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Enrichment of hydrogenotrophic methanogens for biomethanation

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AFFIDAVIT

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Preface

This project was carried out at the Department of Chemical Engineering, Biotechnology and Environmental Technology (KBM), University of Southern Denmark (SDU) from 1st February 2019 to 3rd June 2019 in the KBM laboratories.

The supervisors at the University of Southern Denmark was Associate Prof. Jin Mi Triolo. I would like to thank her for her great help and all the valuable advice throughout the project. I am also thankful to my home university supervisors Prof. Matthäus Siebenhofer, Institute of Chemical Engineering and Environmental Technology, TU Graz for his guidance, scientific suggestions, and encouragement.

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List of Abbreviations

VFA	*Volatile fatty acid*
HM	*Hydrogenotrophic methanogens*
AA	*Acetic acid*
iso BA	*Isobutyric acid*
PA	*Propionic acid*
BA	*Butyric acid*
iso VA	*Isovaleric acid*
VA	*Valeric acid*
LCV	*Lower calorific value*
AD	*Anaerobic digestion*
Conc.	*Concentration*
PSA	*Pressure swing adsorption*
VS	*Volatile solid*
GHG	*Greenhouse gas*

1. Abstract

Increasing demand of energy due to depletion of non-renewable energy sources makes people think about alternative resources of energy. Production of biogas using hydrogenotrophic methanogens is the potential contribution to get this problem under control. Nowadays biogas upgrading technologies have opened a new window for its wider industrial applications. Compared to other renewable resources, microbial assisted biogas technology is the most approachable method that can replace natural gas.

Biogas is a CH_4 rich mixture of gases that is produced anaerobically breaking down organic matter, animal manure or energy crops by following thermophilic or mesophilic temperature. So, with 60-70% CH_4, biogas additionally contains 30-40% CO_2, N_2, water vapor, H_2S, hydrocarbons, NH_3, and other trace elements originating from the effluent. After removing those impurities, the amount of CH_4 could be up to 98%. In the conventional system, CO_2 is removed while advanced technology reuses the CO_2 to produce CH_4. So, reprocessing CO_2 from the process to produce, methane will double the production by saving the environment from the devastating consequences of greenhouse gas (GHG).

Moreover, if the density of hydrogenotrophic methanogens that produce methane can be enriched, the efficiency of the process will be increased too, which has been investigated in this project focusing on the different parameters.

In order to do this, two different inoculums which were biogas plant digested feedstock, and garden sediment was mixed with two mediums, named mineral medium and cattle manure in this fed-batch process. By following the microbial assisted ex-situ technology, different reactor setups were tested to evaluate the impact of the inoculum in a mesophilic temperature range.

Therefore, there were five types of mini reactors installed which were made using inoculum and medium. From the five reactors, three were operated with the same mixture, i.e., biogas plant digested feedstock and cattle manure, but their position in the incubator was different from each other. One of them was standing position, the second one was horizontal position in the incubator and the third one was also standing up but with a filling material inside the reactor. The use of filling material in the reactor was to create more surface area and moisture for the microorganism's enrichment. The fourth sample was a combination of garden sediment with cattle manure and the fifth was biogas plant digested inoculum with a mineral medium. All the samples were flushed with N_2 and filled with H_2 and CO_2 to generate the reaction.

The whole process was carried out for a total of 61 days, divided into two phases. After finishing the first phase which lasted for 30 days, all the reactors were re-cultured to get faster growth of microorganisms as well as methane production. The pressure drop of all samples was recorded and the reactors were fed with fresh nutrients every day.

To calculate the conversion of methane and other products, a gas sample was collected before flushing the sample when the pressure became low and a liquid sample was collected once a week to measure the volatile fatty acid (VFA).

Kurzfassung

Der steigende Energiebedarf aufgrund der Erschöpfung nicht erneuerbarer Energiequellen veranlasst die Menschen, über alternative Energiequellen nachzudenken. Die Produktion von Biogas durch Verwendung von hydrogenotrophen Methanogenen ist ein potenzieller Beitrag, um dieses Problem in den Griff zu bekommen. Heutzutage haben die Technologien zur Aufbereitung von Biogas ein neues Fenster für seine breitere industrielle Anwendung geöffnet. Im Vergleich zu anderen erneuerbaren Ressourcen ist die mikrobiell unterstützte Biogastechnologie die am weitesten verbreitete Methode, um Erdgas zu ersetzen.

Biogas ist ein CH_4-reiches Gasgemisch, welches durch den anaeroben Abbau von organischen Substanzen, Tierdung, oder Energiepflanzen, durch die Einhaltung der thermophilen oder mesophilen Temperatur erzeugt wird. So enthält Biogas mit 60-70% CH_4 zusätzlich 30-40% CO_2, N_2, Wasserdampf, H_2S, Kohlenwasserstoffe, NH_3 und andere aus dem Abwasser stammende Spurenelemente. Nach der Entfernung dieser Verunreinigungen kann die Menge an CH_4 bis zu 98% betragen. In konventionellen Systemen wird das CO_2 entfernt, während dieses bei moderner Technologie zur Herstellung von CH_4 wiederverwendet wird. Bei der Wiederaufbereitung des CO_2 aus dem Prozess zur Herstellung von Methan wird also die Produktion verdoppelt, und die Umwelt vor den verheerenden Folgen des Treibhausgases (THG) bewahrt.

Wenn zudem die Dichte der hydrogenotrophen Methanogene, welche Methan produzieren, angereichert werden kann, wird dadurch auch die Effizienz des Prozesses erhöht, was in diesem Projekt mit Blick auf die verschiedenen Parameter untersucht wurde.

Dazu wurden zwei verschiedene Inokulumsorten verwendet: In Biogasanlagen prozessierte Rohstoffe und Gartensedimente. Diese wurden mit zwei Medien, dem „mineralischen Medium“ und Viehdung, in einem Fed-Batch-Verfahren gemischt. Durch die Anwendung von mikrobiell unterstützter Ex-situ-Technologie wurden verschiedene Reaktoranordnungen getestet, um die Auswirkungen des Inokulums in einem mesophilen Temperaturbereich auszuwerten.

Daher wurden fünf Typen von Mikro-Reaktoren installiert, die mit Inokulum und Medium hergestellt wurden. Von den fünf Reaktoren wurden drei mit derselben Mischung betrieben, (in Biogasanlagen prozessierte Rohstoffe und Viehdung), jedoch deren Position im Inkubator variiert. Einer von ihnen war stehend, der zweite lag horizontal im Inkubator und der dritte war ebenfalls stehend, jedoch mit einem Füllmaterial im Inneren des Reaktors. Die Verwendung von Füllmaterial im Reaktor sollte mehr Oberfläche und Feuchtigkeit für die Anreicherung des Mikroorganismus schaffen. Die vierte Probe bestand aus einer Kombination aus Gartensediment

und Viehdung und die fünfte Probe war ein von der Biogasanlage prozessiertes Inokulum mit einem mineralischen Medium. Alle Proben wurden mit N_2 gespült und anschließend mit H_2 und CO_2 gefüllt, um die Reaktion zu starten.

Der gesamte Prozess wurde insgesamt 61 Tage lang durchgeführt, aufgeteilt in zwei Phasen. Nach Abschluss der ersten Phase, welche 30 Tage dauerte, wurden alle Reaktoren rekultiviert, um ein schnelleres Wachstum der Mikroorganismen sowie schnellere Methanproduktion zu erreichen. Der Druckabfall aller Proben wurde aufgezeichnet und die Reaktoren wurden täglich mit frischen Nährstoffen versorgt.

Um die Umwandlung von Methan und anderen Produkten zu berechnen, wurde eine Gasprobe vor dem Spülen der Probe entnommen, wenn der Druck niedrig wird, und einmal pro Woche eine flüssige Probe zur Messung der flüchtigen Fettsäure (FFS) entnommen.

2. Introduction

The increasing world's population is putting pressure day by day on conventional energy sources such as coal, crude oil, natural gas, etc. to power - industry and other private and public infrastructures. Therefore, to minimize the pressure on finite sources a new path is needed which will also satisfy some important prerequisites such as sustainability, low environmental impact, economic efficiency, etc. Renewable energy like wind power, solar energy, hydroelectric power, bioenergy has widespread use. Due to rapid development, biogas upgrading technologies have received more recognition in the recent market not only for, it is cost-effective method but also for its lower impact on climate change.

The production of biogas from sludge, food waste, animal manure, organic municipal solid waste, industrial waste, agricultural residues, etc. through anaerobic digestion. The anaerobic digestion to generate biogas follows four steps which are hydrolysis, acidogenesis, acetogenesis, and methanogenesis. In the methanogenesis steps, methane is produced utilizing hydrogenotrophic methanogens (HM) reacting with H_2 and CO_2 or acetotrophic methanogens via acetic acid consumption (Nabin Aryala). The pathway of methane production depends on the nature of the substrate and energy source. The substrates follow two pathways. In the first pathway, hydrogenotrophic methanogens can be consumed H_2 and CO_2 at a volume ratio of 4:1 to produce biomethane [**Eqn. (1)**]. In the second pathway, H_2 consuming bacteria homoacetogens can utilize H_2 and CO_2 at a molar ratio of 2:1 to produce CH_3COO^- [**Eqn. (2)**], which can then be consumed by acetoclastic methanogens to generate methane (Rui Xu, 2019). For the high percentage of energy generation and carbon fixation, hydrogenotrophic pathways are more efficient where it can follow reverse oxidation reaction to keep the process continuous (Heng Xua, 2020).

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad (1)$$

$$4H_2 + 2CO_2 \rightarrow CH_3COO^- + H^+ + 2H_2O \quad (2)$$

In the anaerobic digestion (AD) process, along with methane, there are CO_2, NH_3, H_2S, etc. which can damage the reactor via oxidation. To get rid of these toxic gases different technologies have been developed such as amine scrubbing, water scrubbing, pressure swing absorption, chemical washing, and membrane technologies, etc. For the low cost and environmentally friendly process, biological in-situ or ex-situ technology has been investigated and developed over the years for which the bioenergy has reached a mature stage now.

So, it could be presumed that the status of biogas upgrading technologies has already established which is going to provide a better integration in the upcoming years not only in the EU but also in the other countries of the world who are looking for a path to utilize their waste.

3. Aim and hypothesis of the thesis

3.1 Objective

Conventional biogas contains 30-40% CO_2 with other unwanted gas components. The energy content of biogas is determined by the heating value of the methane fraction. So around 40% of CO_2 in the biogas displaces almost half of its energy density and it cannot be injected into a natural gas grid or transported and cannot be used in vehicles because of the quality of the biogas (Persson, 2014). Moreover, to increase the heating value, removal of CO_2 is a solution but releasing the CO_2 in the environment without utilizing it, has a bad impact on nature.

This can be solved via methanation where H_2 and CO_2 are used in the process to produce methane. Though earlier this had been done catalytically with high pressure and temperature, newer studies have shown that hydrogenotrophic methanogens can upgrade biogas using H_2 and CO_2 which is called biomethanation. (Deshusses, 2017).

Therefore, if the density of hydrogenotrophic methanogens is increased it will react with the maximum amount of substrate and upgrade the volumetric amount of methane. So, the enrichment of methanogens was the aim of this project.

3.2 Hypothesis

It is assumed that,

- Enrichment of the inoculum will be towards hydrogenotrophic methanogens
- The concentration of VFA will be decreased end of the phase than initial
- Reactors with high surface area will have a high population of methanogens
- Reactors with filling material will have a high consumption of H_2 and CO_2 as well as high HM density
- The percentage of biomethanation will increase in the second phase

4. Current energy strategies

To ensure the supportable economy, increase the energy efficiency from renewable resources, lower the emission, etc. in Europe, policymakers have proposed a lot of proposals and solutions (European Commission, n.d.). The European Commission has focused more on sustainable resources rather than fossil fuels to make the union more environment-friendly. This part will be highlighted in the current energy scenarios in the EU.

4.1. Energy in EU

The European renewable resources have implemented many policies, programs and initiatives to reach the goal of energy consumption by 2020,2030, and 2050. Among them the EU has set itself a long-term goal by 2050, to reduce greenhouse gas emissions by 20%. To achieve the goals, the EU set the milestone by 2020 and 2030 for its countries (***Fig. 1. 1***).

2020 renewable energy targets (European Commission, n.d.)

- 20% of final energy consumption from renewable sources
- Other EU countries must get at least a 10% share
- Energy savings of up to 20%

2030 renewable energy targets (European Commission, n.d.)

- goal to reduce emissions by at least 40 %.
- the share of renewable energy consumed in the union approaches at least 32 %.

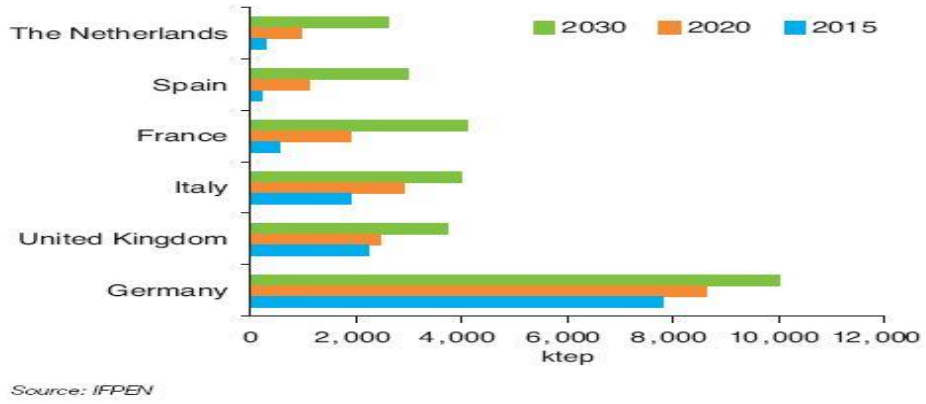

Fig. 1. 1: Potential biogas development in Europe by 2030 (ifp Energies Nouvelles).

4.2. Biomass potential in EU

In 2014, the EU Commission published a report about the use of solid and gaseous biomass fuels in a sustainable way for heat generation and transportation which has been modified by November 2016 and added as reviewed Renewable Energy Directive. From ***Fig. 1. 2***, it has been shown that EU countries need more biomass potential in the Future. According to the European Environmental plan, the EU's primary energy requirement will be at 1.8 billion tonnes oil equivalent (toe) in 2020 and projected biomass availability could contribute 13 % or 236 million toes (European Commission, n.d.). However, there are some uncertainties in the consideration for the actual biomass source due to weather conditions or land availability for energy crops. Some countries like France and Spain have a balance between supply and demand than other countries like the UK and Germany. Therefore, some countries could work as suppliers or provide pretreatment of raw biomass for other countries to increase the source.

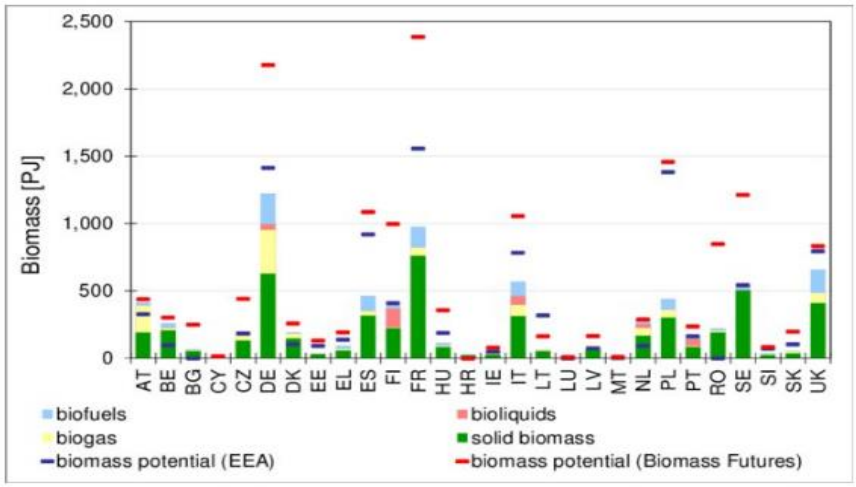

Fig. 1. 2: Overview of biomass in EU countries (Reynaldo Victoria, 2015).

