INLAND NORWAY UNIVERSITY OF APPLIED SCIENCES

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Master Thesis

Effects of temperature, particle size and enzyme addition on biogas production in-vitro

Effekt av temperatur, partikkelstørrelse og tilførte enzym på in-vitro biogassproduksjon

Master in Sustainable Agriculture

2018

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Preface

The basis of this thesis arose from my childhood remembrances of a small biogas digester in my home. We used to generate enough fuel to cook food for our small family from only one buffalo. Since then, I perceived biogas as a very useful fuel source. As I grew old, I came to know it haves not only household importance but also environmental and ecological importance as well. Then after, my interest to study about biogas production stemmed up. Understanding my passion, my supervisor proposed the novel idea to use Moose rumen culture for biogas production test. I became more than happy with a thinking that I may add even one small step towards sustainability by this study. I hope it will be helpful for my readers.

This thesis is prepared as a completion of master degree from Høgskolen i Innlandet, Blæstad in sustainable agriculture. It is my pride to thank all of my helping hands to complete this manuscript. I would like to express my sincere gratitude to my supervisor Dr. Lars Erik Rudd for his continuous guidance, scholarly encouragement and appreciable suggestions during experimentation and writing period. I am really grateful to my co-supervisor professor Dr. Knut Olav Strætkvern for his efforts to design the experiment and for extensive support in data analysis. I like to appreciate Høgskolen i Innlandet administration especially Mr. Fred Håkon Johnsen and Mrs. Elisabeth Røe for their support during my study period. I would also like to remember all known and unknown students of INN for lovely gesture and greetings they provide. Last but not least, thank you guys; Manisha, Rajesh and all.

Dedicated to family and friends.

Namaste

Sanjaya Lamichhane

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Acronyms

MRB: Moose rumen bacteria

v/v: volume/volume

DoE: Design of experiments

g: Gram

AD: Anaerobic digestion

mm: millimeter

ppm: parts per million

EU: European Union

S.D.: Standard deviation

Avg.: Average

TWH/y: Terawatt-hour per year

MODDE: Modelling and design

nm: Nanometer

C/N: Carbon-to-nitrogen ratio

Nm^3/t FM: Normal m^3 per ton of fresh matter

VFA_s: Volatile fatty acids

Temp*Enz: Temperature and enzyme interaction

Abstract

Biogas is an anaerobically produced ecofriendly renewable form of energy which can address the harmful effects associated with conventional source of fossil fuels. The objectives of present study were to examine effects of temperature, particle size and enzymes on biogas production. Moose (*Alces alces*) thrives in woody browse in semi arctic region and it was presumed that it hosts unique micro-organisms capable of producing fibrolytic enzymes. An experiment was conducted in the laboratory of INN, Blæstad. Cow manure with 5% dry matter content and wheat straw with 3 different particle size were treated with 3 dose of Moose rumen bacteria (MRB) culture along with 3 temperature settings. A cubical model was selected according to DoE (Design of experiments) and 11 treatments with different combinations of temperature, particle size and enzymes were tested. 100ml Erlenmeyer flasks were used as reactors, whereas gas was collected in 100 ml syringes. Wheat straw was ground and sieved to 1, 3 and 5mm particle size. MRB was applied at 0, 2.5 and 5% v/v of enzyme and substrate. 20, 30 and 40°C temperature were maintained in room, water bath and in heating cabinet respectively. Specified treatments were applied in 11 reactors provided with 30g manure solution and 3g of wheat straw substrate. Amount of gas collected in all the syringe were measured five times during a 48h period. The data were then analyzed using MODDE pro and MS Excel. The study shows that increase in temperature and enzyme has positive main effects and interaction effects for achieving a high rate of biogas production. However, particle size was found indifferent in biogas rate conformed by the large error bars. The study reveals the positive effect of using of MRB culture on the production of biogas since it contains novel microorganisms able to promote biomass digestion.

Keywords: biogas, temperature, enzyme, moose, particle size

Sammendrag

Biogass er en miljøvennlig energiform som er produsert anaerobt uten de skadelige effektene som er koblet til konvensjonelle fossile energikilder. Målene med dette forsøket var å undersøke effekten av temperatur, partikkelstørrelse og enzymer på produksjonen av biogass. Elg (*Alces alces*) trives på skogsbeite i halvarktiske områder og det ble derfor antatt at dyret kan ha spesielle mikroorganismer som muliggjør produksjon av fibrolyttiske enzym. Et forsøk ble gjennomført i laboratoriet på Høgskolen Innlandet, Blæstad. Husdyrgjødsel fra storfe med 5% tørrstoff og hvetehalm med tre ulike partikkelstørrelser ble behandlet med tre ulike konsentrasjoner av bakteriekultur fra vomma av elg (MRB) og ved tre ulike temperaturforhold. En kubisk modell ble valgt som forsøksdesign hvor 11 behandlinger med kombinasjoner av temperatur, partikkelstørrelse og enzymer ble testet. 100ml Erlenmeyer flasker ble brukt som reaktorer og gass ble samlet opp i 100ml sprøyter. Hvetehalm ble knust og siktet i fraksjoner med partikkelstørrelser på 1, 3 og 5mm. MRB ble tilsatt i 0, 2.5 og 5% v/v av enzym og substrat. Temperatur på 20, 30 og 40°C ble opprettholdt i henholdsvis laboratorierom, vannbad og oppvarma kabinett. De spesifikke behandlingene ble brukt for de 11 reaktorene som ble tilført 30g husdyrgjødsel og 3g av hvetehalm-enzym substratet. Mengden gass som ble samlet opp i de ulike sprøytene ble målt over en 48-timers periode. Data ble deretter analysert gjennom MODDE pro og MS Excel. Forsøket viser at temperatur og enzym begge har positive hovedeffekter og det finnes også en samspillseffekt mellom faktorene i forhold til å oppnå en høy produksjonsrate av biogass. Derimot viste partikkelstørrelse ikke å påvirke produksjonsraten og dette henger sammen med store feilkilder og variasjon. Forsøket viser en positiv effekt av MRB kultur på produksjonen av biogass og dette kan skyldes dets innhold av unike mikroorganismer som øker nedbrytningen av biomassen.

Nøkkelord: biogass, temperatur, enzym, elg, partikkelstørrelse

1. Introduction

1.1 Background

The total energy utilized by the entire human civilization across every country all over the world is called world energy consumption. It is typically measured per year and involves every energy equivalent for humanity's endeavors from all energy sources. Many institutions periodically calculate total utilization of energy and categorize it according to sources. Institutions such as International Energy Agency (IEA), European Environment Agency (EEA) and Energy Information Administration (EIA) are responsible for recording and publishing the patterns and trends of utilization of world energy. According to the below mentioned figure, we could easily notice the heavy reliance over nonrenewable energy sources like oil, coal and gas with increasing trend over years.

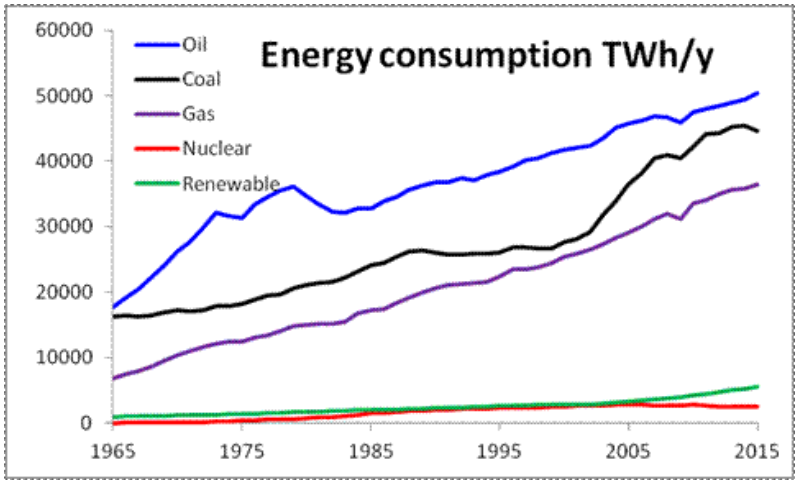


Figure 1 The world's energy consumption (BiophysEco., 2018)

After the commencement of the industrial revolution human activities have resulted in a 40% increase in atmospheric carbon dioxide (CO_2) concentration with a profound increase from 280 ppm in 1750 to 406 ppm in 2017 (ESRL, 2018). Combustion of non-renewable energy sources emits greenhouse gases such as carbon dioxide, nitrogen oxides (NO_x), Sulphur dioxide (SO_2) and volatile organic compounds (WHO, 2018). These gases are produced naturally or by various anthropogenic (human induced) activities. Greenhouse gases in the atmosphere act like a blanket causing trapping of heat; the greenhouse effect. This effect causes a global increase of earth`s temperature and thus could lead to consequences like global warming, increased sea level, less ice and snow, drought and flooding, climate change and

extreme weather incidents (Wellinger, Murphy, & Baxter, 2013). Therefore, to address the harmful effects of nonrenewable energy and to sustain environment and health of humans, animals and plants, there is extreme need for promotion and development of alternative renewable form of energy.

1.2 Potential of biogas in Europe

Agricultural production is responsible for about 33% of total global anthropogenic cause of methane release. In which animal husbandry, rice fields and animal manure comprise 16%, 12% and 5% respectively (Broucek, 2014). Some proportion of methane is released by digestion mechanisms of ruminants (likely 80 Mil ton CH_4 annually), which can rarely be controlled (Broucek, 2014). But methane release from manure, or by other organic materials can be controlled and energetically utilized by the method of anaerobic digestion. The potential of methane discharge from dairy cattle in modern farm is about 0.24m^3 CH_4/kg volatile solids (Broucek, 2014). Through controlled anaerobic digestion of animal excreta, we could eliminate about 1324 Mil ton of raw methane per year (Jørgensen, 2009). Processing of animal and human excrements for anaerobic digestion to produce biogas, certainly advance sanitary conditions in surroundings or in the locality. The use of organic waste for anaerobic treatments greatly reduce substrate for disease causing microbes thus promote health of the vicinity. In rural areas the replacement of firewood by biogas for cooking and heating purpose could also contribute significantly to fight deforestation. Unlike the limited amount of fossil fuels, biogas can be available endlessly as long as there is life and biomass on the earth.

