125.6	1.8	0.119	2.472	0.00000	15.459	0.0078	18.1	283.7
Jar 5	278	107	5	14	11	80.0	1.0	15.0	5.4	15.0	0.0	85.6	1.1	0.028	0.599	0.00000	12.438	0.0047	13.1	292.6
Jar 6	278	144	34	16	228	48.8	1.0	14.0	49.0	23.0	0.3	70.2	1.4	1.733	1.053	0.00017	6.223	0.0063	9.0	327.0
Jar 7	278	93	8	16	184	74.8	1.0	15.0	7.4	14.9	0.2	69.6	0.9	0.062	0.680	0.00007	9.457	0.0041	10.2	291.2
Jar 8	278	111	18	22	43	59.0	1.0	14.0	20.0	24.4	0.0	65.4	1.1	0.374	1.535	0.00000	7.009	0.0048	8.9	287.8
Jar 9	278	78	1	10	4	88.0	1.0	15.0	0.8	7.8	0.0	68.6	0.8	0.001	0.223	0.00000	10.973	0.0034	11.2	294.9
Jar 10	298	426	46	38	347	14.7	1.0	14.0	196.0	161.9	1.5	62.4	4.3	8.754	16.392	0.00106	1.550	0.0173	26.7	303.3
Jar 11	278	155	24	33	418	41.6	1.0	14.0	37.2	51.2	0.6	64.5	1.6	0.929	4.821	0.00060	4.869	0.0068	10.6	267.4