4.3. Bioenergy in Austria

The importance of bioenergy as a domestic source of energy has become the mainstream in Austria to reach the target of 100 % renewable resources by 2030. At present 29.9% gross consumption is covered from bioenergy, with 39% solid biomass followed by 33% hydropower. (Jellinek, 2018). With a share of 82% in 2017, heat generation was the biggest market for bioenergy where the energy consumption increased 130 PJ in 2005 to 207 PJ in 2017. Since 1970 the use of bioenergy increased five times in Austria which is now more than 56% in share. But it is noticeable that consumption has also increased by 37% over that period. To follow the EU rules Austria needs to attain a 34% share of renewable energy by 2020 which was 32.6% until 2017 (***Fig. 1. 3***). But there is uncertainty to achieve the goal because the share of other renewable resources is fluctuating 5 to 10% over the decades (Österreich Biomasse - Verband, n.d.)

Austria has developed the ''Energy Strategy for Austria'' to contribute to the targets 20-20-20 set by EU which are as follows (Jellinek, 2018).

- 20% increase in energy efficiency
- 34% share of renewable energy
- Reduction of greenhouse gas emissions by 6% in non-ETS sectors
- Reduction in greenhouse gas emissions of at least 20% below 1990 levels

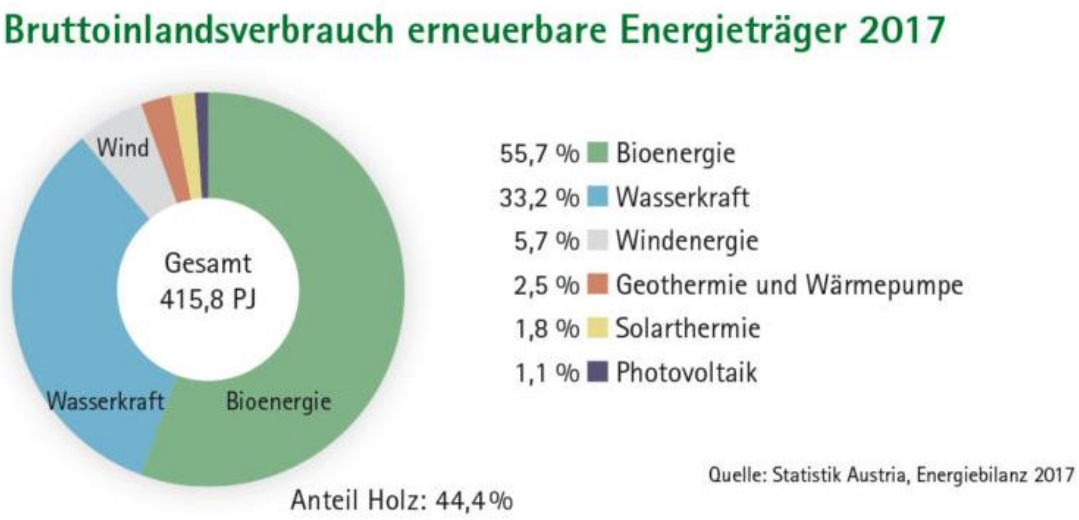

Fig. 1. 3: Austria's gross domestic consumption of renewable energy sources in 2017.

4.4. Biogas in Denmark

Like other EU countries, Denmark has also announced its plan to achieve 100% renewable energy for the heat generation and transport sectors by 2050 (Ministry of Climate). To achieve this goal Denmark now has more investment in biogas plants. At present Denmark is covering around 75% of energy for heating from renewable resources. There are more than 20 biomass plants in Denmark fueled with wood, wood chips, biodegradable waste as well as straw as the source of biomass to produce green energy. However, Denmark has set a goal to reach 35% of energy consumption with renewable resources and 50% from wind energy by 2020 (Ministry of Climate). The following figure shows an overview of Danish energy consumption from different sources up to 2020. So, it has been expected that Denmark will increase renewables consumption from around 136.5 PJ in 2012 to 173PJ in 2020 (***Fig. 1. 4***).

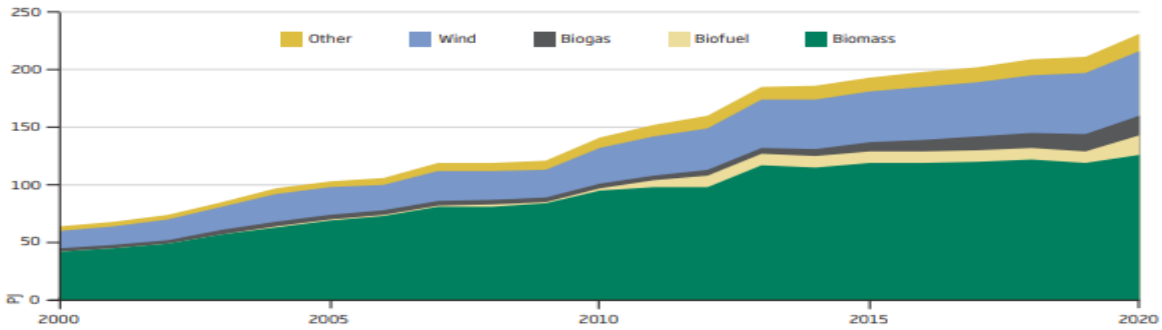

Fig. 1. 4: Overview of Danish Energy consumption from 2000 to 2020 (FROM SUSTAINABLE BIOMASS TO COMPETITIVE BIOENERGY).

5. Background of biogas upgrading

Biogas upgrading is a process where methane gas is produced removing CO_2, H_2 and other contaminates to make it comply with the natural gas standard. This process is also called anaerobic digestion which is a unique process to generate renewable energy from organic waste. However, raw biogas contains water and toxic gases which cannot be used due to corrosion. Upgraded biogas without any contaminates has high calorific value so it can be stored in the natural gas grid to supply and transport (***Fig. 1. 5***).

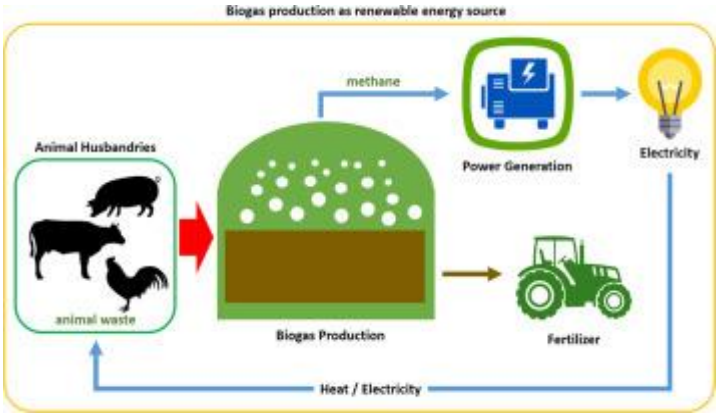

Fig. 1. 5: Biogas production as renewable energy sources (Munawar Khalil, 2019)**.**

Biogas mainly contains methane and carbon-di-oxide as a product where the percentage of the two gases comparatively depends on the nature of the substrate and the pH. Besides these two-gases, it contains N_2, which is from air saturated in the effluent, water vapor, O_2 due to leakages in the sample, H_2S from some waste which contains sulfate, NH_3 from proteinogenic waste, hydrocarbons, and siloxanes (Irini Angelidaki, 2018). To inject the biogas into the natural gas grid, it must be free from pollutants and chemically conditioning to meet the standard value. There are physical and chemical methods to remove the pollutants which have several technical burdens to fulfill the requirement. More importantly, losing CH_4 content during the process can increase the GHG and minimize the total energy efficiency. Therefore, for upgrading the biogas, enrichment of hydrogenotrophic methanogens following the biological method has been demonstrated to be an effective way. Though the hydrogenotrophic methanogens are slow growers but increasing the contact of substrate with methanogens, this drawback could possible to resolve (Yeo-Myeong Yun, 2017). A complete list of all the gas in biogas has been shown below (

Table 1) with their average amount after primary treatment.

Table 1: Percentage of all components in the Biogas (Irini Angelidaki, 2018)

Name of the component	***Average amount***
CH_4	*50–70%*
CO_2	*30–50%*
N_2	*0–3%*
H_2O	*5–10%*
O_2	*0–1%*
H_2S	*0–10,000 ppmv*
NH_3	*0,01–2,5 mg/m³*
Hydrocarbon	*0–200 mg/m^3*
Siloxanes	*0–41 mg/m^3*

6. Feedstock for biogas production

Feedstock such as livestock waste, straw, energy crops, and discarded fruits and vegetables are the main components that contribute to the production of biogas. The high efficiency of methane depends on the composition, homogeneity, and biodegradability of different waste in the digester. So, waste should be optimized cautiously in AD to get a high yield of gas. It has been shown that organic waste such as food waste produces more energy than industrial waste, sewage or manure (Munawar Khalil, 2019). However, with organic waste using another substrate for co-digestion increases the yield of methane gas and also improves nutrients balance and minimizes the percentage of toxic compounds. The preferred use of a basic substrate is pig or cow manure with co-fermentation of biogas crop (Rebecca Sebola, 2014). High strength waste easily co-digested with low strength waste such as food waste co-digested with pig manure (***Fig. 1. 6***).

The energy from biomass which is also known as biochemical methane potential is measured by CH_4 NL (kg/vs-1) mostly for animal manure and food waste.

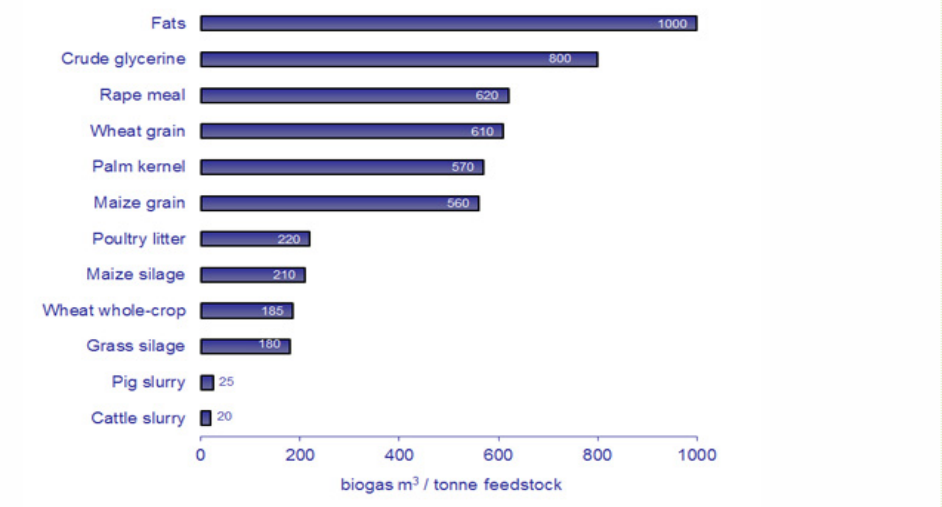

Fig. 1. 6: Biogas from various feedstocks (Denise Nicholls, n.d.)**.**

7. Anaerobic digestion technology

7.1. Operational parameters

The anaerobic digestion process depends on the environmental factors and process parameters. Among other parameters, nutrients, temperature, pH of the samples are most important which should be tracked during the production of methane in a biogas reactor. The influence of any of

the parameters in the reactor can inhibit the whole process or can lower efficiency. Some of the parameters will be described in the following paragraphs.

7.1.1. Nutrients

In AD processes, micronutrients or macronutrients are needed to increase the growth of microorganisms. Carbon, nitrogen, hydrogen, sulphur, phosphorus are the main nutrients that must be in the feedstock in a balanced amount. It has been recommended that a 16-25/1 ratio between carbon and nitrogen should be used for anaerobic digestion (Safoora Mirmohamadsadeghi, 2019). Carbon is needed for cell growth and nitrogen for protein synthesis. Phosphorus is also a necessary nutrient that is passing energy via energy carriers in microorganisms' activities (Safoora Mirmohamadsadeghi, 2019). There are some other trace elements such as cobalt, iron, sulphur, nickel, zinc, etc. required to keep the microorganisms alive. Besides the cell growth, iron can react with H_2S by precipitating iron (II) sulphide and protect the biogas reactor from corrosion (Safoora Mirmohamadsadeghi, 2019). Moreover, cobalt is necessary to stabilize the AD process and nickel for the optimal growth of methanogens. Therefore, all the nutrients are playing an important role in the AD process. So, the substrate must contain these trace elements in the required amount. On the contrary, due to high concentration of these elements, the process can be damaged too. So, selecting the right concentration for the trace elements is the first process to estimate the absolute combination of them in the method (Safoora Mirmohamadsadeghi, 2019).

7.1.2. Temperature

AD process follows either thermophilic (55-70) ºC or mesophilic (32-45) ºC temperature conditions. It is very important to continue with stable temperature because some microorganisms are unstable at elevated temperature. In the process, mesophilic conditions can vary between ±3º (Safoora Mirmohamadsadeghi, 2019). But it could be crucial for the microorganisms if the temperature increases between 40-45 ºC because of irreversible destruction of the microorganisms (Safoora Mirmohamadsadeghi, 2019). On the contrary, except variation with temperature thermophilic operation shows higher degradation rate than mesophilic to make the process more efficient. It has less solubility of oxygen, less impact due to ammonia accumulation and can operate at lower retention time (Safoora Mirmohamadsadeghi, 2019). Experiments indicate that thermophilic low loading rate yields more gas than the mesophilic digestion. However, mesophilic digestion has the advantage of higher stability in temperature fluctuation.

7.1.3. pH

There is an optimum pH range which is 6 to 8.5 to get high yields of methane (Safoora Mirmohamadsadeghi, 2019). The effect of the high pH value influences the digestion process and dissociates important compounds. So, lower pH value is required for the process. However, to maintain the optimum pH conditions there are two natural buffer systems going on in the process.

As shown in **Eqn. (3)** a natural buffering process between CO_2 and H_2O is occurring at an equilibrium rate to prevent a shift to lower pH value (Safoora Mirmohamadsadeghi, 2019). Another is shift from ammonia to ammonium equilibrium composition which prevents the higher pH value [**Eqn. (4) & (5)**]. The reactions are given below.

$$CO_2 + H_2O \rightleftarrows H_2CO_3 \rightleftarrows HCO^{3-} + H^+ \rightleftarrows CO_3^{2-} + 2H^+ \quad (3)$$

$$NH_3 + H^+ \rightleftarrows NH_4^+ \quad (4)$$

$$NH_4^+ + OH^- \rightleftarrows NH_3 + H_2O \quad (5)$$

These two buffer systems keep the process at an optimum operational value. Though, temperature fluctuation, high organic loading rate and high degradability of feedstock can change the buffer systems (Safoora Mirmohamadsadeghi, 2019).

7.1.4. Retention time

Retention time is another important factor in the AD process. The rate of methane production depends on the length of the retention time. The higher the retention time, the less production of volatiles from solids. However, lower retention time can reduce the investment cost. It has been studied that 75% of biogas can be produced from within 10 to 15 days of retention time. If the retention time is decreased, the organic loading rate should be increased to get a higher amount of biogas. Moreover, lower retention time also could affect the concentration of microorganisms in the biogas plant if the plant doesn't have recycle systems. So, minimum retention time is required to prevent the removal of biomass in shorter lengths (Safoora Mirmohamadsadeghi, 2019).

Retention time is characterized by two parameters, one of them is hydraulic retention time which is a ratio of digester volume/flow rate of the digester. The other is solid retention time, which shows the average time of microorganisms, retains in the digester (Safoora Mirmohamadsadeghi, 2019).

8. Anaerobic digestion biochemical process

Anaerobic digestion is the process to produce biogas by breaking down organic matter such as energy crops, agricultural waste, wastewater sludge, plant biomass, animal manure, etc. This digestion followed both thermophilic and mesophilic methanogenic process. Mesophilic digestion ranges in temperature from (35-45) ºC whereas the thermophilic digestion is ranging from (55-70) ºC. Due to the high energy requirement for heating and lower process stability in thermophilic digestion, mesophilic digestion is broadly used in the biogas plant. However, AD is a very complex

process that is divided into four phases (a) hydrolysis (b) acidogenesis (c) acetogenesis and (d) methanogenesis. A process flow of degradation of organic matter has been shown in ***Fig. 1. 7.***

Hydrolysis: Organic micro molecules such as carbohydrates, lipids, proteins, etc. are depolymerized to produce cellular enzymes. Then those produced monomers and oligomers undergo a hydrolysis reaction to produce amino acids, sugar, and long-chain fatty acids.