According to European Biomass Association (AEBIOM), production of biomass related energy could be increased to 220 Mtoe (million tons of oil equivalent) in 2020 from 72 Mtoe in 2004 (Seleiman, 2014). It also states that 20-40 Mha (million hectare) of land area can be utilized in EU alone to grow energy crops for biomass without affecting European food supply. Biomass resources that exist on our earth, gives different estimates of potential global biogas production when calculated by different experts. German Institute for Energy and Environmental Research (IFEU) estimated that Europe has feasibility to replace the total use of natural gas by biogas and bio-methane if supplied to the existing national grid (figure 2).

Figure 2 Potential corridors for biogas production (yellow) in Europe and supply in natural gas grid (Thrän et al., 2007)

A report from Enova (an enterprise owned by ministry of climate and environment) in coordination with Østfoldforskning and NMBU (Norwegian university of life sciences) assessed that the energy potential of Norway from waste and residues is around 6 TWh (terawatt-hour) per year (Raadal, Schakenda, & Morken, 2008). Among which 2.3 TWh per year is the actual realistic potential that can be achieved by 2020 (Sletten & Maass, 2013). Despite this huge potential, only 0.5 TWh/year is utilized in Norway for generation of biogas (Sletten & Maass, 2013) (figure 3).

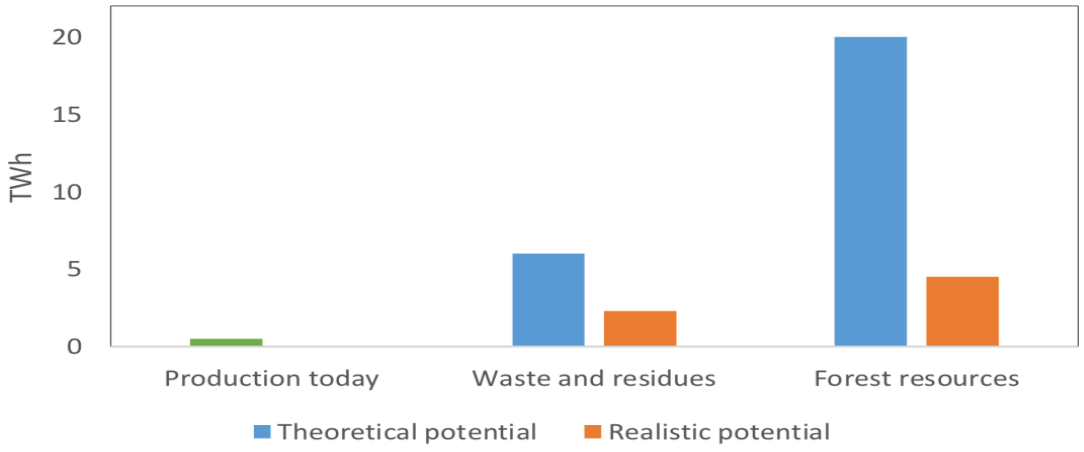


Figure 3 Norwegian biogas potential (Huseby, 2015)

1.3 Biogas as a renewable energy

Renewable energy characterizes that type of energy which comes from such sources that are not depleted significantly by their use. Wind, tide, sunlight, biomass and hydroelectricity are some examples of renewable energy. These could be the answer to the ill effects created by burning fossil fuels.

In general, biogas is composite of different gases generated by the disintegration of organic matters in the absence of oxygen. Biogas is a gaseous mixture of methane and carbon dioxide with small amounts of hydrogen sulphide (H_2S) and trace gases. Wide range of organic matters originating from household and industrial waste, manure, plants debris, sludge, sewage or any other biodegradable feedstocks have potential to produce biogas (Schnurer & Jarvis, 2010). Biogas is considered as a renewable source of energy because production-and-use cycle of biogas is continuous with no net carbon dioxide gain (McKendry, 2002). Biogas has higher caloric value than ordinary petroleum gas such as ethane, propane and butane (Wellinger et al., 2013). Biogas can be used directly for the purpose of combustion, heating and electricity generation or could be liquefied to bio-methane for use as a vehicle fuel. The process of biogas digestion also produces high value digestate, which is less odorful and have a higher agricultural value to use as a fertilizer (Ward, Hobbs, Holliman, & Jones, 2008). At present, there are small, large as well as industrial scale biogas plants in operation worldwide. Small biogas plants for household purpose with only a capacity of 7-800 liters could be used as sufficient for cooking fuel and lightings in homes (Jørgensen, 2009). Millions of people from less developed regions of Asia and Africa are already using household digesters for their home purpose. Primarily, an effective digester can generate 200-400 m^3 of biogas with 50-75% methane content per ton of dry input (Jørgensen, 2009). Composition of biogas and the potential of different substrate to yield biogas is given in table 1 and figure 4.

Table 1 Composition of biogas (Wellinger et al., 2013)

Gas	Percent composition
Methane	50-80%
Carbon dioxide	25-50%
Nitrogen	0-10%
Hydrogen	0-1%
Hydrogen sulphide	0-3%
Oxygen	0-2%

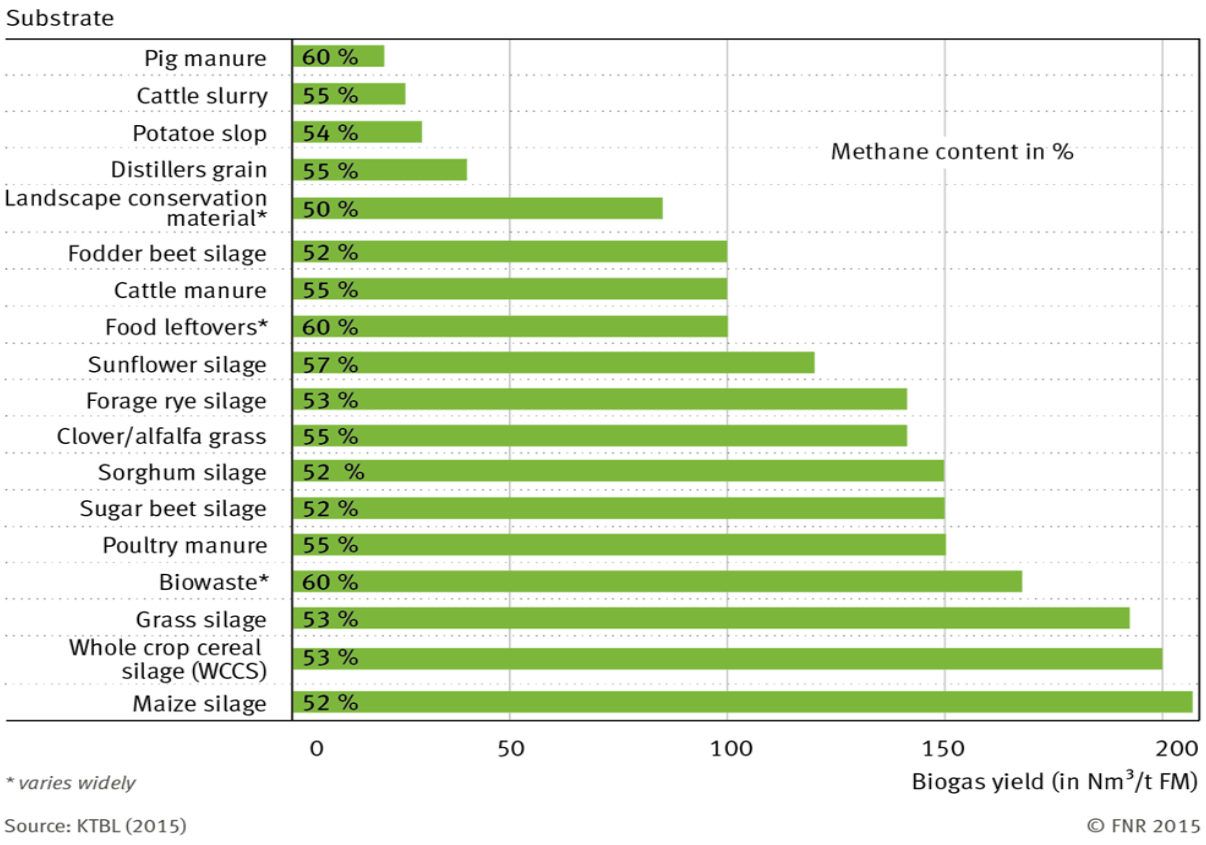


Figure 4 Potential of different substrate to yield biogas (FNR, Erdmann, & Kirchmeyr, 2015; KTBL, 2015) (Nm^3/t FM: normal m^3 per ton of fresh matter)

1.4 Biomass (Lignocellulose)

Plant cells and walls are composed of complex structures of polysaccharides, glycolytic proteins and lignin. Most of the cell wall biology in plant ranges from 38-50% cellulose, 17-32% of hemicellulose and 15-30% lignin (Sánchez, 2009). Which is collectively called as lignocellulose. The most prolific organic material in the earth is lignocellulose. Annual production of lignocellulosic biomass all around the world is estimated to be around $1*10^{10}$ million tones (Sánchez, 2009; Zhang & Zhao, 2010). The main component cellulose, is made up of glucose molecules chained by β-1,4 linkage where hemicellulose is composed of 5- or 6- carbon sugar molecules such as glucose, galactose, arabinose, mannose and xylose (Zhang & Zhao, 2010). And third, lignin is made of major phenolic compounds; coniferyl alcohol, sinapyl alcohol and coumaryl alcohol (Heeg et al., 2014). Because of the structure of lignocellulosic substrates, they exhibit low degradability (40–60%) in anaerobic digesters (Sánchez, 2009). In comparison with the degradation of lignocellulose in rumen of ruminant, AD systems are only 20% efficient (Nair et al., 2005). It is therefore of interest to closer investigate e.g. enzymes or other mechanisms from the digestion system of ruminants.

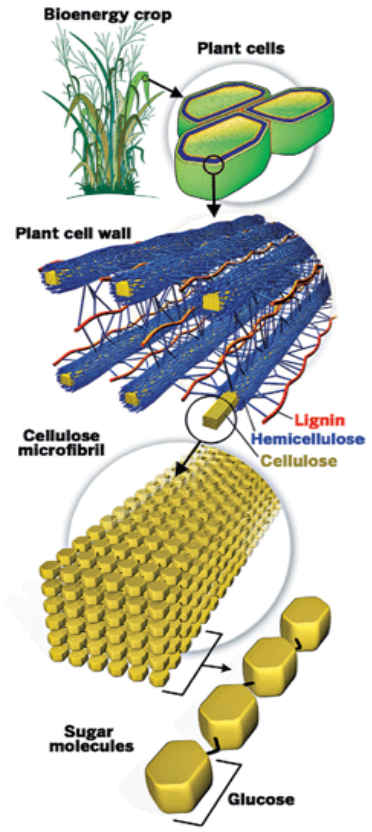


Figure 5 Complex structure of lignocellulose (Ritter, 2008)

1.5 Moose and enzyme

Moose (*Alces alces*), is a large ruminant in Cervidae (deer) family. Moose are generally native to northern latitude territories such as northern United States, Canada, Russia and Scandinavia. As a diet, they largely browse woody vegetation i.e. pine, ash, willow, birch, maple, etc. The stomach of moose, like other ruminants is specialized four chambered viz. rumen, reticulum, omasum and abomasum. Moose mostly consume deciduous or coniferous leaves, twigs, stems and also strips bark from trees. This feed contains large amount of lignin, tannins, polymers of cellulose and hemicellulose which is very harsh to digest since they largely act as barrier for absorption of nutrients (Ishaq, Kim, Reis, & Wright, 2015). Rumen and reticulum of the stomach fosters a large consortia of microbial population (bacteria, archaea, fungi, protozoa). Those microbiota plays a significant role to break down binds of nutrients and makes them readily available for absorption. The enzymes secreted by microbes like esterase, lignanse and cellulase helps them to carry out the digestion process at a higher rate (Bayané & Guiot, 2011). The bacterial isolates and culture from a male moose was found to consist of 21 strains of *Streptococcus bovis*, 9 strains of *Butyrivibrio fibrisolvens*, 7 strains of *Lachnospira multiparus*, 2 strains of *Selenomonas ruminantium* and many more (Ishaq & Wright, 2012).

Therefore, we hypothesized that, ruminal fluid of moose might host novel microorganisms possibly of interest in biogas production. Those microbes would have a wide array of enzymatic action, capable of producing useful metabolites and probably have an unique potential to break down lignocellulose and hence, increase gas yield.

1.6 Process of anaerobic digestion

Anaerobic digestion is the process of decomposition of organic matter by activities of microorganisms without the presence of oxygen. Primary main product of AD is biogas and effluents. Microbial activities and different secreted or induced enzymes hydrolyze insoluble organic polymers such as cellulose, hemicellulose and lignin to simpler soluble and degradable organic products (Henze, Loosdrecht, Ekama, & Brdjanovic., 2008). Followed by other different physiological and chemical change, AD leads to production of gaseous combination of methane, carbon dioxide and other trace gases (McKendry, 2002).

The biogas formation process from AD is the result of steps of linked processes, often characterized by the four stages hydrolysis, acidogenesis, acetogenesis and methanogenesis

(Wellinger et al., 2013). Initial material is broken down into simpler and smaller units of intermediate products. Individual steps of AD are carried out by specific group of microorganism.

1.6.1 Hydrolysis

In first step of AD, polymers (complex organic matters) are decomposed in simpler particles of mono or oligomers. Complex molecules of carbohydrates, fats, proteins and nucleic acids are altered into glucose, glycerol, purines and pyridines (Heeg et al., 2014). In the biogas reactor, hydrolytic microorganisms produce hydrolytic enzymes due to which degradation of complex compounds takes place (Donoso-Bravo, Retamal, Carballa, Ruiz-Filippi, & Chamy, 2009).

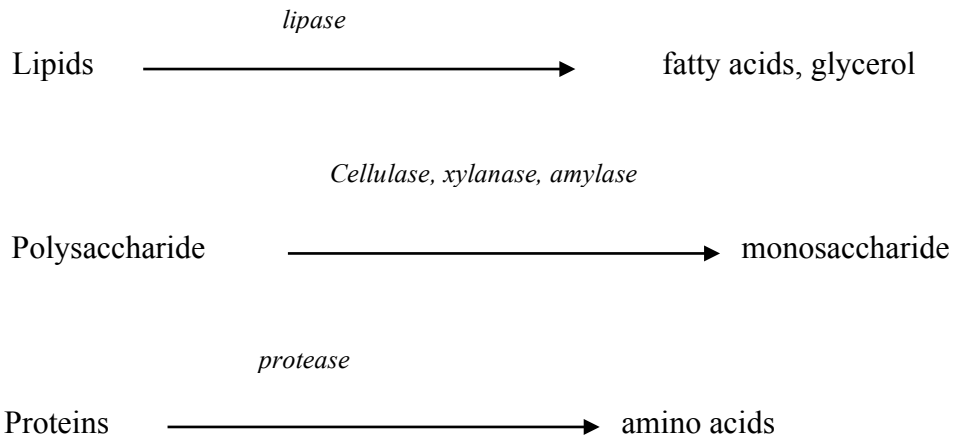


Figure 6 Hydrolysis reactions

Bacterial groups such as *Acetivibrio, Bacteriodes* and *Clostridium* plays active role in hydrolysis of polysaccharides and cleave macromolecules into simpler ones (Venkiteshwaran, Bocher, Maki, & Zitomer, 2015).

1.6.2 Acidogenesis

In this step, the intermediate products from hydrolysis are further converted by fermentative (acidogenic) microorganism into methanogenic substance. Fatty acids, sugars and amino acids are converted into acetate, CO_2, H_2, alcohols and Volatile fatty acids (Wellinger, 2013; Henze et al., 2008). Acidogenesis, is a rapid process containing many pathways producing many intermediate and end products. Diversity of microbial consortium reaches to its maximum in

this stage of AD. Genera of microbes like *Acetobacterium, Enterobacterium*, and *Eubacterium* acts as fermenting agents in this step of process (Venkiteshwaran et al., 2015).

1.6.3 Acetogenesis

In this step of AD, the products from acidogenesis are converted into methanogenic substrates. In symbiosis with oxidation performing bacteria along with methanogens, VFA_s and alcohol are converted into acetate, hydrogen and carbon dioxide (Heeg et al., 2014). *Clostridium, Syntrobacter, Syntrophus* and *Syntrophomonas* are some examples of bacterial genera that along with other methanogens play crucial role in acetogenesis (Heeg et al., 2014; McInerney et al., 2008).

1.6.4 Methanogenesis

Methanogenesis, final steps in AD is carried out in accordance with methanogenic bacteria. 70% of total methane is formed by conversion from acetate, while the remaining 30% is formed by hydrogen (H_2) and carbon dioxide (CO_2) (Merlino et al. 2013).

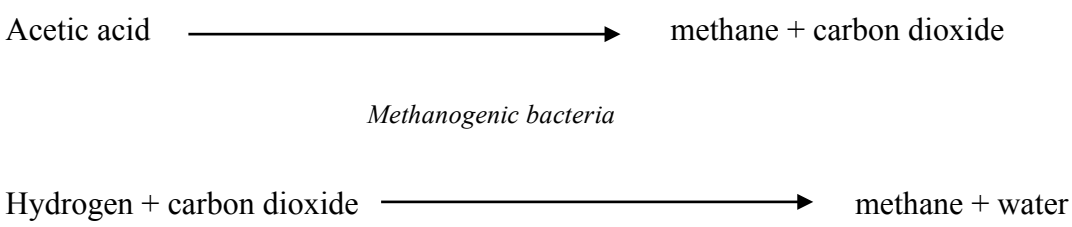


Figure 7 Methanogenesis reactions

It is the slowest and critical step in entire AD. Process of methanogenesis has six major pathways for conversions of substrate into methane compound. Those six major substrates are acetic acids, carbon dioxide, formic acid, dimethyl sulphate, methylamine and methanol (Slonczewski & Foster 2013). The process and the composition of the final product is strictly influenced by operating conditions. Substrate composition, hydraulic retention time, temperature, pH, type of microbes are critical factors that influence the duration of digestion and level of methane production. Different species of genus *Methanobactor* produce methanolytic enzymes that plays crucial role during commencement of this step (Venkiteshwaran et al., 2015).

1.7 Other factors influencing gas production

The production of biogas is influenced by many factors such as temperature, particle size, substrate composition, enzyme, C/N ratio, hydraulic retention time, etc.; with one parameter in analogy with other (Angelidaki & Ellegaard, 2002; Dobre, Nicolae, & Matei, 2014; Noraini, Sanusi, Elham, Sukor, & Hamid, 2017). Due to the synergy between factors, if any factor is limited we can adjust other factors to compensate the effect (Wellinger et al., 2013). There are 3 temperature region in which AD takes place viz. psychrophilic (0-15°C), mesophilic(15-45°C) and thermophilic (45-65°C) (Kardos et al., 2011). Choice and control of temperature have solid influence in the quantity and quality of biogas formulation (Dobre et al., 2014).

Size of particles in the substrate defines the surface area where the effect of enzymes and microorganisms takes place. Raw and coarse particles yield less in comparison to fine particles. Physical treatments like grinding could reduce the size of particle significantly (Yadvika, Sreekrishnan, Kohli, & Rana, 2004).

2. Objectives

This research was focused on the production of biogas under different conditions. Different values of temperature, size of particle (PS) and dose of enzyme were tested in various combinations.

The objectives were to

1) Investigate main effects of temperature, enzyme and PS independent with each other on biogas production.
2) Study the interaction effects of temperature, enzyme and PS in relation with each other on rate of biogas production.