Acidogenesis: All the products from the hydrolysis process converted into short-chain fatty acids, carbon dioxide, hydrogen, and acetic acids by fermentative bacteria.

Acetogenesis: Acetogenic bacteria further convert the acetic acids and other products to hydrogen and, carbon dioxide which is the main substrate to produce methane.

Methanogens: In the methanogenesis, methane can be produced either by hydrogenotrophic methanogenic archaea using carbon dioxide and hydrogen or acetoclastic methanogenic archaea using acetic acids. In hydrogenotrophic methanogens, CO_2 converts into CH_4 where H_2 is used as an external source of electrons which is also known wood-ljungdahl pathway (Nabin Aryala). On the other hand, in the acetoclastic methanogenic process, homoacetogenic bacteria convert CO_2 to acetate and then acetate produces methane. But this process is very sensitive to O_2 concentration, lower pH, feedstock impurities and other environmental parameters of the system.

However, in the AD process, all the four steps should be in the equivalent position otherwise the whole process could fail due to the high acidity.

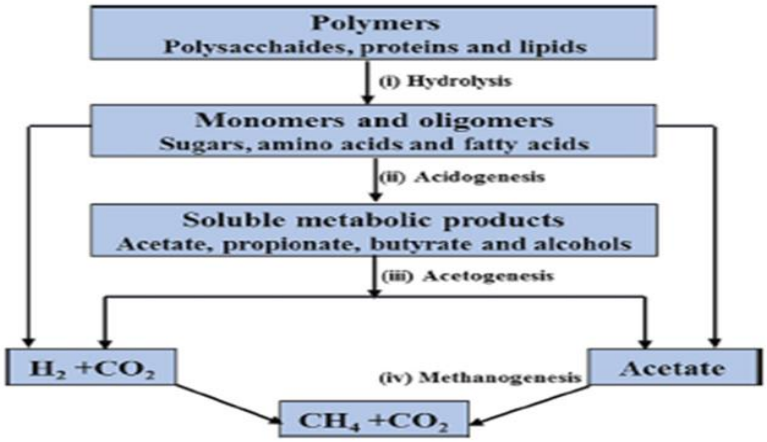

Fig. 1. 7: Conventional anaerobic process of organic matter (Nabin Aryala)

9. Thermodynamics analysis of carbon dioxide utilization

Carbon dioxide is considered a greenhouse gas which is the greatest threat to the environment. The source of carbon dioxide is either from the chemical process or from the combustion of fuel. So, capturing or utilizing this greenhouse gas could be a better option to protect the environment as well as in the economic point. Carbon dioxide could be chemically converted into other molecules such as formic acid, methanol, methane, etc. as an energy source, known as Sabatier reaction. Due to the high energy content and demand for methane, it is now preferable to utilize CO_2 in biogas production [**Eqn. (6)**].

$$CO_2 + 4H_2 \rightleftarrows CH_4 + 2H_2O \qquad \Delta H = -165\ kJ/mol \tag{6}$$

CO_2 could be collected from various sources such as industrial flue gas or biochemical process. To convert the CO_2 into other chemical species hydrogen is required which can be collected by water electrolysis, steam reforming of natural gas or biological process (raw material). Conversion of CO_2 is constrained into four products HCOOH, CH_4, CH_3OH, and H_2CO with 1:4 M of CO_2: H_2 (***Fig. 1. 8***). The capture of carbon dioxide can be calculated using the following **Eqn. (7)** (Ahmed M. Yousefa, 2019).

$$CO_2 \text{ Captured Ratio } (\%) = \frac{m^A_{co_2}feed - m^A_{co_2}cleaned}{m^A CO_2 feed} \tag{7}$$

Where, $m^A_{Co_2 feed}$ is the mass flow rate of CO_2 in the feed biogas (kg/s), which is an input. $m^A_{co_2 Cleaned}$ is the mass flow rate of CO_2 in the cleaned/upgraded gas (kg/s).

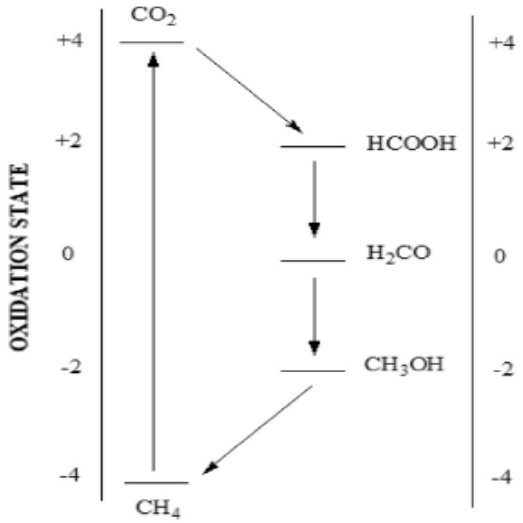

Fig. 1. 8: *Different stages of conversion of CO_2 to the product* (Rachid Hadjadj, 2019).

The influence of variation in concentration of feed gas (i.e., CO_2) is an important parameter to ensure the high quality of biomethane. In that case, thermodynamic principles can be used to calculate the limit of production and composition of biogas by following Gibbs free energy minimization method for this equilibrium reaction. This Gibbs' free energy will range a minimum value at equilibrium state. Gibbs free energy [**Eqn. (8)**] can be expressed in terms of enthalpy (ΔH), and entropy (ΔS).

$$\Delta G = \Delta H - T\Delta S \quad (8)$$

If the enthalpy is positive in the system, heat has to be added and when it is negative, heat must be removed from the system. When Gibbs free energy is negative, the system has the potential to work and for the positive value, requires work.

For the specific temperature and pressure, the Gibbs free energy is expressed as (Hadi Ghaebi, 2019)

$$G = \sum_{i=1}^{Nc} n_i \mu_i \quad (9)$$

Where, n_i is the number of equilibrium moles for species i and μ_i is the chemical potential

$$\text{Here, } \mu_i = \mu_i^0 + RT \ln\left(\frac{f_i}{f_i^0}\right) \quad (10)$$

μ_i is the chemical potential of component i at the reference temperature and pressure, f_i is the fugacity of the ith pure component in the reference state and f_i^o is the fugacity of component I in the mixture.

$$f_i = \phi_i \cdot y_i \cdot P \quad (11)$$

Where y_i is the molar fraction and $\emptyset_i$ is the fugacity coefficient of component i. Considering $\emptyset_i$ = 1 at high temperature and low-pressure.

Therefore, Gibbs free energy [**Eqn. (9)]** can be rewritten as

$$G = \sum_{i=1}^{Nc} n_i \cdot \left(\mu_i^0 + RT \ln\left(\frac{\phi_i \cdot y_i \cdot P}{P^0}\right)\right) \quad (12)$$

10. Biogas upgrading technologies

Biogas upgrading technologies refer to the removal of all the contaminants from the raw biogas to get biomethane in good quality. It involves multiple steps from gas separation to dry the biogas. Upgraded biogas not only reduces greenhouse gas (GHG) but also emits less hydrocarbons, carbon monoxide, and nitrogen oxide. There are many physical and chemical biogas upgrading technologies that have been developed which are mainly based on absorption, pressure swing

adsorption, cryogenic separation, and membrane separation. But all these technologies are conventional and removes all contaminants such as CO_2, H_2S, moisture, etc. Instead, microbial assisted technologies use the CO_2 together with H_2 to produce biomethane.

In this section microbial and other conventional technologies have been described.

10.1. Absorption

Absorption is the process where components of the gas-phase dissolve into the liquid phase passing through an interfacial region. In absorption, suitable solvent plays an important role, considering nonhazardous nature, volatility, etc.

The absorption process is a simple biogas upgrading method that is divided into Physical Scrubbings such as water scrubbing, organic scrubbing, and Chemical Scrubbing like amine scrubbing.

10.1.1. Physical absorption method using water scrubbing

Water scrubbing is a common process to remove CO_2 which is accounted for around 80-90% removal of CO_2 from biogas (Qie Sun, 2015). The raw biogas is flowed into the scrubber at 6-10 bars up to 40 °C from the bottom of the tank, while water flows from the top (Kui Zhou, 2017) (***Fig. 1. 9***). To ensure the maximum removal rate of CO_2 and reduce energy consumption, a packing is used in this process. The efficiency of this process depends on the solubility of CO_2 in the water phase compared to CH_4 (Kui Zhou, 2017). H_2S can also be removed using this technology due to better solubility of the gas in water than CO_2. However, H_2S is toxic so gas pre-separation is needed for it. After absorption, water-rich CO_2 desorbs from the wash-liqour by releasing the CO_2 into a strip gas at higher temperature or lower pressure than ambient. In this process, CH_4 losses due to the solubility of this gas in the water is around 3-5% theoretically (Qie Sun, 2015). However, the air stripping process also consumes some energy in the end.

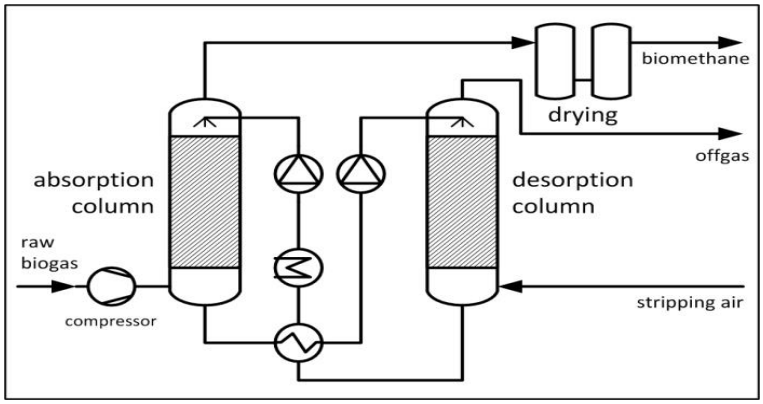

Fig. 1. 9: Activated carbon filtration coupled with water scrubbing for the upgrading of biogas (bio.methan.at, n.d.).

10.1.2. Physical absorption method using organic solvents

This process is like water scrubbing, except that an organic solvent can be used instead of water. The most common mixture of organic solvent is methanol and dimethyl ethers of polyethylene glycol. Furthermore, due to the low freezing point of the organic mixture, this process can be operated at less than -20 °C which doesn't need extra heat supply (Kui Zhou, 2017). However, the pipelines and equipment should be stainless steel to protect it from corrosion. On the other hand, the solubility of H_2S in methanol is higher than CO_2, which requires extra heat to remove and recycle the organic solvents. The higher the concentration of H_2S in the raw biogas, the more heat is required to remove it. So, it has been recommended to remove the H_2S before injecting the biogas in the solvent. In this process, at first, the raw biogas is compressed to 7-8 bars and cooled at around 20°C and the organic solvent is restored at 1 bar by heating up to 80°C (Irini Angelidaki, 2018) (***Fig. 1. 10***). The final CH_4 content using this technology is around 98% ((Irini Angelidaki, 2018).

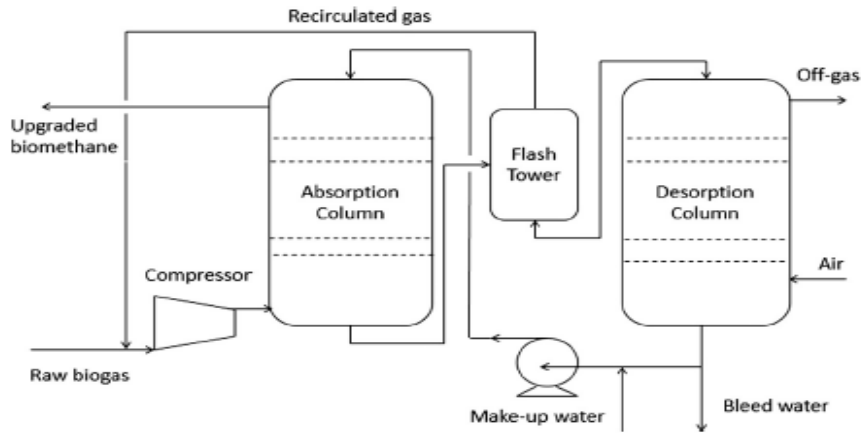

Fig. 1. 10: Simplified process flow diagram of a typical organic physical scrubbing process (Imran Ullah Khana, 2017)**.**

10.1.3. Chemical absorption method using amine solutions

The chemical absorption process consists of an absorber and a stripper where amine solution is used to bind CO_2 from the raw biogas (***Fig. 1. 11***). The advantage of this process is that H_2S gas can also be removed along with CO_2. In the column, raw biogas at 1-2 bars is supplied from the bottom of the tank while an amine solution flows from the top (Irini Angelidaki, 2018). In the plant, CO_2 collected by an exothermic reaction is directed into the stripper unit for recycling. The stripping column contains a boiler that provides a temperature of 120-160°C to break the chemical bonds and produce a vapor stream (Irini Angelidaki, 2018). The vapor stream is finally condensed and cooled, containing the CO_2 to be released.

There are some disadvantages of this process, such as toxicity of the solvents which is harmful to the human body and the environment, the heat required to restore the chemical solution, the high cost of amine solution and loss of the solution during evaporation. Therefore, aqueous alkaline salts are preferred rather than amines to minimize the effect (M. Yoo, 2013). Using this technology 99% methane content can be achieved (Irini Angelidaki, 2018).

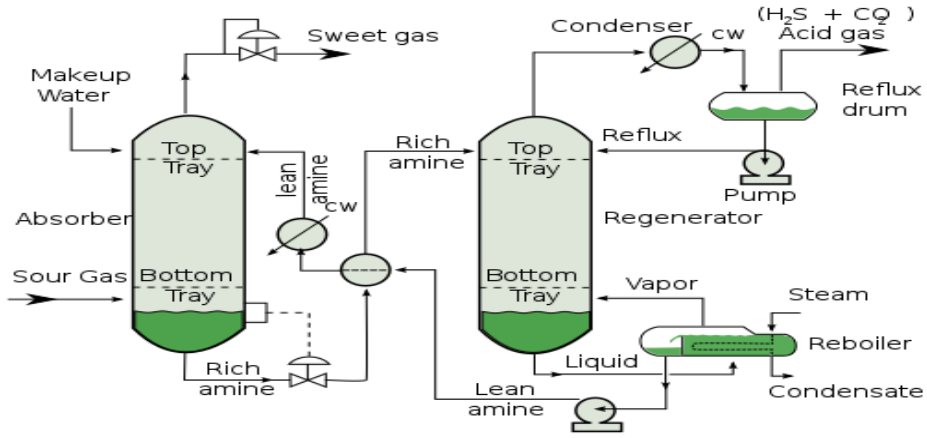

Fig. 1. 11: Amine gas treatment process (Raminagrobis, n.d.)**.**

10.1.4. Pressure swing adsorption

The pressure swing adsorption process (PSA) is based on the mechanisms to separate gas according to the molecular size and the affinity of the gas to the sorbent material. CH_4 molecules are larger in size than CO_2 or N_2, so PSA technology can be used to disperse CH_4 from other contaminated gas. This technology depends on the properties of pressurized gas. High pressure is recommended in the adsorption process, on the other hand, decreased pressure will release the gas. PSA is divided into four different steps which are adsorption, blow-down, purge and pressurization (Irini Angelidaki, 2018). At first, raw biogas at 4-10 bars is injected into the column from where all the unwanted gases are entrapped in the adsorbent except CH_4 and methane is collected from the top of the tank (***Fig. 1. 12***). When the adsorbent is saturated, the gas will flow into the next column, while the adsorbent is regenerated by the desorption process and captured gas is unconfined. The released gas mixture contains methane. However, the adsorption of H_2S is irreversible in this technology, therefore H_2S must be removed prior to the injection of the gas into the adsorbent column (Qie Sun, 2015). The advantages of this method are low investment, equipment compactness, safety, and simplicity. Using this method, up to 96% of methane collection can be possible. (Irini Angelidaki, 2018).