The main hypothesis of the present study is that, temperature of the digester, particle size of the substrate and dose of rumen culture (enzyme) at low, medium or in high level have different rates of biogas generation.

3. Material and methods

In present study, effects of temperature, particle size and enzyme concentration on gas production were investigated. Temperature was set to low, medium and high at 20°C, 30°C and 40°C respectively. Particle size (PS) was set to low (<1mm), medium (3mm) and high (>5mm). Similarly, proportion of enzyme was set to low (0% v/v), medium (2.5% v/v) and high (5% v/v). Concentrated moose rumen bacterial culture was used as an enzyme. To ensure equal volume of liquid input inside the digester 5% v/v (1.5ml) of distilled water was added in digester of no enzyme supplement. Likely, 2.5% v/v (0.7ml) of distilled water was added by the pipette to the digester having 2.5%v/v enzyme to replenish the amount of liquid. Since the main objective was to study effects of these 3 variables, the amount of manure and straw were kept constant in all experiments. Considering 100 ml capacity of our reactor, every flask was provided with 30 gm manure and 3 gm straw mixture (10:1 ratio) (standard VDI 4630).

In order to carry out this research study, following complementary steps were done.

3.1 Design of test

Design of the experiment was performed in accordance to MODDE Pro software. Three different factors were portrayed along x, y and z-axis according to DoE (Design of the experiments). 8 different treatments were selected at the edges of cube. 3 of treatments were taken from the center points at the middle value. As a whole 11 treatments were obtained as shown in the figure.

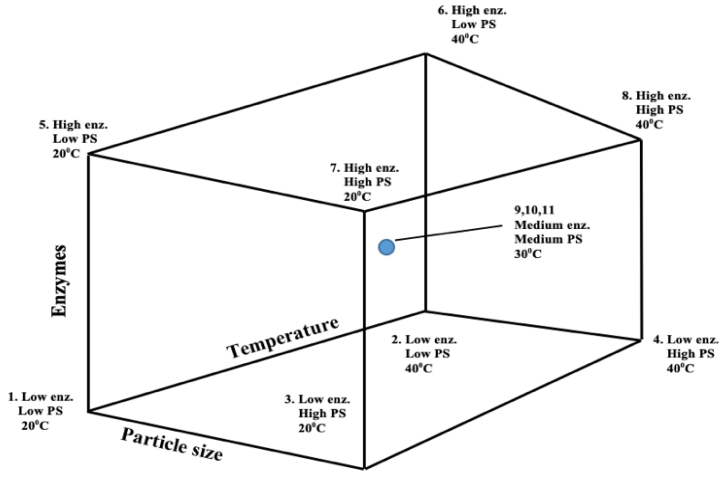


Figure 8 Design of tests

Table 2 Test condition of reactors

No of Reactor	Temp (°C)	Particles size (mm)	Enzymes (%v/v)
1	20	1	0
2	40	1	0
3	20	5	0
4	40	5	0
5	20	1	5
6	40	1	5
7	20	5	5
8	40	5	5
9	30	3	2.5
10	30	3	2.5
11	30	3	2.5

3.2 Preparation of substrate

Wheat straw and cow dung manure were used as a substrate for our test to produce biogas in the laboratory. Dry wheat straw was collected from a local field around Høgskolen i Innlandet, Blæstad. To find different particle size, straw was ground and then extracted from sieve with different mesh size. Particles that passed through mesh size 1mm were used as low(<1mm), particles that passed mesh size 4mm and stocked in the 3 mesh were used as medium (3mm). Lastly, particles that pass mesh size 6mm and stocked in 5mm mesh plate were used as large PS (>5mm). Fresh cow dung was also collected from one nearby commercial dairy farm. Here, cow dung provided the inoculum for the microbes to produce biogas. Percentage of dry matter presented in the manure was calculated by drying the samples to constant weight in a muffle furnace at 103°C (standards EN 12880 and APHA 2540 B). Stable standard 5% DM (dry matter) was achieved in the manure solution by adding neutral water in the mixture.

3.3 Maintenance of temperature

For our test, 3 different temperature variables were selected. For high temperature, 40°C constant temperature was maintained inside a heating cabinet. For stable 30°C, a sous-vide cooking device with heating element, thermostat and stirring mechanism was used to maintain constant temperature during the experiment in a water bath. And for testing at 20°C, constant room temperature was maintained by external automated room heater equipped with thermostat. The outdoor temperature was lower than 20°C throughout the whole test period.

3.4 Preparation of test

11 different reactors were prepared for testing. After mixing of manure, straw and enzymes according to specified mixture, it was then fitted with rubber cork for insulation of gas. Syringes were fitted in all of the flasks with a bore in corks to measure gas yield. The whole setup was made leakage proof by applying glue in the connections. Piston of the syringe exerts some frictional force within its wall. To ensure comparable friction, all the syringes were lubricated with silicone gel applied on the gasket of the syringe piston to minimize friction. Then after, syringes were tested with weights. Syringes were held upright and small weight (20g) was tied in the piston with a very fine small thread. Small weights were continuously added until the piston just started to slide down i.e. friction of piston. Among many syringes, only those syringes with same frictional weight were selected for test (120g in our case).

3.5 Collection and culture of enzyme (MRB)

Bacterial isolate from moose rumen secreting cellulase activity, i.e. CMCase (=endo beta 1,4 glucanase) and xylanase was used for the test. The isolate was prepared from rumen content of a road-killed Moose at the University of Bergen (prof. Vidar Bakken) but was proprietary to the company TransHerba AS, Elverum. Thus, the actual identity of the isolated organism was not disclosed but was given the name 'MRB 4' for practical reference. Master seed stock of the organism was stored at -80°C. The protocol for growth and enzyme enrichment was done according to Thapa (2018). The MRB4 was pre-cultured in Anaerobic Basal Broth (ABB) from Oxoid. Pre-culture of MRB 4 was done in ABB and stirred at 30 °C for 20 hours. Optical density (OD) of the culture after 20h was 0.56 when measured at 600nm. 5 ml of the pre-culture was transferred to 100 ml ABB and incubated at 37°C for 17 h to OD_{600nm} 0.55. 20% sterile cellobiose was then added to the culture to a final concentration 0.4%. Addition of cellobiose was done to activate (trigger) the production of the CMCase in MRB 4. The MRB 4 was cultured further for 3 h to final OD 0.86. The enzyme activity at that stage was typically 0.4-0.5 CMCase units per ml (Thapa, 2018).

The activated culture (85ml) was transferred to a 10 cm dialysis tubing (Spectrapor 12-14kDa), sealed in both ends and concentrated by dialysis by immersion in solid PEG (polyethylene glycol). Dialysis in PEG was carried out in cold room (4°C) for 4.5 h and the concentrate was recovered. Collected volume was 30 ml, i.e. 2.9x concentration. The concentrated culture

(containing bacteria and enzyme) was stored cold until used next day in biogas experiment. The concentrated enzyme preparation was dispensed at 0.75 or 1.5 ml to selected AD reactor mixtures according to the experimental plan (DoE).

3.6 Recordkeeping

After setup, all the flasks were numbered according to table 2 and kept at their respective place of temperature settings. Gas yield was noted twice in a day to find the pattern and rate of gas yield. A simple and easy to use scheme was maintained to note every detail during experiment.

3.7 Testing of gas

After the second day (48h) some of the syringes got all filled up with the gases. The experiment was then stopped. A burning test was conducted after the completion of the experiment. The gas from all reactors burnt with clear blue flame conforming the formation of methane gas.

3.8 Statistical analysis

The result of biogas yield in all of the reactors were statistically analyzed by using MODDE Pro software (MKS UMETRICS, Umeåa Sweden) and MS Excel version 15.26.

4. Results

4.1 Biogas collected in reactors

Gas collected in 11 different reactors fitted with designated treatments and mixture composition when analyzed after the completion of the test showed various volume of gas collection. Among them reactors no. 6 and 8, first achieved maximum limit of gas collection i.e. 100ml. Both of the reactors were provided with same amount of 5%v/v enzyme kept in 40°C but with different particle size. The linear projection of the achieved data shows the rate of biogas production.

Table 3 Amount and rate of gas production (temp. in°C, enzyme v/v and PS in mm)

	Gas volume (ml)		Reactor no										
Day	Time	hrs	1	2	3	4	5	6	7	8	9	10	11
1	12:00	0	0	0	0	0	0	0	0	0	0	0	0
1	21:45	10	0	5	0	3	0	0	0	15	10	10	5
2	08:15	20	0	5	0	9	0	20	5	35	10	15	5
2	02:15	26	3	5	0	13	5	35	5	45	13	18	15
3	08:15	44	5	5	0	15	5	80	5	90	40	45	40
3	12:15	48	**5**	**5**	**0**	**20**	**5**	**100**	**7**	**100**	**45**	**50**	**45**
		Rate (ml/hr)	**0.1231**	**0.0699**	**0**	**0.39**	**0.1246**	**2.17**	**0.138**	**2.12**	**0.933**	**1.05**	**0.989**
	Settings	temp	20	40	20	40	20	40	20	40	30	30	30
		enz	0.0	0.0	0.0	0.0	5.0	5.0	5.0	5.0	2.5	2.5	2.5
		particle	1	1	5	5	1	1	5	5	3	3	3

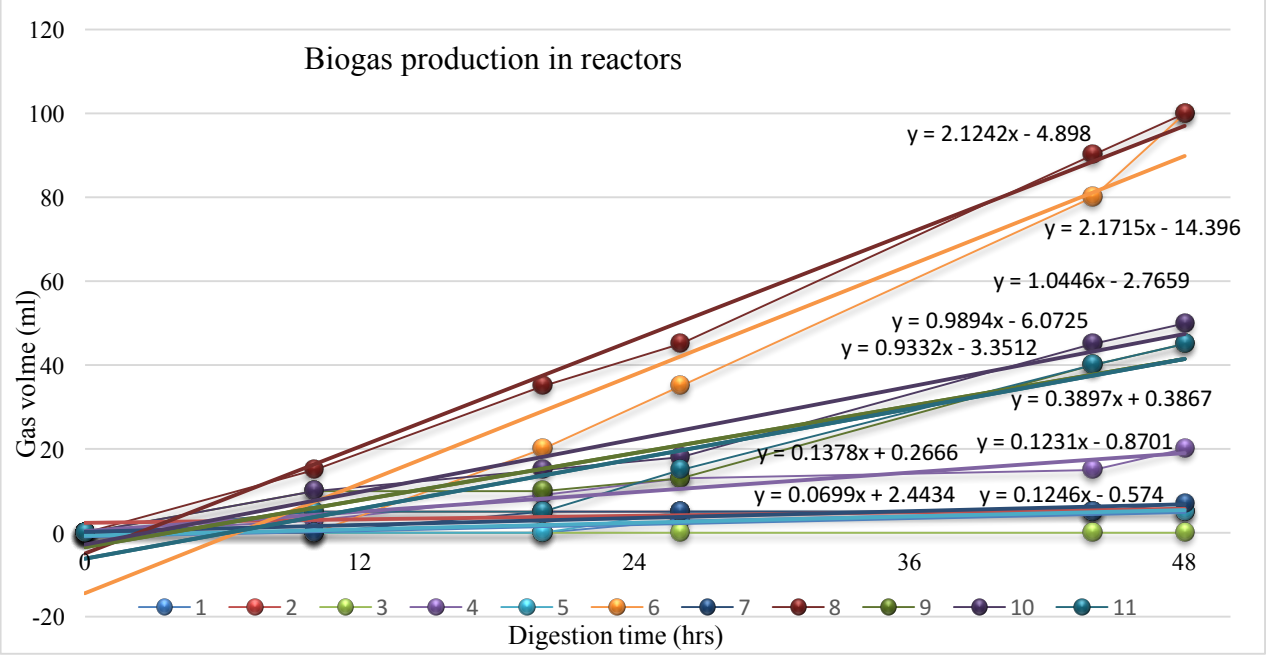


Figure 9 Linear representation plot

4.2 Nature of data

To study the nature of our data, it was examined under two models, i.e. biogas rate model and accumulated gas model. Following replicate plot and probability plot were graphed.

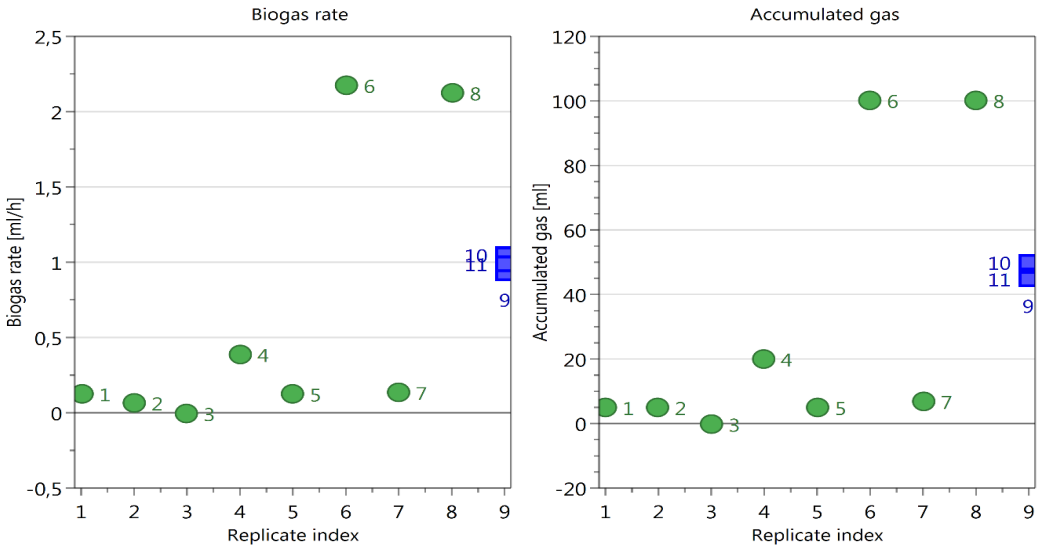


Figure 10 Replicate plots

Replicate plot shows the variation in results for all experiments for a quick raw data inspection. Fig (10) displays the spreading of data points (max-min) compared to the three replicates (9,10,11). Replicates are very close indicating that observed responses 1-8 also have the same degree of variance.

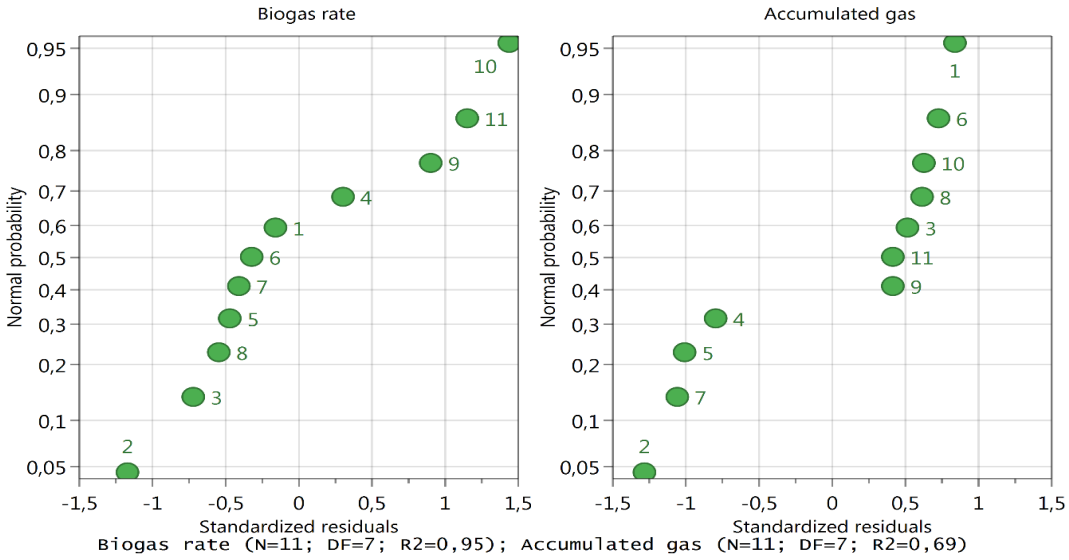


Figure 11 Probability plots

Ideally the points should fall on a diagonal- more or less linear, which will indicate that experimental responses have a random (and normal) distribution i.e. no bias. Here we see some deviations from ideal curves indicating model problems or outliers.

4.3 Test of model validity

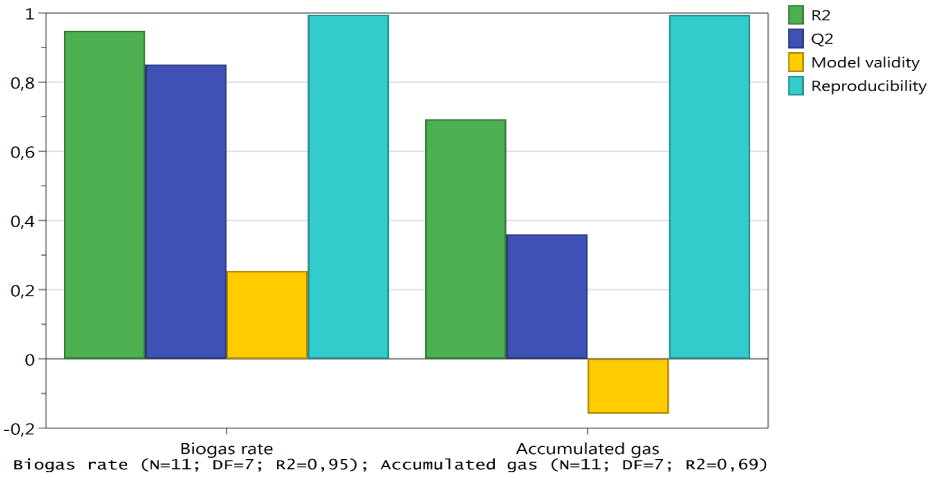


Figure 12 Model validity test

The bar graph briefly illustrates properties of the prediction model made from the data. R2 shows the fitness of model and Q2 shows an estimate of precision of future prediction. A model with R2 of 0.95 is considered a perfect significant model. Here, biogas rate model has R2 and Q2 close to 1 which denotes the fitness of the model. To be 1 or 100% fit R2 and Q2 should be close in size or the difference between them shouldn't be more than 20%.

All we can say is that biogas rate data modelling have the best modelling outcome. The low and even negative yellow bar reflects the deviations described in the probability plot (Fig. 11). Here reproducibility is close to 1 for both of the models. It is the ability of an entire process to be duplicated and it ensures reliability of the methodology.

4.4 Interaction of temperature, particle size and enzyme

When interaction between two different parameters, independent from the third variable was graphed, different graphs showing the following results appeared.