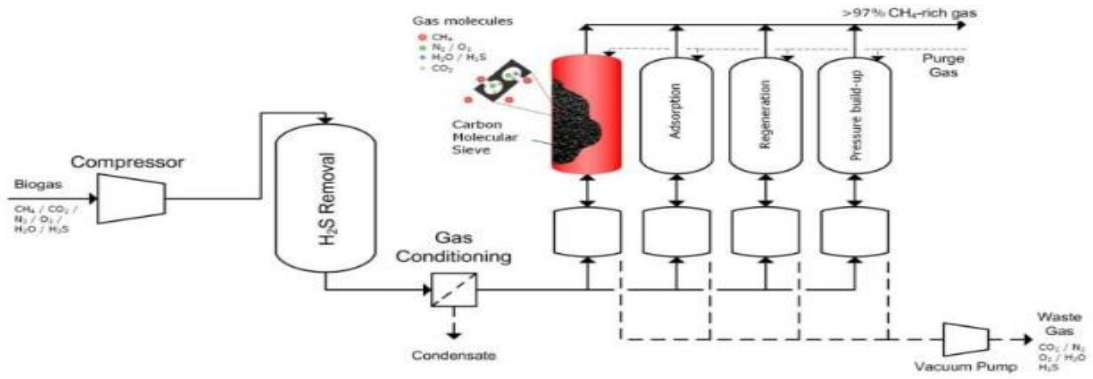

Fig. 1. 12: Pressure swing adsorption process diagram (Matthew D. Ong, 2014).

11. Microbial assisted biogas upgrading technologies

The biological process divides into an in - situ and ex-situ process which is performed by an external reactor under hydrogenotrophic methanogens. In an in-situ methanation process, hydrogen and organic substrate are added into the digester to produce biogas. On the contrary, in an ex-situ process hydrogen, nutrients and hydrogenotrophic methanogens are used as external sources.

11.1. In-situ biological upgrading technology

In this process, H_2 is injected into the reactor which reacts with CO_2 to produce CH_4 by the action of methanogenesis. The in-situ process can generate up to 99% CH_4 if it is controlled under an optimal condition, i.e., pH. The increasing pH can help to remove bicarbonate, which is produced when CO_2 is dissolved in the liquid phase during digestion [**Eqn. (13)**]. From the following reaction, it has been shown that CO_2 reacts with the liquid phase and dissociates in H^+ and HCO^{3-}. Therefore, CO_2 can decrease the H^+ and establish an optimum pH for the fermentation process.

$$H_2O + CO_2 \leftrightarrow H^+ + HCO^{3-} \quad (13)$$

For the in-situ process, a pH of 8.5 is the optimum parameter for both thermophilic and mesophilic operation, which can be maintained by co-digestion with acetic acid (Irini Angelidaki, 2018). To solve this problem, the co-digestion of manure with whey wastewater can maintain the optimal pH in the biogas upgrading process. Moreover, there is a possibility of oxidation of VFA and alcohol if the concentration of H_2 in the reactor is very low (D.J. Batstone, 2002). On the other hand, the high amount of H_2 can degrade VFA to generate lactate, ethanol, etc. For this reason, the process can be imbalanced or damaged due to excess acidification. Therefore, injecting the right concentration of hydrogen can make this methanogen process effective to upgrade biogas.

11.2. Ex-situ biological upgrading technology

The ex-situ process is based on the theory that the injection of CO_2 and H_2 from an external source into the reactor to generate CH_4 (***Fig. 1. 13***). This method has numerous advantages over the in-situ process, such as stability of the biogas upgrading process, biomass autonomous process, no degradation of an organic substance, etc. The efficiency of this process can be from 79% to 98% depending on the biogas reactor (Irini Angelidaki, 2018). However, ex-situ technology has higher H_2 consumption rates than in-situ. It has been examined that thermophilic temperature can convert 60% of H_2 and CO_2 into bio methanation than mesophilic (G. Luo, 2012). Besides, time is required to increase the density of microorganisms, so that the efficiency could be higher by fermenting CO_2 and H_2 gases.

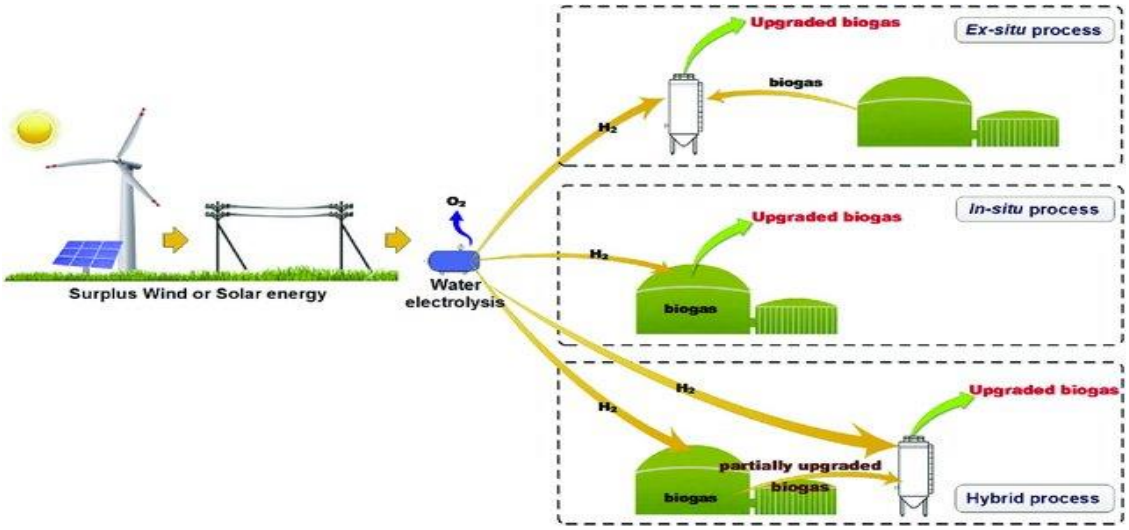

Fig. 1. 13: In-situ, ex-situ and hybrid biological biogas upgrading technologies (Irini Angelidaki, 2018)**.**

12. Material and methods

Enrichment experiments of HM have been described in this part. The experiment was carried out for two months and data collected on everyday basis. The outcome from the experiment has been described in the result and analysis part.

12.1. Reason to choose ex-situ method

The in-situ process gives the opportunity to implement the AD process without the post gas treatment where the H_2 gas is injected directly into the reactor. But it could lead to the methanogen's low performance and degrade VFA especially when the concentration of CO_2 is low. However, a recent study has shown that H_2 gas can penetrate only less than 1mm saturation

into the active microorganisms which indicates that a small portion of HM will uptake the H_2 in the process (Nabin Aryala). However, the consumption of H_2 via homoacetogenesis using CO_2 has less impact on the substrate pH because the removal of two carbonic acids is somehow balanced by the production of one acetic acid [**Eqn. (3)**]. But the limitation of carbon- source could inhibit the target to reach the CH_4 production and mass transfer might be affected for the availability of the other by-products.

On the other hand, in the ex-situ process, both substrates are injected from the outside as an external source which is demanding for extra volume and less production of CH_4 than in-situ process. But a research group has studied the different reactor types with the ex-situ process, and they have ended up with a result from a minimum 89% to 99% CH_4 production. It was noticeable that increasing pH in this method did not inhibit the methanation process, which is indicating that the adjustment capability of microorganisms is highly flexible (Nabin Aryala). Considering all of these advantages the ex-situ process has been chosen over the in-situ process.

12.2. Inoculum

The inoculum used in this project was collected from the Fangel Biogas plant and it was a mesophilic biogas plant. After collecting the inoculum all the solid parts were removed to separate the liquid part. Then the liquid part was kept for three days to ensure methane production in the liquid. Afterward, the liquid was sieved for one more time before using in the experiment to remove any unwanted solid particles.

12.3. Packing material

In this project, a packing material was used, is consists of polyurethane foam (PUF) which has a surface area of 600 m^2 m^{-3}.

12.4. Experimental procedure

This project was a fed-batch process that had been done on a lab-scale. There were five different samples that were duplicated and prepared with biomass from a biogas plant digested feedstock, garden sediment, cattle manure and mineral medium. The aim of this experiment was to enrich methanogens to increase the efficiency of the conversion rate of CH_4 by using H_2 and CO_2. A flow chart of the whole process has been added in **Table 2**.

12.4.1. Phase 1 (First cultivation)

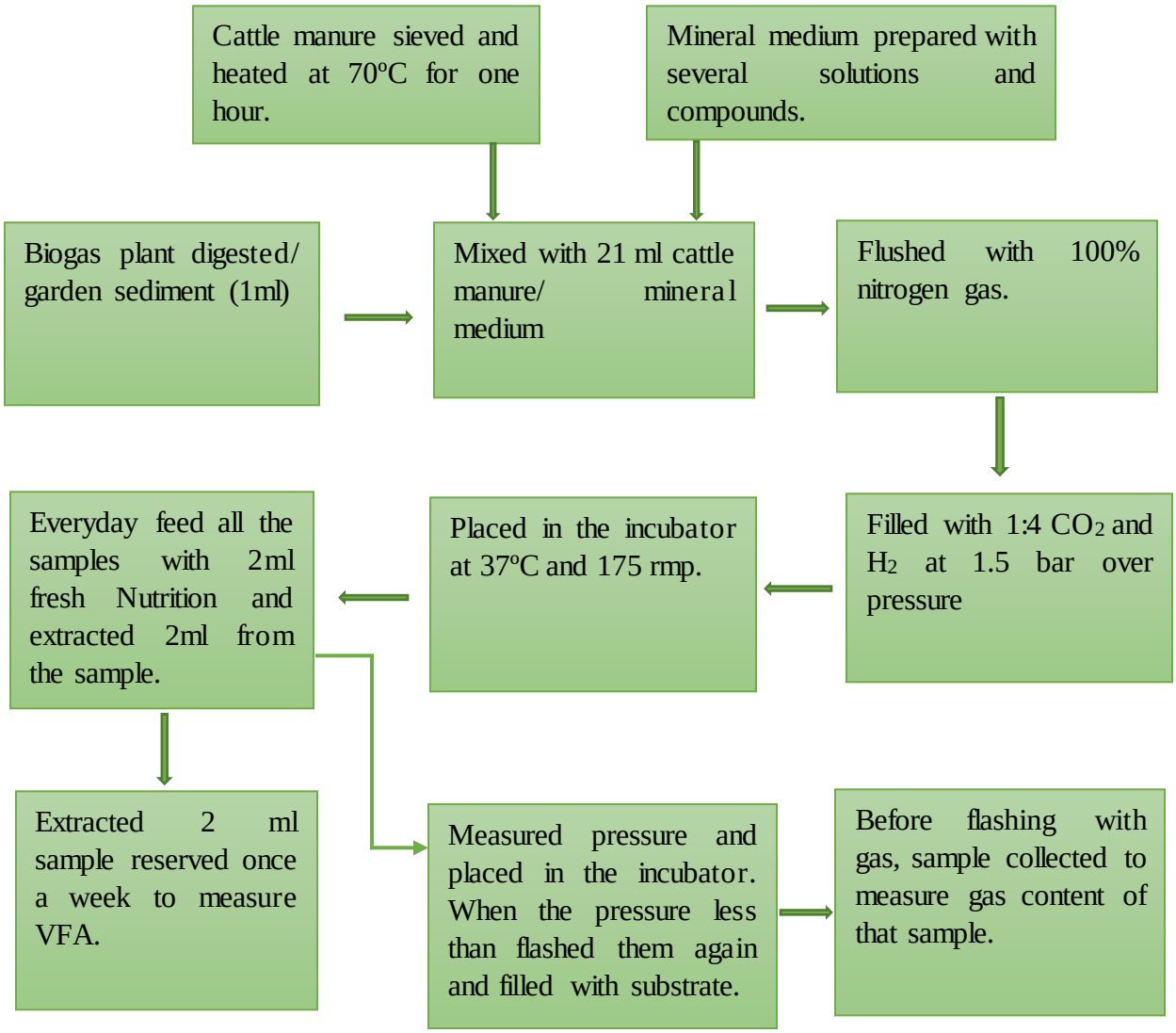

Table 2: Flow chart of the experiment.

From the flow chart (***Table 2***), at first 250ml mineral medium which was a mixture of five different solutions and other solid compounds was prepared. Cattle manure from biomass plant was filtered and heated at 70ºC with 260 rpm for 1.5 hours in 500 ml beaker. Then, five mini reactors were taken, and 1 ml of inoculum and 21 ml of mineral medium were transferred into a 110 ml bottle. Afterward, all the samples were flushed with 100% nitrogen for around 3 to 4 minutes and filled with 80 % H_2 and 20% CO_2 with overpressure (1.5 bar). Then the samples were placed in an incubator at 37ºC with 175 rpm. In one of the samples, there was packing material as a filling. Every-day, 2 ml liquid medium was discarded from all the samples and 2 ml fresh medium was added. The discarded medium was preserved to test the VFA. Pressure drop was noted using a manometer every-day and when the pressure became stable, all the samples were flushed and filled again. Before flushing, gas samples were collected and preserved to test GC.

All the five samples were duplicated, therefore 10 samples were prepared for this project. This phase one culture process was run for one month.

The started-up procedure has demonstrated in ***Fig. 1. 14*** and a list has been added below (***Table 3***) mentioning the position and mixture of inoculum and medium of all samples.

Table 3: Overview of all the samples with position, inoculum, and medium

Sample Name	***Inoculum***	***Nutrition***	***Position in the incubator***	***Filling material***
S	*Biogas plant digest*	*Cattle manure*	*Upright*	*Without*
D	*Biogas plant digest*	*Cattle manure*	*Horizontal*	*Without*
F	*Biogas plant digest*	*Cattle manure*	*Upright*	*With*
L	*Garden Sediment*	*Cattle manure*	*Upright*	*Without*
WC	*Biogas plant digest*	*Mineral medium*	*Upright*	*Without*

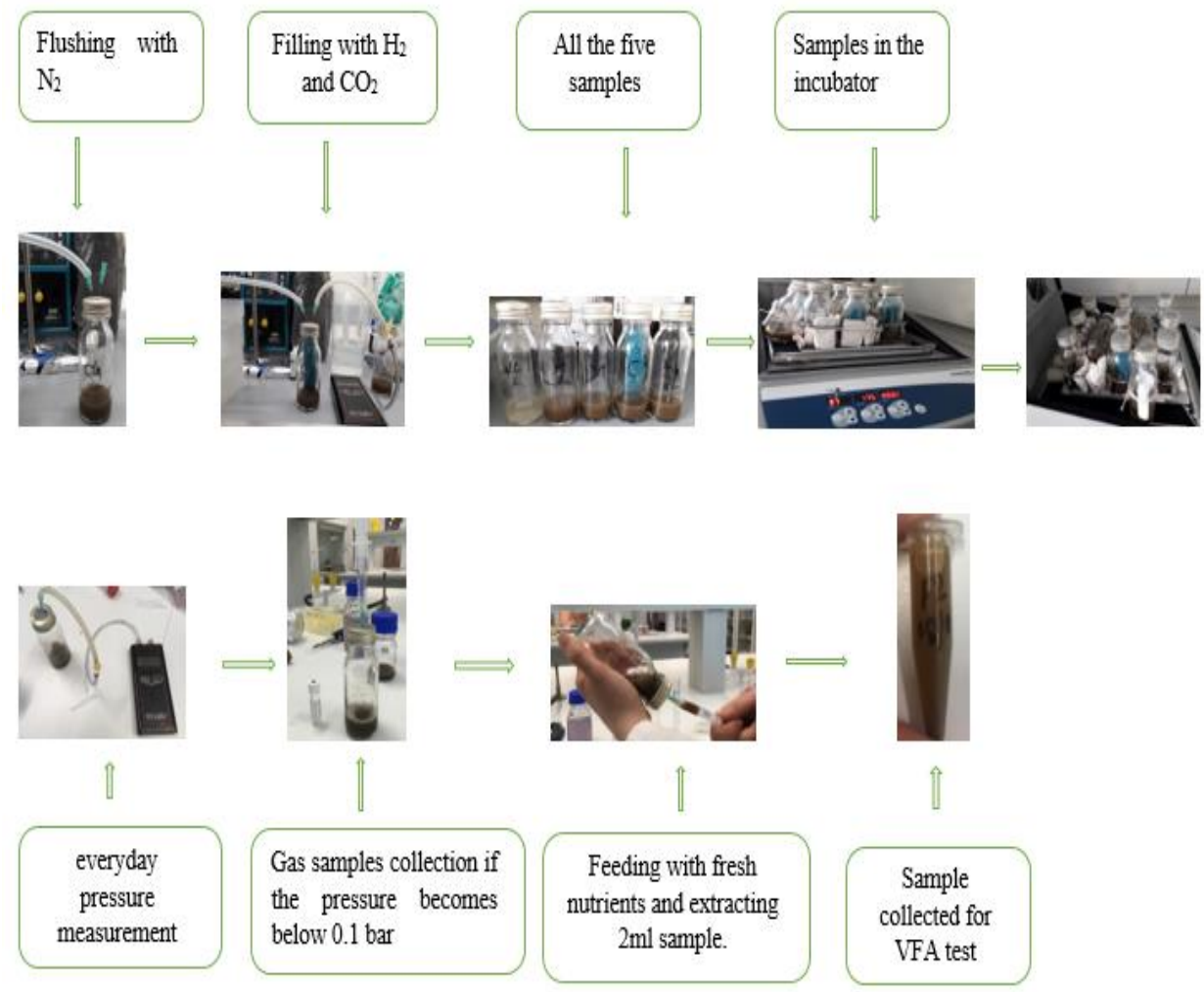

Fig. 1. 14: Overview of the experiment.