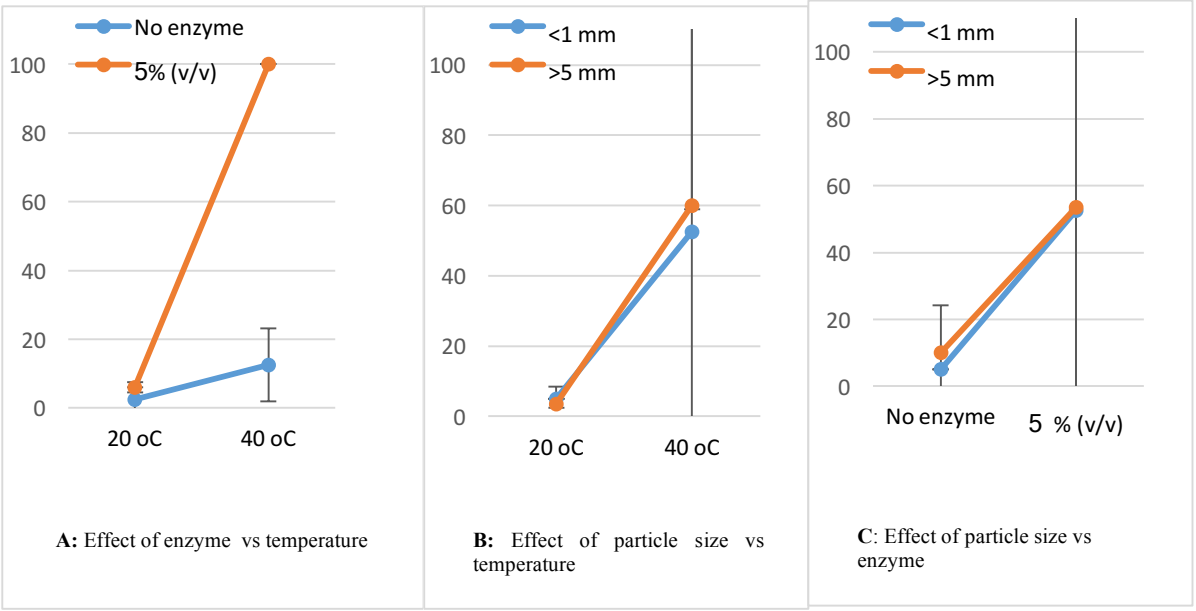
Table 4 Interaction between factors

Temperature vs enzyme	20°C	40°C
0%	5	5
0%	0	20
Avg.	2.5	12.5
S.D.	3.5	10.6
5%	5	100
5%	7	100
Avg.	6	100
S.D.	1.4	0.0

Temperature vs particle size	20°C	40°C
<1mm	5	5
<1mm	5	100
Avg.	5	52.5
S.D.	0.0	67.2
>5mm	0	20
>5mm	7	100
Avg.	3.5	60
S.D.	4.9	56.6

Enzyme vs particle size	0%	5%
<1mm	5	5
<1mm	5	100
Avg.	5	52.5
S.D.	0.0	67.2
>5mm	0	7
>5mm	20	100
Avg.	10	53.5
S.D.	14.1	65.8

(values were taken from the main table 3. Only the corner conditions are included)



A: Effect of enzyme vs temperature

B: Effect of particle size vs temperature

C: Effect of particle size vs enzyme

Figure 13 Interaction plots

4.4.1 Temperature vs enzyme

Temperature has a significant positive effect which is attenuated even more with the enzyme supplement. High temperature along with high dose of enzyme was found highest producing with 0 deviation from the mean (SD). The line graph signifies a strong interaction effect between the two factors on biogas accumulation.

4.4.2 Temperature vs particle size

The positive effect of temperature is again demonstrated, however irrespective of the particle size. The contribution from particle size is not significant since the error at 40°C (standard deviation) is larger than the observed effect itself. Parallel lines signify that particle size doesn't have any contribution to biogas accumulation.

4.4.3 Particle size vs enzyme

Again the positive effect of enzyme supplement to biogas accumulation is demonstrated, but the particle size is of no significance, shown by the large error and by the connecting lines with the same slope.

4.5 Main effects

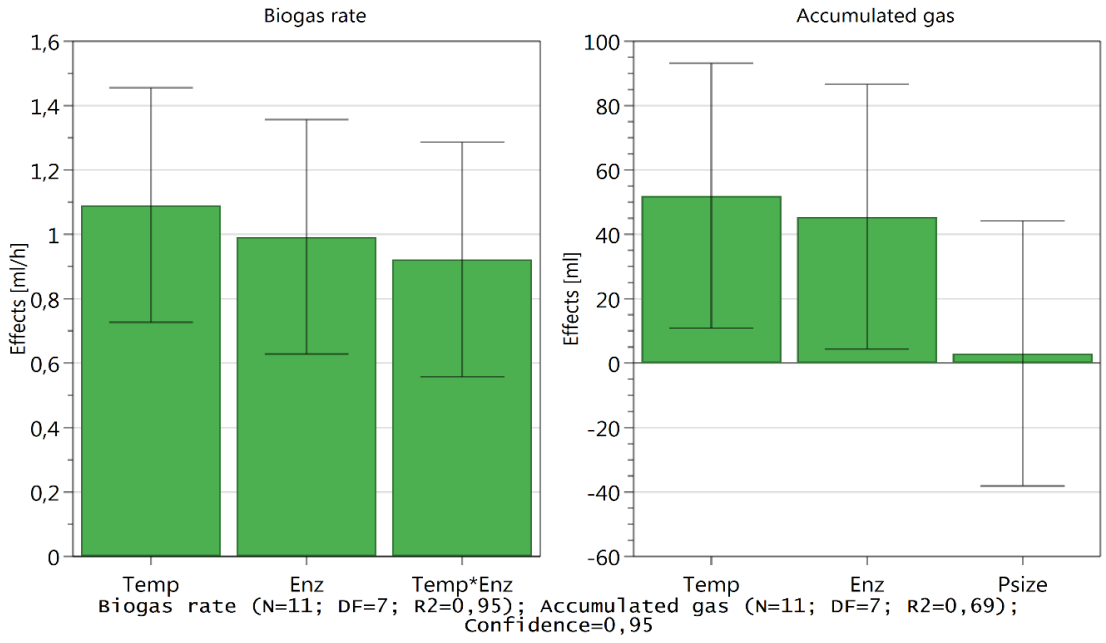


Figure 14 Main effect plots

This is the main result. Bars are calculated main effects and their standard deviation (error bars). For biogas rate increased temperature and enzyme addition have significant positive effect. The Temp*Enz factor also shows a positive interaction effect. Right panel also shows the same main effects of temperature and enzyme but having more error connected, although still significant. Particle size however, in this outcome, had no effect and the error is large.

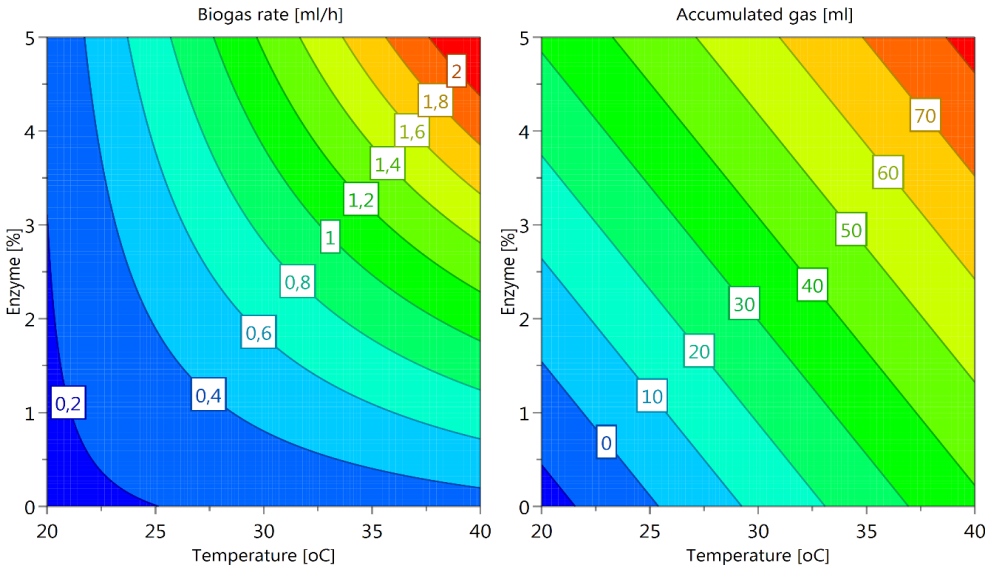


Figure 15 Contour plot of temperature and enzyme

This is result diagram or contour plot (heat diagram). Based on the data a response surface is modelled by multilinear regression (MLR). Responses can be predicted for a given set of conditions (Temp and Enzyme) shown as iso-lines or color bands. Both panels point to upper right corner as a 'hot spot' for best biogas conditions. Left panel is slightly curved, and reflects the interaction term of the two factors. Right panel have a linear form, i.e. interaction is not so pronounced. The contours surfaces are shown at particle size of 3 mm (no difference between the three size categories (contour plot, right panel)).

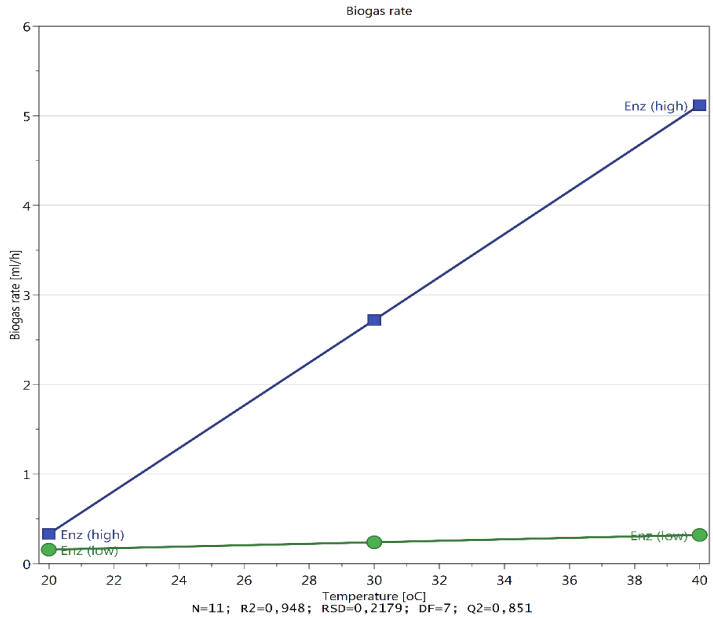


Figure 16 Interaction between temperature and enzyme

This is an interaction plot of temperature vs low (0%) and high (5%) enzyme addition, and their effects on the biogas rate. Both factors have a positive correlation but 'enzyme' has the major contribution. The intersection of lines is a clear evidence of interaction (synergy) between the two factors. Same as the effect plot in fig. 14, this is a quantitative representation of how much temperature (+20 degrees) and enzyme (+5%) contributes to increase in biogas formation rate.

By the whole analysis we resulted that, for high rate of biogas production

1) Increase in temperature has positive effect
2) Increased enzyme has positive effect
3) Temperature and enzyme addition interaction has positive effect
4) Change in particle size from 1 to 5 mm has indifferent effect

5. Discussion

Most of the earlier studies conducted in biogas production, are studies about methane production potential of sewage sludge, municipal waste, agricultural waste and food waste. Different studies were also conducted with mono or co-digestion of substrate for comparing the quantity, quality and rate of biogas produced. Formerly, researchers have used different enzymes and microbial solution of bacteria and fungi to study the activity. But, there is almost no any study have done yet about utility of moose enzyme for the study of biogas production.

5.1 Temperature effect

The temperature in fig. (14) showed the clear evidence of its positive main effect. Low errors bars (SD) associated with the temperature makes it main responsible independent factor for high biogas production. As the temperature increase towards 40°C from 20°C the increase rate of biogas production occurred.

Bergland, Dinamarca, and Bakke (2015) studied temperature effect on biochemical process of AD such as particle disintegration and substrate hydrolysis in a pilot experiment of 220-liter sludge bed reactor. Dairy manure when treated for 4 months in varying temperature 25°C, 30°C and 35°C resulted that temperature has 3.4 % per degree increase in rate at 25 – 30 ° C and 1.6 % per degree increase in rate at 30 – 35 °C. Similarly, Donoso-Bravo et al. (2009) conducted batch test at a temperature range between 15-45°C with glucose, starch and acetic acid as substrate for acidogenesis, hydrolysis and methanogenesis respectively. The obtained result showed that temperature strongly influences all anaerobic processes with highest effect on the steps of acidogenesis. Assuming 5% decrease from operational temperature of the reactor, 50% slower kinetics of acidogenesis and 10% slower in hydrolysis rate occurred.

Increase in temperature is responsible for weaker hydrogen bonds between crystalline cellulose and the structural complexes in the biomass (Wellinger et al., 2013). Increase in temperature induce increased rate of biochemical reaction and thus increases the yield of biogas (Merlino et al. 2013). According to thumb rule, for every 10°C rise in temperature the rate of biochemical reaction will be doubled within certain limits. Which works in the case of AD process also (Jørgensen, 2009). As temperature increases, the substrate will be less viscous and have higher solubility which potentially increase the process of AD.

5.2 Enzyme effect

Same as temperature, enzyme was also found to have positive independent effect for increase biogas production. When the dose of enzyme increases it was found to increase biogas rate significantly.

Same as ours, digestion of cattle manure under cellulolytic strains of mixed consortium and actinomycetes have been observed to improve biogas production in range of 8.4-44% when studied by Parawira (2012). Ishaq et al. (2015) performed fibrolytic test to examine biochemical potential of moose rumen microbiota to digest complex plant carbohydrates such as cellulose, cellobiose, xylan, starch and lignin. When 31 fibrolytic isolates were tested, 15 of them were found capable to digest all types of investigated plant components. They also suggested that those microbes have huge application to be used in agriculture and industrial sector.

Cellulose and xylan are the major constituents of lignocellulose (Rao &Li, 2017). The MRB culture have cellulases and xylanase activity (Thapa, 2018). Cellulases breaks large cellulose molecules into smaller mono or polysaccharides (Saini, Saini, & Tewari, 2015). Xylanase cleave the xylosidic linkages in xylan and reduce polymerization of biomass making it easy to degrade (Saini et al., 2015). And thus reduced substrate yield more biogas.

5.3 Effect of particle size

In our work, PS does not found to have much effect on the rate and amount of gas collected. Though, many research suggested PS also have influence on gas production. Mshandete, Björnsson, Kivaisi, Rubindamayugi, and Mattiasson (2006) from the substrate size of (2, 5, 10, 30, 50, 70 and 100mm) although found variable rate of gas production, the gas produced from 2 and 5 mm are almost equal. Large size of feedstock possesses less surface area for microbial activity and eventually could cause less methane yield and clogging of the digester (Sharma, Mishra, Sharma, & Saini, 1988; Wellinger et al., 2013).

Our study added that, despite a role of PS in biogas production, 1 mm to 5mm of straw substrate will yield relative same rate of biogas.

5.4 Combined effect of temperature and enzyme

Treatments of temperature and enzyme combination was found best to improve biogas production shown by fig. (13A, 14 and 16). Contour plot (fig.15) showed that as the settings approaches towards maximum temperature and enzyme conditions, rate of biogas production continued rising. It identified red color bands from the combination of high settings of temperature towards 40°C and enzyme application at 5%v/v were the best conditions of biogas generation from wheat and manure substrate.

Methane forming bacteria which takes part in AD are divided into 3 categories: *Cryophiles, Mesophiles, Thermophiles*. Growth of bacteria corresponds with change in temperature (Dobre et al., 2014). Same as our study Angelidaki & Ellegaard (2002) found growth of mesophiles up to 40°C was increasing.

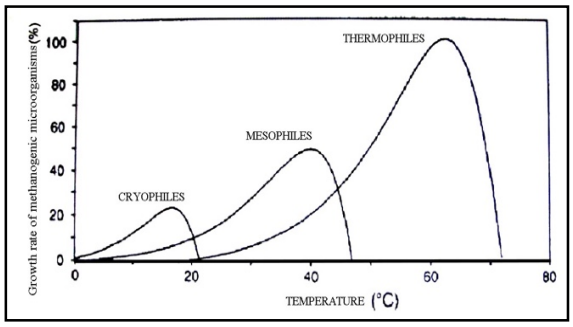


Figure 17 Growth rate of methanogens according to temperature (Angelidaki & Ellegaard, 2002)

Bohn, Siversson, Batstone, Björnsson, and Mattiasson (2001) in the study of anaerobic digestion of agricultural waste under low temperature condition revealed that efficiency of degradation of propionate and acetate significantly decrease with decrease in temperature which resulted low enzymatic activity with ultimate low biogas yield. Thermophilic microflora in comparison to psychrophilic and mesophilic microflora have greater capacity to use several sources of carbon to convert into methane gas or the intermediate products in the process (Converti, Del Borghi, Zilli, Arni, & Del Borghi, 1999). The hydrogen converting bacteria like *Methanobacterium thermoautotrophicum,* which generate methane and water molecules from CO_2 and H_2 has increased activity at high temperature range (Converti et al., 1999). Acetoclastic bacteria which converts acetic acid to methane gets slower in activity as the temperature drops towards psychrophilic conditions (Lettinga et al., 1999; Nozhevnikova et al., 2000). Hupfauf et al. (2018) proposed 45°C have a maximum efficiency on biogas

production when studied AD of cattle slurry and maize straw at 10-55°C. From 10°C towards 45°C hydrogenotrophic to acetoclastic methanogenesis transition was observed and the trend was reversed from 45°C to 55°C.

All of these studies when taken as reference, we can justify the positive combined effect of temperature and enzyme.

5.5 Model analysis

It was found from our experiment that; accumulated gas model doesn't gave best fit for the data. It is because the time length in which substrate remains inside the digester is an important factor for gas production. For complete degradation of organic materials, the hydraulic retention time (HRT) should be higher (Yadvika et al., 2004). HRT is the total time spent by the substrate inside the digesting plant. A shorter HRT will give rise to high biogas production rate but low overall degradation (Wellinger et al., 2013). Ezekoye, Ezekoye, and Offor (2011) performed a test to study the effect of retention time on biogas production from chicken droppings and cassava peels. They found that droppings from poultry produce biogas in much faster rate than the cassava peels within short retention time but after complete digestion cassava peels yielded more gas. They concluded that variation in temperature of biogas affects the retention time of substrate. Hence, the complete digestion will occur within different HRT. Therefore, for batch reactor while analyzing the biogas potential it will be better to consider biogas production rate rather than the accumulated gas collected at last.

6. Conclusion

In our study, temperature and enzyme were found to have positive independent main effects in biogas production. The rate of production was even more supported when temperature and enzyme were applied in combination. It confirmed that the culture from rumen of Moose had some vital organisms which produce certain valuable substance that will eventually produce high biogas from the digester. The interaction effects showed that those organisms and/or the enzymes were reinforced with increase in temperature. Particle size did not show any significant relation to the rate of biogas production. Our study added that, despite of role of PS in biogas production, 1 mm to 5mm of straw substrate will yield relative same rate of biogas. 40°C temperature and 5%v/v enzyme application was found to have high rate of gas production among all combinations.

Effects of MRB culture in biogas production when confirmed by our study, it is recommended that the rumen of the moose host enzyme producing bacteria that are useful to degrade the lignocellulose of the biomass. Research institutions or the stakeholders are suggested to perform the detailed study of microbiota of moose rumen. Isolates from the moose rumen should be studied in detail to extract the actual responsible microbial population. And their effectiveness should be studied ex-vitro (in actual biogas plant).

References

Angelidaki, I., & Ellegaard, L. (2002). *Anaerobic digestion in Denmark: Past, present and future.* Paper presented at the 7th FAO/SREN-Workshop. Department of Chemical Enzymology, Chemistry Faculty. Moscow State University.

Bayané, A., & Guiot, S. R. (2011). Animal digestive strategies versus anaerobic digestion bioprocesses for biogas production from lignocellulosic biomass. *Reviews in Environmental Science and Bio/Technology*, 10(1), 43-62.

Bergland, W. H., Dinamarca, C., & Bakke, R. (2015). *Temperature effects in anaerobic digestion modeling*. Linköping Electronic Conference Proceedings, 261-269.

BiophysEco. (2018). *Statistical Review of World Energy* (67th edition). London, U.K.

Bohn, I., Siversson, B., Batstone, D., Björnsson, L., & Mattiasson, B. (2001). *Anaerobic digestion of agriculture residues under psychrophlilic conditions.* Paper presented at the Proceedings of the 9th World Congress Anaerobic Digestion. Belgium: IWA Antwerpen. p. 2-6.

Broucek, J. (2014). Production of methane emissions from ruminant husbandry: a review. *Journal of Environmental Protection, 5*(15), 1482.