12.4.2. Phase 2 (Re-cultivation)

After one month, all the samples were re-cultivated. For the re-cultivation 1 ml sample was taken from old samples and transferred into a new reactor. In this way, 10 new reactors were prepared with mineral medium and cattle manure as done in phase 1. Then all the samples were flushed with 100% N_2 and filled with 80% H_2 and 20% CO_2. Afterward, samples were placed in the incubator following the position as they were in phase 1. Every day, the pressure drop was measured, and 2 ml of the sample was discarded, and 2 ml of the fresh sample was added. In this way, the data were collected for around one month. The collected liquid samples from the discarded liquid were analyzed and the conversion rate of the gas sample as well.

12.5. Mineral medium preparation

A list of all the solutions and ingredients to prepare the mineral medium is given below in **Table 4.** Preparation of A, B, C, D, and E solution has been added in the appendix.

Table 4: Solution and ingredients to prepare mineral medium

Solution / ingredients	***Volume / weight***
A	*10 ml*
B	*2 ml*
C	*1 ml*
D	*1 ml*
E	*1 ml*
yeast	*2 g*
Trypticase	*2 g*
Cysteine	*0.5 g*
$NaHCO_3$	*2.6 g*
$Na_2S.9H_2O$	*25 mg*
Water	*974 ml*

12.6. VFA measurement

The concentration of VFA was examined on gas-chromatograph (GC) (7890B, Agilent Technologies, Santa Clara, CA, USA) with a flame ionization detector and 30 m x 0.25 mm x 0.25 µm column (HP-INNOWax, Agilent Technologies, Santa Clara, CA, USA).

To analyze VFA, 1ml of the sample was weighed and 10% phosphoric acid (purity 85%) was added into it. Then the samples were centrifuged for 20-25 minutes and filtered before storing in a 2 ml vial for the VFA analysis.

12.7. Gas content measurement

The gas vials from the experiments were analyzed on a gas chromatograph (GC) (7890A, Agilent Technologies, Santa Clara, CA, USA) which was equipped with a thermal conductivity detector and a 30m x 0.53mm column (Carboxen® 1010 PLOT, Fused Silica Capillary Column, 30m x 0.53mm).

13. Calculation

In the reactor, volumes and molar amounts of H_2, CO_2 and N_2 injected were determined as shown below.

Mass balance over the system:

The reaction:

$$CO_2 + 4\,H_2 \rightarrow CH_4 + H_2O \qquad (14)$$

V_0 = Volume of gas space

$V_{reactor}$ = Volume of the whole mini reactor =110 ml

V_{sample} = Volume of the sample in the reactor = 21 ml

$V_0 = (110 - 21)\ mL = 0.089\ L$

Calculation of the total injected volume:

$V_{total} = V_0 * (P_{inj} - P_{atm}) = 0.089\ L * (1.5\ bar - 1\ bar)$ =0.044592 L =44.5 ml

P_{inj} = The injected pressure after flushing

P_{atm} = Atmospheric pressure.

Calculation of the injected volume of CO_2 and H_2 in the reactor

$V_{H_2} = V_{CO_2H} * \frac{4}{5} = 35.6$ ml

$V_{CO_2} = V_{CO_2H} * \frac{1}{5} = 8.9$ ml

Calculation of the injected molar amount:

$V_{standard} = 22.4L$

T_0 = The temperature at 0 ºC

T_{20} = The temperature at 20 ºC

n_H = Injected molar amount in H_2

$n_H = \frac{V_{H_2}}{V_{standard}} * \frac{T_0}{T_{20}}$ =0.0014 moles = 1.48 milimoles

n_{CO2} = injected molar amount of CO_2

$n_{CO_2} = \frac{1}{4} * n_H = 0.00037$ moles= 0.37 mili moles

The molar amount of nitrogen with a ration 5:4:1 (N_2: H_2: CO_2)

With a 100 % conversion of the hydrogen to methane:

The molar amount of methane

$n_{CH_4} = \frac{1}{4} * n_H = 0.00037$ moles = 0.37 mili moles

GC Calculation

In the gas content, there were O_2, CO_2, N_2, and CH_4. From the area of the gas total area and new area have been calculated. Then a response factor [**Eqn. (15)**] was calculated to estimate the percentage of the area of CH_4 and CO_2 without nitrogen and oxygen.

Total area = ($^{A}CH_4$ + $^{A}CO_2$ + $^{A}N_2$ + $^{A}O_2$)

New area = Total area – ($^{A}O_2$ + $^{A}N_2$)

$$\text{Response factor } f = \frac{Total\ area}{New\ area\ (without\ nitrogen\ and\ \text{oxygen})} \quad (15)$$

New area for CH_4= Area of CH_4 from GC * factor

New area for CO_2= Area of CO_2 from GC * factor

Comparing with the standard curve, the volume of CH_4 and CO_2 were calculated and then the percentage of contents following the **Eqn. (16)** and **Eqn. (17).**

$$\%CH_4 = \frac{\%\text{GC CH4}}{(\%\text{GC CH4} + \%GC\ H2 + \%\ GC\ CO2)} \quad (16)$$

$$\%CO_2 = \frac{\%\text{GC CO2}}{(\%\text{GC CH4} + \%\ \text{GC H2} + \%\text{GC CO2})} \quad (17)$$

14. Results

In this section, the results from the GC and VFA have been described. VFA results of the cattle manure have been added in ***Table 5***. However, there is no VFA in the mineral medium.

As all the five samples were duplicated, so results have been made based on the average of the respective samples. From the GC result, it has been seen that there was no H_2 present in any reactors.

Table 5: Concentration of VFA in cattle manure

Name of the acids	***Conc. (g/L)***
Acetic acid (AA)	*6.23928298*
Propionic acid (PA)	*1.28179248*
Isobutyric acid (iso BA)	*0.22880698*
Butyric acid (BA)	*0.62654731*
Isovaleric acid (iso VC)	*0.23192925*
Valeric acid (VA)	*0.065729*

The result is discussed in two parts for two phases. To make samples comparable, sample S was considered as a reference sample which is with biogas plant digested feedstock and cattle manure in a standing positing in the incubator. So, at first, the reference scenario was elaborated and related to the other four samples according to position, filling material, inoculum, and nutrients to investigate the influence of these parameters on biomethane production.

Both GC and VFA results have demonstrated for all the samples. In the VFA test, there were six different acids (***Table 5***) in every sample where the concentration of ethanoic acid was higher compared to others. In the GC result, the percentage of CH_4 and CO_2 were calculated based on the volume of 89 ml which was the vacant space of the reactor i.e., 110 ml was the total volume of reactor and 22ml was the solution.

14.1. First phase

Reference scenario

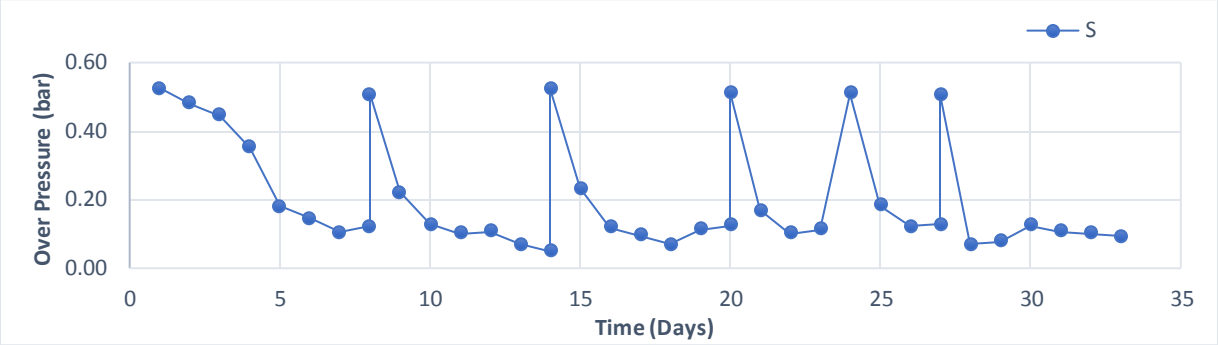

Fig. 2. 1: Overpressure (bar) of reference scenario S over 33 days.

The first phase was carried out for 33 days. ***Fig. 2.1*** is showing the overpressure of sample S over the period which was with biogas plant digested inoculum and cattle manure. After one- or two-days, pressure dropped when methanogens started producing biomethane using H_2 and CO_2.

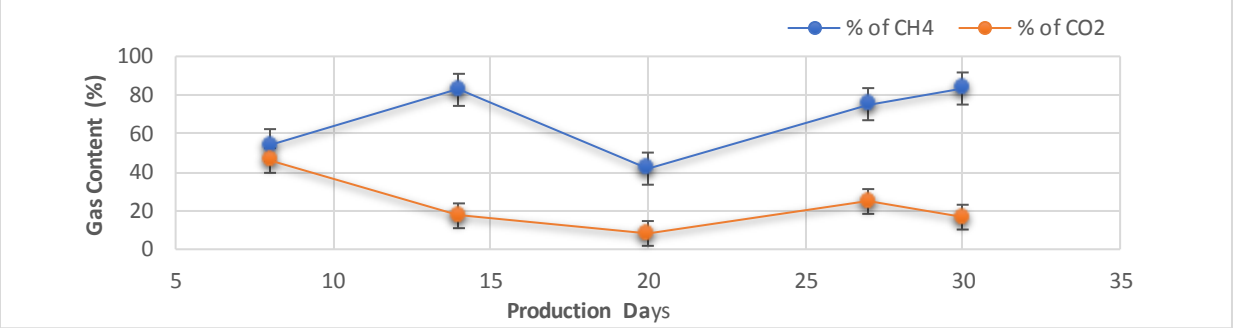

Fig. 2. 2: Gas composition of reference scenario S in the first enrichment.

From ***Fig. 2.2***, it is noticeable that the percentage of methane was initially a small amount. This is possible because of the low density of hydrogenotrophic methanogens. More specifically it can be said that due to high concentration H_2 and CO_2 were producing more ethanoic acid via homoacetogenesis. On day 20 the methane production became very low which could have happened due to the experimental error because the concentration of VFA did not increase (***Fig. 2. 2***) at the same time but decreased. Moreover, the percentage of CO_2 fluctuated with methane production.

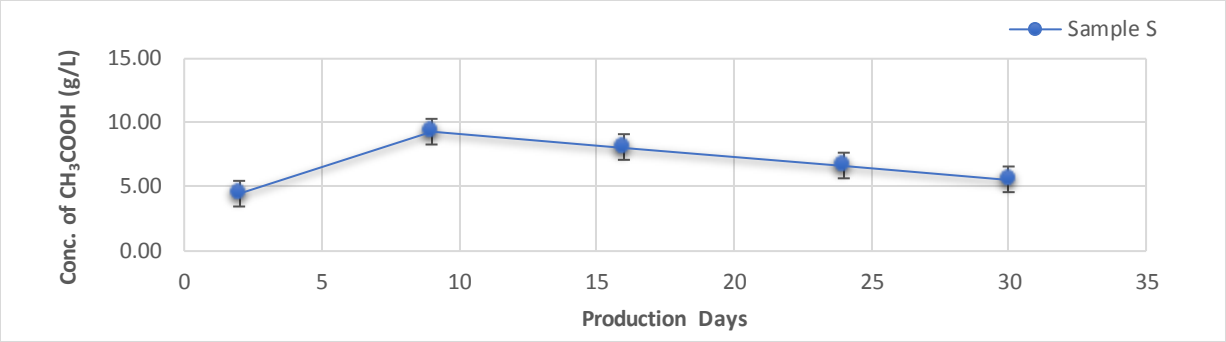

Fig. 2. 3: Acetic acid concentration of the reference scenario S in the first enrichment.

Fig. 2.3 is indicating that between 1 to 10 days the concentration of CH_3COOH was high and then it decreased which demonstrates that the production of acetogenic methanogens declined and reaction shifts to the hydrogenotrophic methanogens. So, the overall percentage of methane from this reactor was more than 80% in the end.

Comparison between position

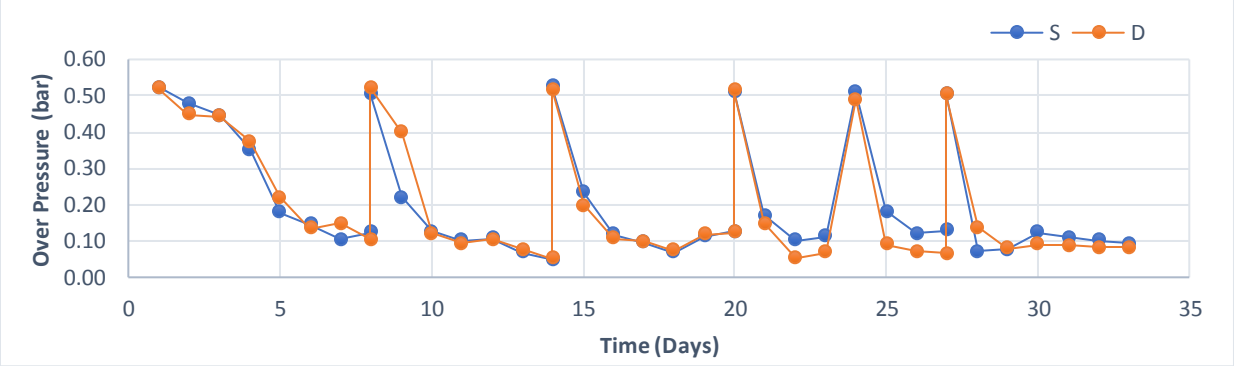

Fig. 2. 4: The overpressure scenario influence on the standing (S) and horizontal position(D) of the samples in the incubator.

Sample D and reference sample S both were with similar nutrition and inoculum, but their positions were different in the incubator where sample D was in the laying down and sample S was standing. ***Fig. 2.4*** is showing the everyday pressure scenario of both samples.

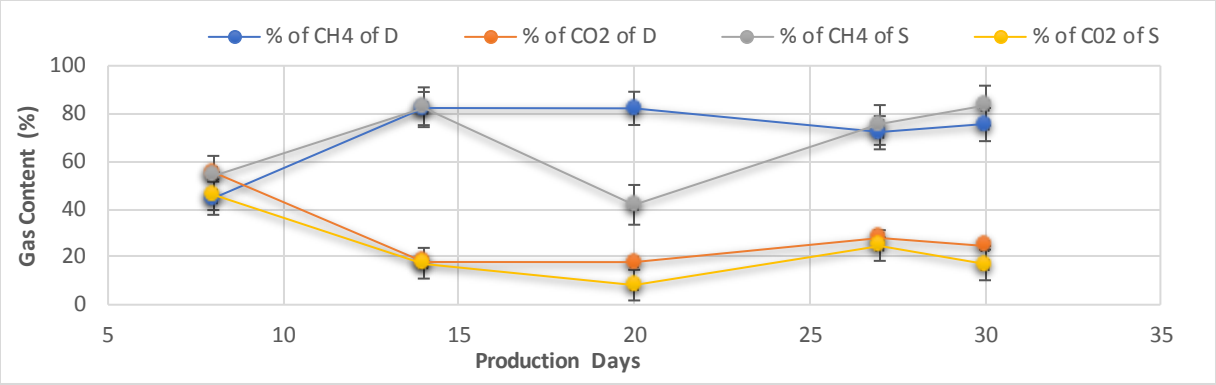

Fig. 2. 5: Comparison of GC results between the different positions of sample D and reference sample S.