Converti, A., Del Borghi, A., Zilli, M., Arni, S., & Del Borghi, M. (1999). Anaerobic digestion of the vegetable fraction of municipal refuses: mesophilic versus thermophilic conditions. *Bioprocess Engineering, 21*(4), 371-376.

Dobre, P., Nicolae, F., & Matei, F. (2014). Main factors affecting biogas production-an overview. *Romanian Biotechnological Letters, 19*(3), 9283-9286.

Donoso-Bravo, A., Retamal, C., Carballa, M., Ruiz-Filippi, G., & Chamy, R. (2009). Influence of temperature on the hydrolysis, acidogenesis and methanogenesis in mesophilic anaerobic digestion: parameter identification and modeling application. *Water science and technology, 60*(1), 9.

Earth System Research Laboratory. (2018). Trends in atmospheric carbondioxide. Global monitoring division. Retrieved from https://www.esrl.noaa.gov/gmd/ccgg/trends/global.html

Ezekoye, V., Ezekoye, B., & Offor, P. (2011). Effect of retention time on biogas production from poultry droppings and cassava peels. *Nigerian Journal of Biotechnology, 22*, 53-59.

FNR, K. S., Erdmann, G., & Kirchmeyr, F. (2015). D4. 2| *Report on current and future sustainable biomass supply for biomethane production.*

Heeg, K., Pohl, M., Sontag, M., Mumme, J., Klocke, M., & Nettmann, E. (2014). Microbial communities involved in biogas production from wheat straw as the sole substrate within a two-phase solid-state anaerobic digestion. *Systematic and applied microbiology, 37*(8), 590-600.

Henze, M., van Loosdrecht, M. C., Ekama, G. A., & Brdjanovic, D. (Eds.). (2008). Wastewater characterization. *Biological wastewater treatment: Principles modelling and design*, IWA publishing. p 33-52.

Hupfauf, S., Plattner, P., Wagner, A. O., Kaufmann, R., Insam, H., & Podmirseg, S. M. (2018). Temperature shapes the microbiota in anaerobic digestion and drives efficiency to a maximum at 45° C. *Bioresource technology, 269*, 309-318.

Huseby, H. H. (2015). *Biogas upgrading: techno-economic evaluation of different technologies based on Norwegian potential of raw materials.* (Master's thesis, Norwegian University of Life Sciences, Ås).

Ishaq, S. L., Kim, C. J., Reis, D., & Wright, A. D. G. (2015). Fibrolytic bacteria isolated from the rumen of North American moose (Alces alces) and their use as a probiotic in neonatal lambs. *PloS one, 10*(12), e0144804.

Ishaq, S. L., & Wright, A.-D. G. (2012). Insight into the bacterial gut microbiome of the North American moose (Alces alces). *BMC microbiology, 12*(1), 212.

Jørgensen, P. J. (2009). Biogas-green energy. *Faculty of Agricultural Sciences, Aarhus University*. Retrieved from http://www.lemvigbiogas.com/BiogasPJJuk.pdf

Kardos, L., Juhasz, A., Palko, G., Olah, J., Barkacs, K., & Zaray, G. (2011). Comparing of mesophilic and thermophilic anaerobic fermented sewage sludge based on chemical and biochemical tests. *Applied Ecology and Environmental Research, 9*(3), 293-302.

Lettinga, G., Rebac, S., Parshina, S., Nozhevnikova, A., Van Lier, J. B., & Stams, A. J. (1999). High-rate anaerobic treatment of wastewater at low temperatures. *Applied and environmental microbiology, 65*(4), 1696-1702.

McInerney, M. J., Struchtemeyer, C. G., Sieber, J., Mouttaki, H., Stams, A. J., Schink, B., Rohlin L. & Gunsalus, R. P. (2008). Physiology, ecology, phylogeny, and genomics of microorganisms capable of syntrophic metabolism. *Annals of the New York Academy of Sciences, 1125*(1), 58-72.

McKendry, P. (2002). Energy production from biomass (part 2): conversion technologies. *Bioresource technology, 83*(1), 47-54.

Merlino, G., Rizzi, A., Schievano, A., Tenca, A., Scaglia, B., Oberti, R., Adani F. & Daffonchio, D. (2013). Microbial community structure and dynamics in two-stage vs single-stage thermophilic anaerobic digestion of mixed swine slurry and market bio-waste. *Water research*, *47*(6), 1983-1995.

Mshandete, A., Björnsson, L., Kivaisi, A. K., Rubindamayugi, M. S., & Mattiasson, B. (2006). Effect of particle size on biogas yield from sisal fibre waste. *Renewable energy, 31*(14), 2385-2392.

Noraini, M., Sanusi, S. N. A., Elham J., Sukor M. Z., & Halim K. (2017). Factors affecting production of biogas from organic solid waste via anaerobic digestion process: a review. *Solid state science and technology*. 25. 29-39.

Nozhevnikova, A., Rebak, S., Kotsyurbenko, O., Parshina, S., Holliger, C., & Lettinga, G. (2000). Anaerobic production and degradation of volatile fatty acids in low temperature environments. *Water science and technology, 41*(12), 39-46.

Parawira, W. (2012). Enzyme research and applications in biotechnological intensification of biogas production. *Critical reviews in biotechnology, 32*(2), 172-186.

Raadal, H. L., Schakenda, V., & Morken, J. (2008). Potensialstudie for biogass i Norge. *Østfoldforskning AS and NMBU*, 55.

Rao, M. P. N., & Li, W.J. (2017). Microbial Cellulase and Xylanase: Their Sources and Applications. *Advances in Biochemistry & Applications in Medicine*. Chapter 4.

Ritter, S. K. (2008). Lignocellulose: A complex biomaterial. *Plant Biochemistry, 86*(49), 15.

Saini, J. K., Saini, R., & Tewari, L. (2015). Lignocellulosic agriculture wastes as biomass feedstocks for second-generation bioethanol production: concepts and recent developments. *3 Biotech, 5*(4), 337-353.

Sánchez, C. (2009). Lignocellulosic residues: biodegradation and bioconversion by fungi. *Biotechnology advances, 27*(2), 185-194.

Schnurer, A., & Jarvis, A. (2010). *Microbiological handbook for biogas plants*. Swedish Waste Management U*, 2009*, Swedish Gas Centre Report 207.1-74.

Seleiman, M. F. (2014). *Towards sustainable intensification of feedstock production with nutrient cycling.* (Doctoral dissertation, University of Helsinki, Finland).

Sharma, S. K., Mishra, I., Sharma, M. P., & Saini, J. (1988). Effect of particle size on biogas generation from biomass residues. *Biomass, 17*(4), 251-263.

Sletten, T. M. & Maass, C. (2013). *Underlagsmateriale til tverrsektoriell biogass-strategi. Oslo: Klimaog forurensningsdirektoratet* (Material for cross-sectoral biogas strategy. Oslo: Climate and Pollution Agency). 245 pp.

Slonczewski, J. L., & Foster, J. W. (2013*). Microbiology: An Evolving Science: Third International Student Edition*: WW Norton & Company.

Thapa, D.S. (2018). Cultivation conditions for a moose (Alces alces) rumen bacterial isolate secreting cellulases. MSc thesis in Applied and Commercial Biotechnology. Inland Norway University of Applied Sciences. Hamar, p. 66.

Thrän D., Seiffert M., Müller-Langer F., Plättner A., Vogel A. (2007). *Possibilities of a European one-way strategy*. Institute of Energy and Environment. Leipzig. Germany.

Venkiteshwaran, K., Bocher, B., Maki, J., & Zitomer, D. (2015). Relating anaerobic digestion microbial community and process function: supplementary issue: water microbiology. *Microbiology insights, 8*, MBI. S33593.

Ward, A. J., Hobbs, P. J., Holliman, P. J., & Jones, D. L. (2008). Optimisation of the anaerobic digestion of agricultural resources. *Bioresource technology*, 99(17), 7928-7940.

Wellinger, A., Murphy, J. D., & Baxter, D. (2013). *The biogas handbook: science, production and applications*: Elsevier.

World Health Organization. (2018). *Household air pollution and health*. Retrieved from http://www.who.int/en/news-room/fact-sheets/detail/ambient-(outdoor)-air-quality-and-health.

Yadvika, Sreekrishnan, T., Kohli, S., & Rana, V. (2004). Enhancement of biogas production from solid substrates using different techniques—a review. *Bioresource technology,* 95(1), 1-10.

Zhang, Z., & Zhao, Z. K. (2010). Microwave-assisted conversion of lignocellulosic biomass into furans in ionic liquid. *Bioresource technology*, 101(3), 1111-1114.

Appendix

Figure 1: Wheat straw at different PS

Figure 2: 5% DM manure solution in reactors

Figure 3: Reactors at 20°C

Figure 4: Reactors at 30°C

Figure 5: Reactors at 40°C

Table 1: Scheme of the experiment

Start time: 24 Jan 12pm

Flask no.	1st day (24 Jan)		2nd day (25Jan)		3rd day (26Jan)	
	Time	Gas yield (ml)	Time	Gas yield (ml)	Time	Gas yield (ml)
1	21:45	0	8:15	0	8:15	5
			2:15	3	12:15	5
2	21:45	5	8:15	5	8:15	5
			2:15	5	12:15	5
3	21:45	0	8:15	0	8:15	0
			2:15	0	12:15	0
4	21:45	3	8:15	9	8:15	15
			2:15	13	12:15	0
5	21:45	0	8:15	0	8:15	5
			2:15	5	12:15	5
6	21:45	0	8:15	20	8:15	80
			2:15	35	12:15	100
7	21:45	0	8:15	5	8:15	5
			2:15	5	12:15	7
8	21:45	15	8:15	35	8:15	90
			2:15	45	12:15	100

9	21:45	10	8:15	10	8:15	40
			2:15	13	12:15	45
10	21:45	10	8:15	15	8:15	45
			2:15	18	12:15	50
11	21:45	5	8:15	5	8:15	40
			2:15	15	12:15	45