Due to the horizontal position of sample D, it had more surface area for which it could produce more hydrogenotrophic methanogens than the reference sample. However, ***Fig. 2.5*** is indicating the opposite scenario. The percentage of methane content in sample D was around 78% which was less than the reference sample. Although it did not fluctuate like sample S over the period.

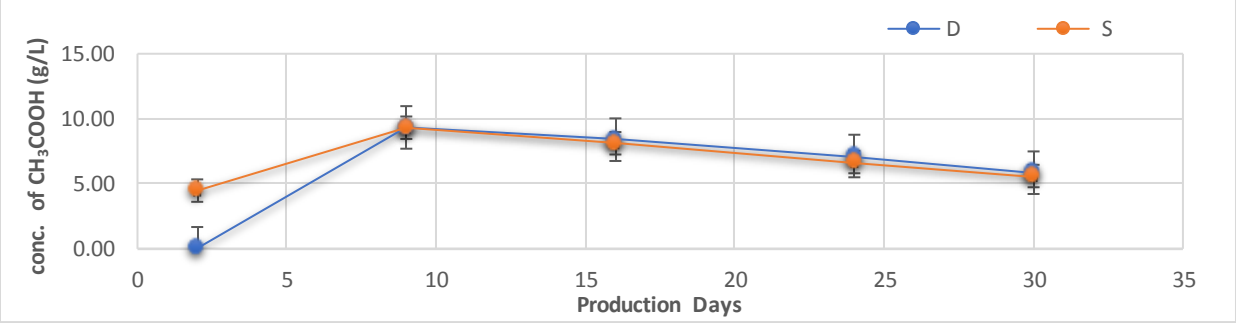

Fig. 2. 6: Comparison of acetic acid concentration between the different positions of sample D and reference sample S.

Sample D was a mixture of cattle manure which contains 6.24 g/L acetic acid. So, it had high production initially which could mean that acetic acid was produced via homoacetogensis methanogens and methane was produced via acetoclastic methanogens. From ***Fig. 2. 6***, the trend of sample D representing the concentration of ethanoic acid increased at first and decreased after 10 days.

Comparing with the reference sample, it can be concluded that acetoclastic methanogens did not inhibit the enrichment of the hydrogenotrophic methanogens in sample D.

Comparison based on filling material

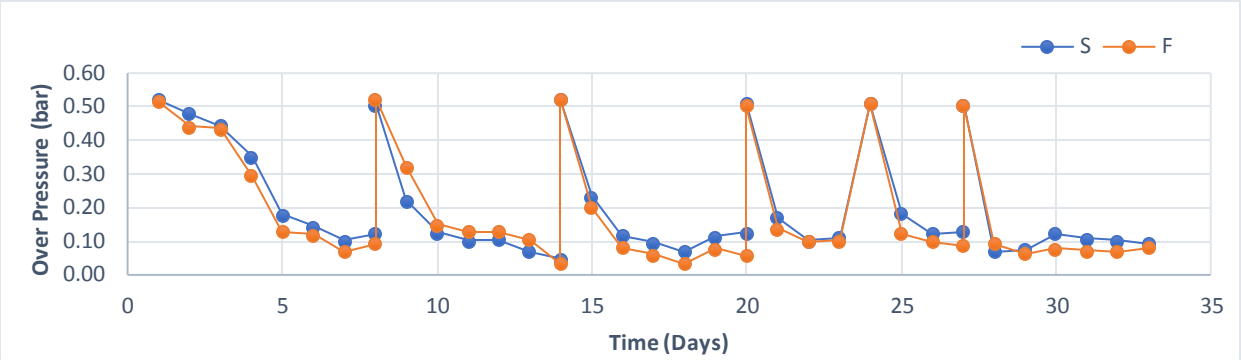

Fig. 2. 7: The overpressure scenario influenced using a filling material (F) and without filling material (S) in the sample.

A pressure dropped scenario of samples with filling material and without filling material is showing in ***Fig. 2.7***. Although sample F and the reference sample S both had a similar inoculum and medium, sample F contained a filling material inside it, for which the microorganisms got more moisture and surface area.

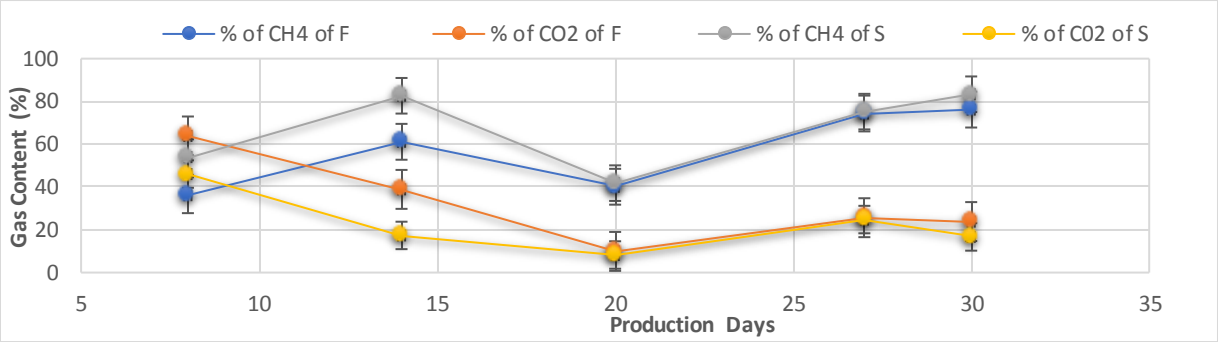

Fig. 2. 8: Comparison of GC result between with and without filling material in the sample F and reference sample S.

Therefore, it was expected to get a high percentage of gas content in sample F with filling material than the reference sample. In ***Fig. 2.8***, the curve for the sample F (with filling material) shows a lower percentage of methane production than the reference. Moreover, it was not stable over the period where it increased after 20 days but still lower than the reference sample.

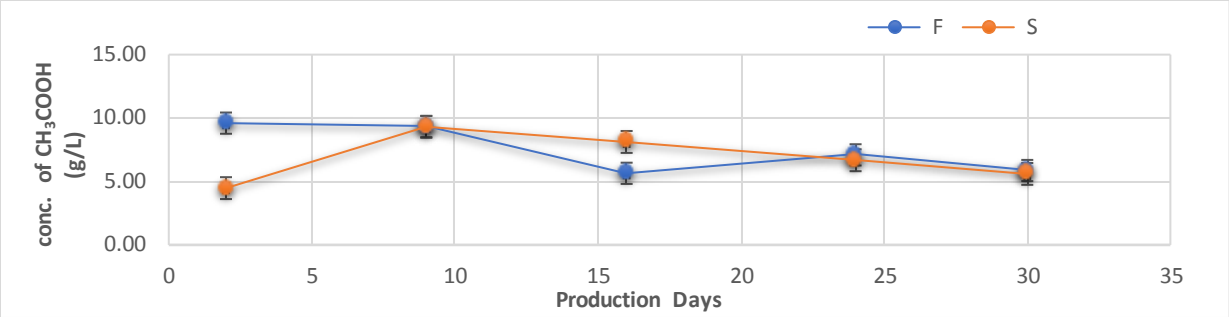

Fig. 2. 9: Comparison of acetic acid concentration between the sample F with filling material and without filling material reference sample S.

Low methane production in sample F was observed, perhaps due to the high concentration of VFA in the reactor, which is visible from ***Fig. 2.9***. Moreover, the concentration of VFA was projected to decrease after 10 or 15 days but it fluctuated in sample F with the filling material.

Comparison between two different inoculums

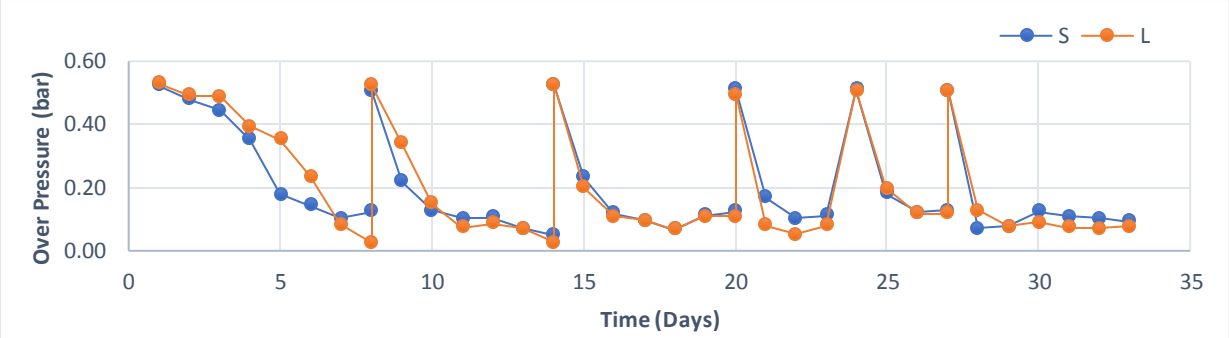

Fig. 2. 10: The overpressure scenario influenced by the Inoculum from the garden sediment (L) and fangel biogas plant(S).

Fig. 2.10 is showing pressure drop overview of the samples with two different inoculums. In sample L, garden sediment was used as an inoculum which was not processed like the reference sample S with biogas plant digested. So, it could be assumed to have higher methane production rate from the reference sample S than the sample L with garden sediment.

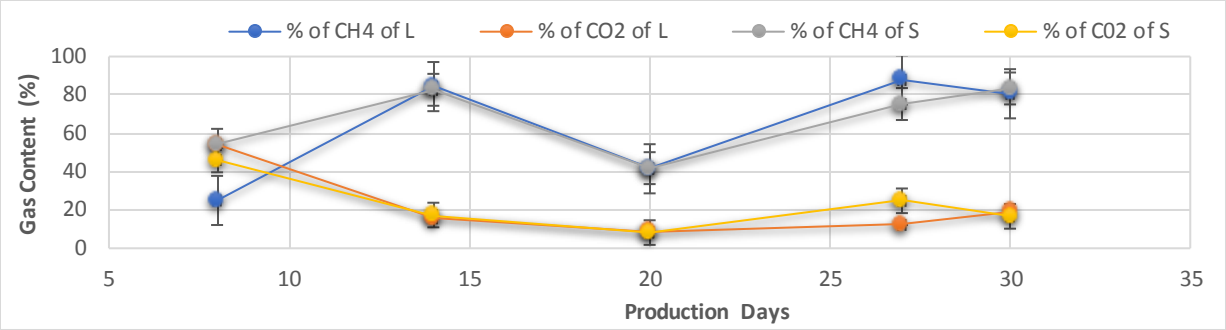

Fig. 2. 11: Comparison of GC result between the use of two different inoculums in the sample L and reference sample S.

Fig. 2.11 illustrates that the reference sample had a high percentage of methane production on day 14, which declined afterward and reached around 42% CH_4 on day 20. Nonetheless, at the end of the phase, it showed more than 80% CH_4 content which was comparable with the reference sample.

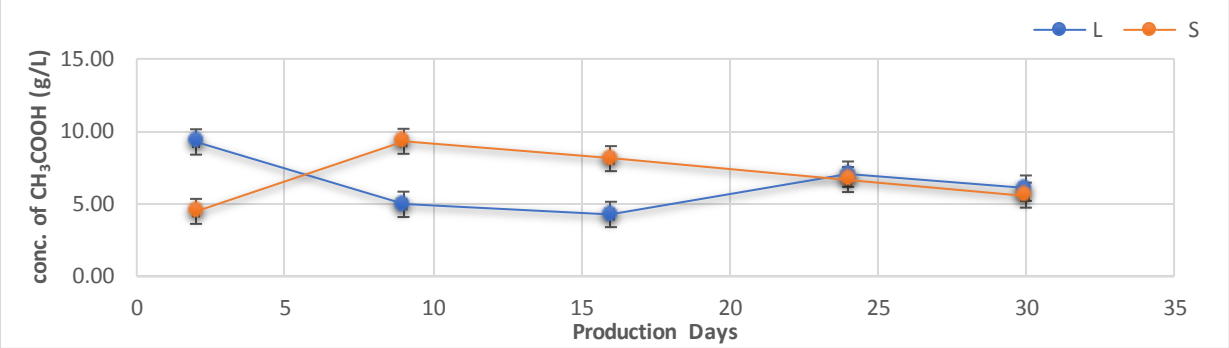

Fig. 2. 12: Comparison of Acetic acid conc. between the use of two different inoculums in the sample L and reference sample S.

For the unprocessed inoculum in sample L, it was assumed that the conc. of VFA will be higher than the reference sample. The trend of sample L in ***Fig. 2.12*** is showing a fluctuated result of ethanoic acid in the cycle as it was expected. Nevertheless, it was still lower than the reference sample. Comparing between GC and VFA results of sample L it is difficult to relate the density population of acetoclastic and hydrogenotrophic methanogens as well as the methane content. It can not be resolved that the production of gas was inhibited by the acetoclastic microorganisms.

Comparison between nutrition

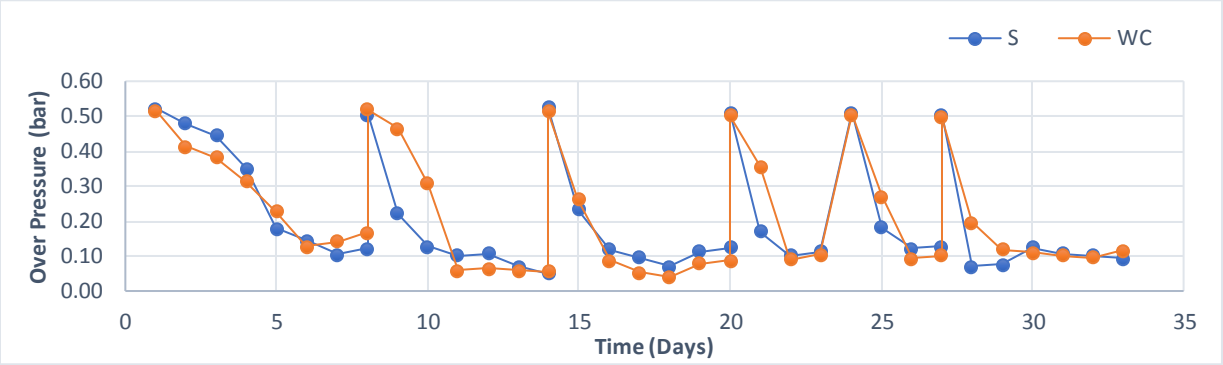

Fig. 2. 13: The overpressure scenario influenced by the nutrition of the sample with cattle manure (WC) and mineral medium (S).

The scenario of pressure drop of the samples with cattle manure and mineral medium is shown in ***Fig. 2.13***. The nutrition of sample WC was a mineral medium where the reference sample was the cattle manure while the inoculum was similar in both samples.

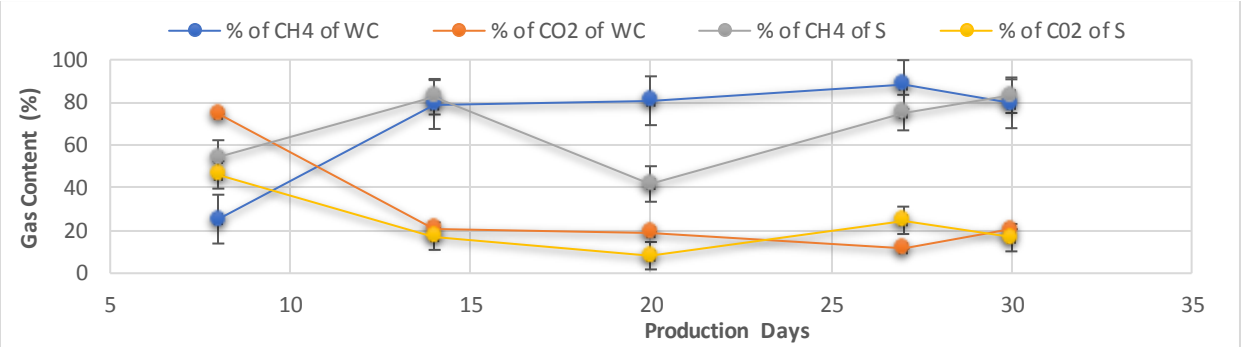

Fig. 2. 14: Comparison of GC result between the use of different nutrients in the sample WC and reference sample S.

The percentage of methane in sample WC was low which is 20% at first, and it increased gradually after 10 days. It formed more than 80% methane at the end of the cycle which was higher than the reference sample (***Fig. 2. 14***). More importantly, it did not fluctuate like the reference sample.

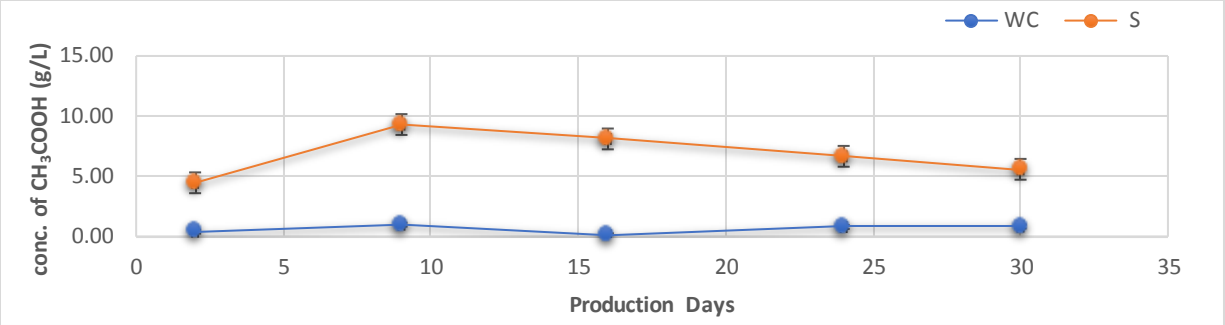

Fig. 2. 15: Comparison of acetic acid concentration between the use of different nutrients in the sample WC and reference sample S.

Additionally, sample WC was made of the mineral medium which did not have any VFA from the nutrients but from the inoculum. ***Fig. 2.15*** demonstrates that there was a huge difference between the concentration of ethanoic acid in sample WC and reference sample S. It could be assumed that the mineral medium of sample WC did not accumulate VFA in the reactor for which the production of the hydrogenotrophic methanogens, as well as the gas content, did not hamper.

14.2. Second phase (Re-cultivated)

The second period was carried out to compare with the enrichment of hydrogenotrophic methanogens with phase one. The faster growth of hydrogenotrophic methanogens was noticeable in the second phase.

Reference scenario

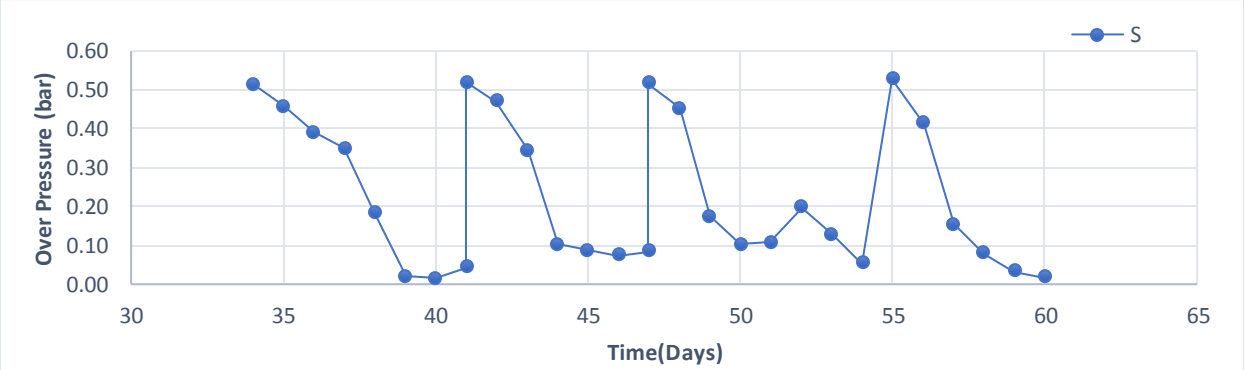

Fig. 3. 1: The overpressure scenario of the reference sample S.

Fig. 3.1 is illustrating the overview of pressure drop in reference sample S which was with biogas plant digested inoculum and cattle manure as nutrition.

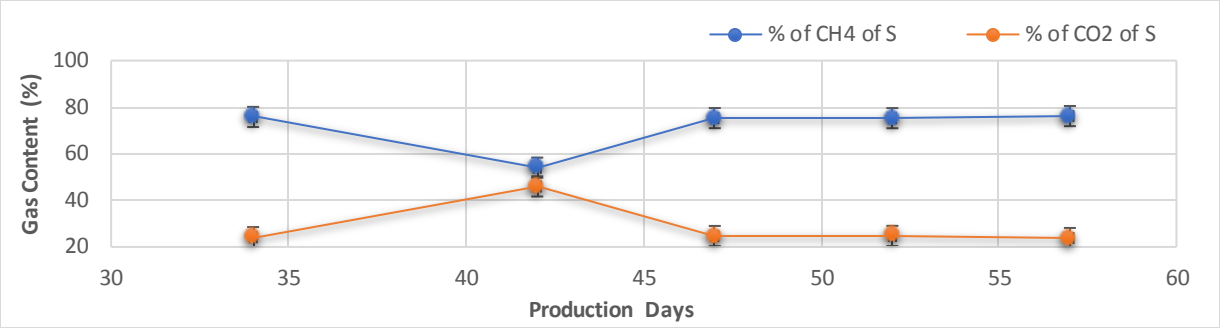

Fig. 3. 2: GC result of the reference sample S in the second enrichment.

In the second phase, the methane content was average overall 76% (***Fig. 3. 2***). After day 40 it decreased to 60% which could be addressed by the presence of another microorganism rather than hydrogenotrophic methanogens in the reactor. It started increasing again after day 45 and reached the maximum amount of methane content. Depending on the percentage of methane, it is assumed that the density population of hydrogenotrophic methanogens was higher in the second phase than the first phase (***Fig. 2. 2***).

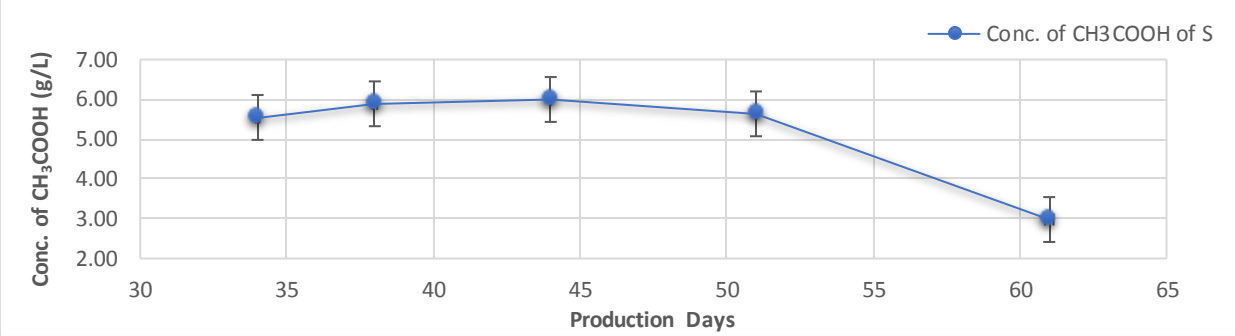

Fig. 3. 3: Concentration of acetic acid of the reference sample S in second enrichment.

The concentration of ethanoic acid (***Fig. 3. 3***) was also lower than in the first cycle and became around 3 g/L at the end which was 6 g/L in the first phase (***Fig. 2. 3***).

Comparison between position

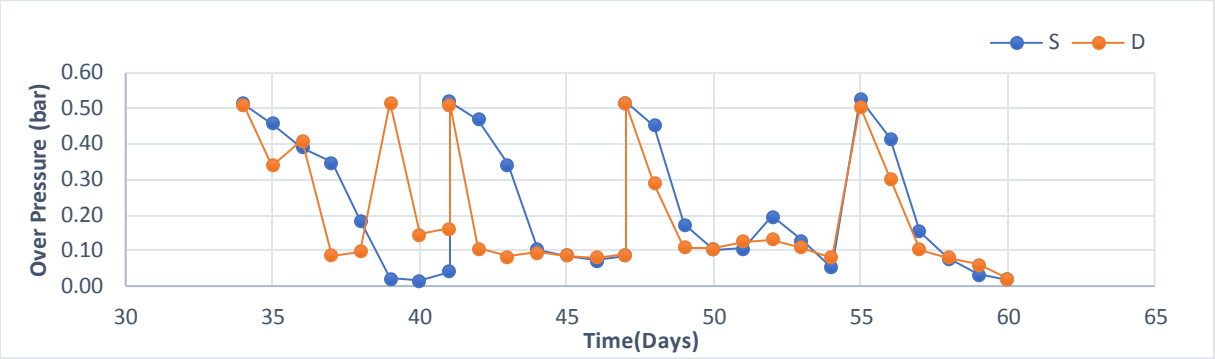

Fig. 3. 4: The overpressure scenario influence on the standing and horizontal position of the samples in the incubator.

Fig. 3.4 is demonstrating the scenario of pressure drop, based on the comparison of reference sample S in the standing position and sample D in laying down position in the incubator.

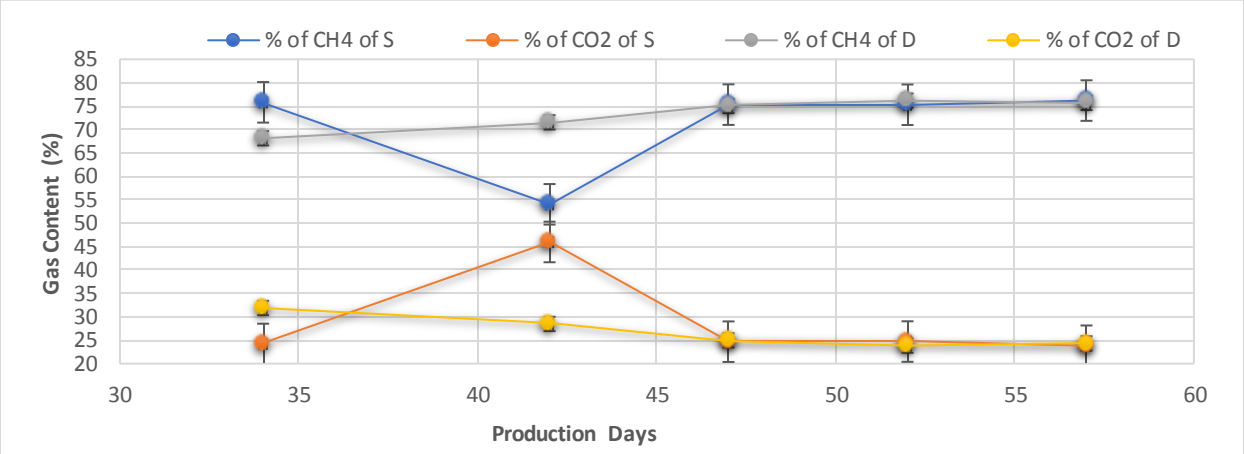

Fig. 3. 5: Comparison of GC result between the different positions of sample D and reference sample S.

The enrichment of hydrogenotrophic methanogens in the re-cultivation stage was assumed to faster in sample D for the higher surface area than the reference sample which can be verified from the percentage of methane content (***Fig. 3. 5***). Though sample D was stable and increased gradually in the whole cycle, the percentage of methane content was 75% at the end of this phase which was less than the first phase (***Fig. 2. 5***) but similar compared with the reference sample.

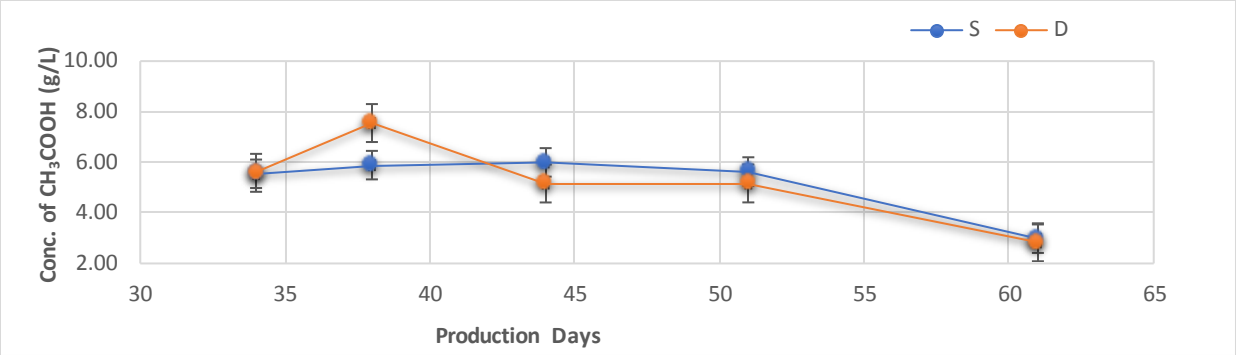

Fig. 3. 6: Comparison of acetic acid concentration result between the different positions of sample D and reference sample S.

From the VFA result (***Fig 3.6***) it is shown that initially, the concentration of CH_3COOH increased to more than 7 g/L due to homoacetogenesis. Though it reduced after 40 days which was less than even the reference sample.

Comparison between operation with and without filling material

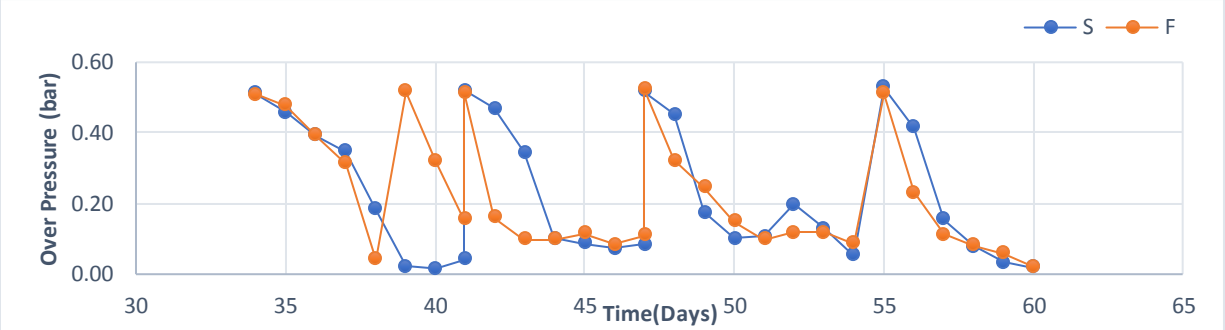

Fig. 3. 7: The overpressure scenario influenced using filling material (F) and without filling material (S) in the sample.

Fig. 3.7 is demonstrating the pressure drop scenario of the samples with (sample S) and without filling material (sample F) in the re-cultivation phase.

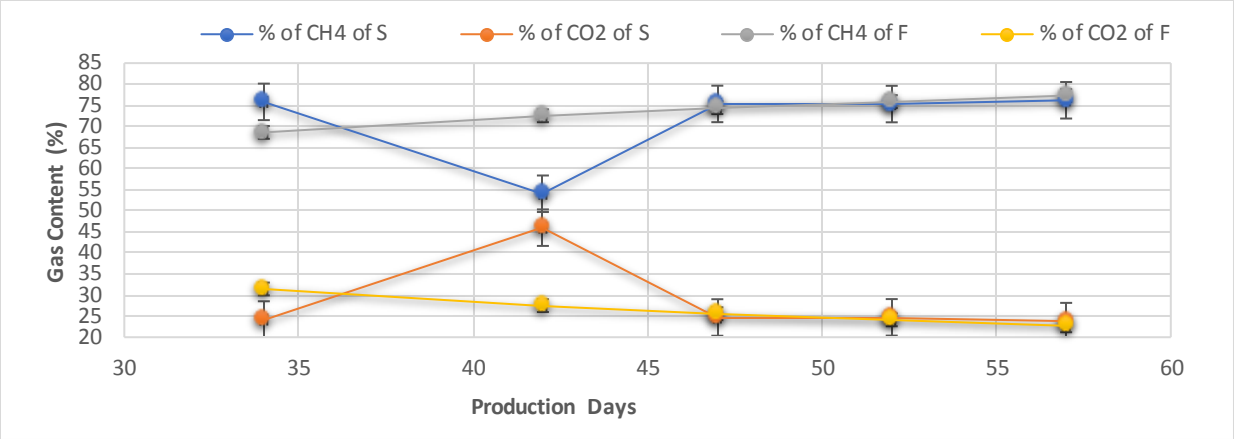

Fig. 3. 8: Comparison of GC result between the use of with and without filling material in the sample F and reference sample S.

However, around 70% of methane (***Fig. 3. 8***) was in sample F and 76% was in the reference sample initially. At the end of the phase, sample F produced about 77% where the reference sample also gave the same result. Comparing with the first phase (***Fig. 2. 8***), there is no significant difference in the methane contents, so, it could be said that the filling material did not work as it was expected.

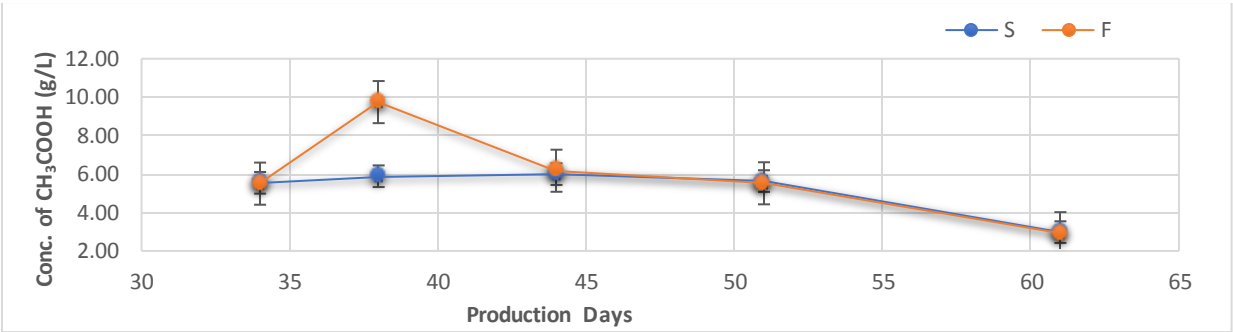

Fig. 3. 9: Comparison of acetic acid concentration between the use of with and without filling material in the sample F and reference sample S.

On the other hand, concentration of CH_3COOH increased in sample F initially, which might be possible for homoacetogenesis methanogens. So, VFA contributed an initial volume of methane in that sample via acetoclastic methanogens. After day 43 concentration of CH_3COOH started to drop and at a certain point concentration of acid in both samples became very close being 2.92 g/L and 2.98 g/L for sample F and S respectively (***Fig. 3.9***).

Comparison between different inoculum

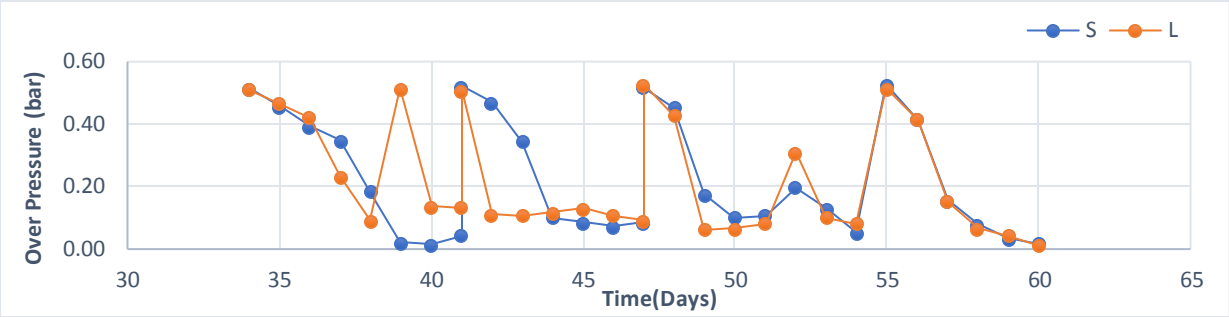

Fig. 3. 10: The overpressure scenario influenced by the Inoculum from the garden sediment (L) and fangel biogas plant (S).

The overview of pressure drop for the sample with garden sediment and biogas plant digest is stated in ***Fig. 3.10***.

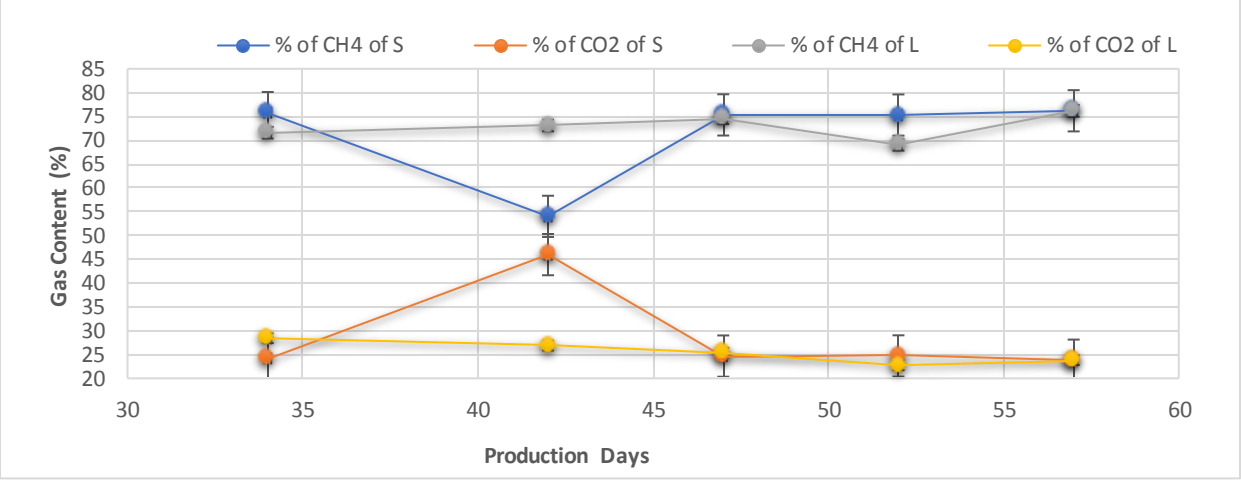

Fig. 3. 11: Comparison of GC result between the use of two different inoculums in the sample L and reference sample S.

From ***Fig. 3.11***, it is noticeable that sample L with garden sediment contributed more than 70% methane in the reactor, and it was almost stable over time compared with the reference sample. Compared with the first phase (***Fig. 2. 11***), in this cycle Sample L, did not fluctuate until 48 days and shown 75% methane end of the phase which is indicating the higher and faster growth of methanogens.

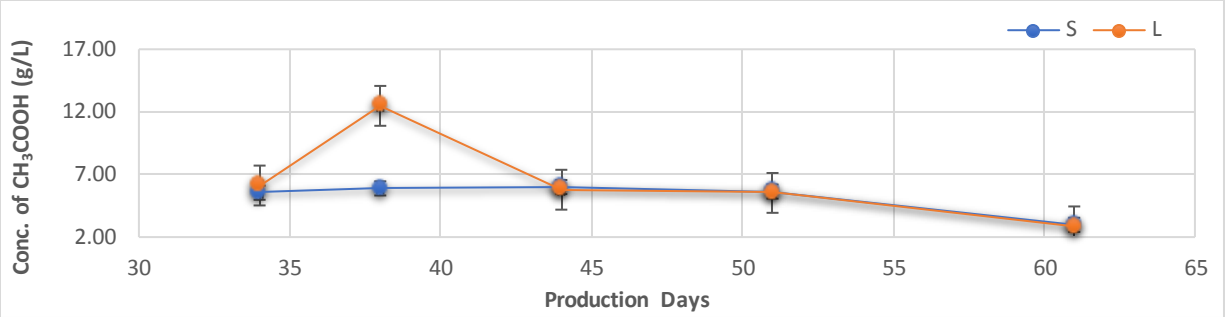

Fig. 3. 12: Comparison of Acetate concentration between the use of two different inoculums in the sample L and reference sample S.

The concentration of CH_3COOH increased for the first few days in the sample with garden sediment but not for the reference sample. Though the mineral medium in sample L and S were cattle manure so it was expected to have high concentration of VFA in both samples. In the reference sample, the maximum conc. was 5.54 g/L whereas sample L had 12.48 g/L which was double than the first phase (***Fig. 3. 12***).

Comparison between nutrients

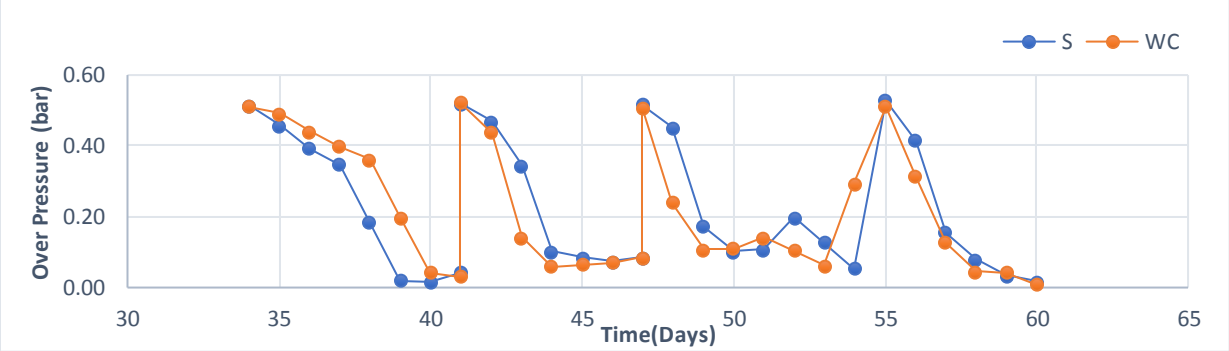

Fig. 3. 13: The overpressure scenario influenced by the nutrients of the sample with cattle manure (WC) and mineral medium (S).

The pressure drop difference between the samples with two different nutrition is illustrated in ***Fig. 3.13***. In this phase, the production of methane was unstable initially for sample WC, where in the first cycle it increased over the period (***Fig. 2. 14***).

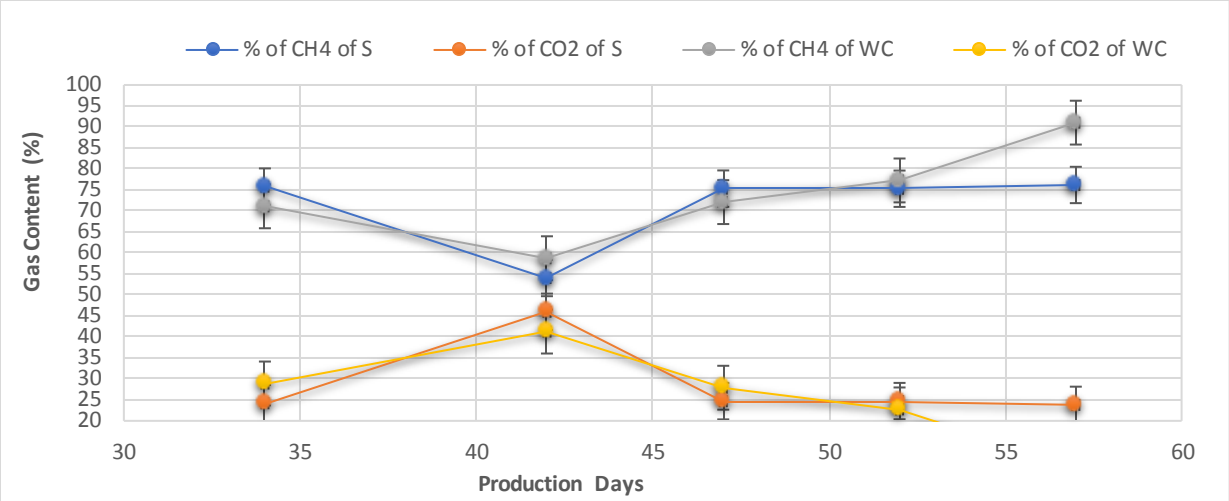

Fig. 3. 14: Comparison of GC result between the use of different nutrients in the sample WC with reference sample S.

The percentage of methane in sample WC was at first 71% and it became 90% at the end of the phase (***Fig. 3. 14***), which was not only the highest amount compared to the reference sample but also other samples. It could be considered that due to the different nutrients in sample WC, it produced a higher density of hydrogenotrophic methanogens rather than other microorganisms.

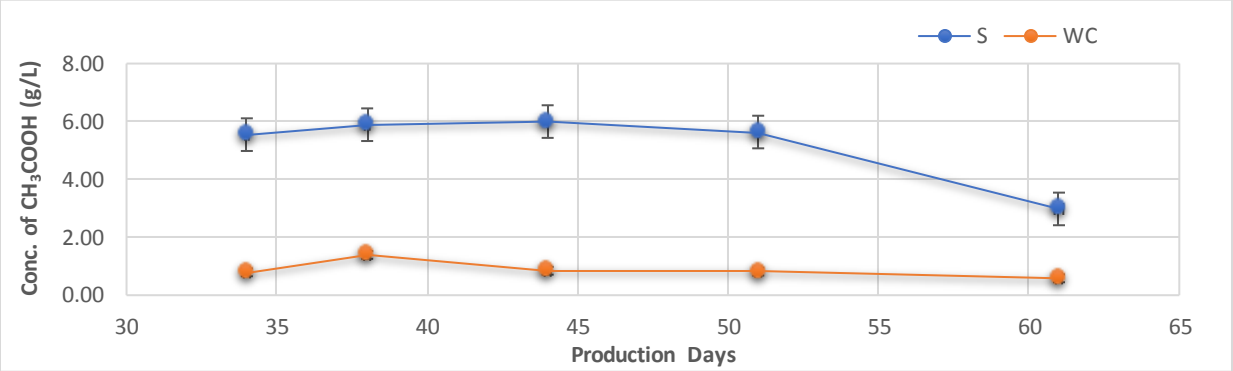

Fig. 3. 15: Comparison of Acetate conc. between the use of different nutrients in the sample WC and reference sample S.

Therefore, ethanoic acid was also less than 1 g/L at day 61 (***Fig. 3. 15***) in the sample WC, which was the lowest amount compared with the reference and other samples.

15. Discussion

Table 6: An overview of the whole project with maximum and minimum percentage of CH_4, CO_2, and CH_3COOH in all the samples for both periods.

Objective ⟹ Enrichment of HM					**Retention Time ⟹ total of 61 days**							
Temperature ⟹ 37 °					**Method ⟹ microbial assisted Ex-situ technology**							
	Phase 1 (Cultivation)						**Phase 2 (Re- cultivation)**					
Sample	CH_4(%)		CO_2(%)		CH_3COOH (g/L)		CH_4 (%)		CO_2(%)		CH_3COOH (g/L)	
Name	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min
S(Reference)	83.35	41.8	46.04	8.20	9.31	4.48	76.18	54.01	45.98	24.20	6.00	2.98
D(Position)	82.18	44.57	55.43	17.82	9.34	0.04	76.15	68.12	31.88	23.84	7.55	2.84
F(Filling)	77.3	36.16	63.85	9.89	9.60	5.64	77.3	68.56	31.44	22.70	9.74	2.92
L(Inoculum)	87.79	24.96	54.11	8.54	9.27	4.27	76.16	69.05	28.42	22.76	12.48	2.86
WC(Nutrition)	88.36	25.34	74.66	11.67	1.01	0.12	91.03	58.74	41.26	8.97	1.39	0.58

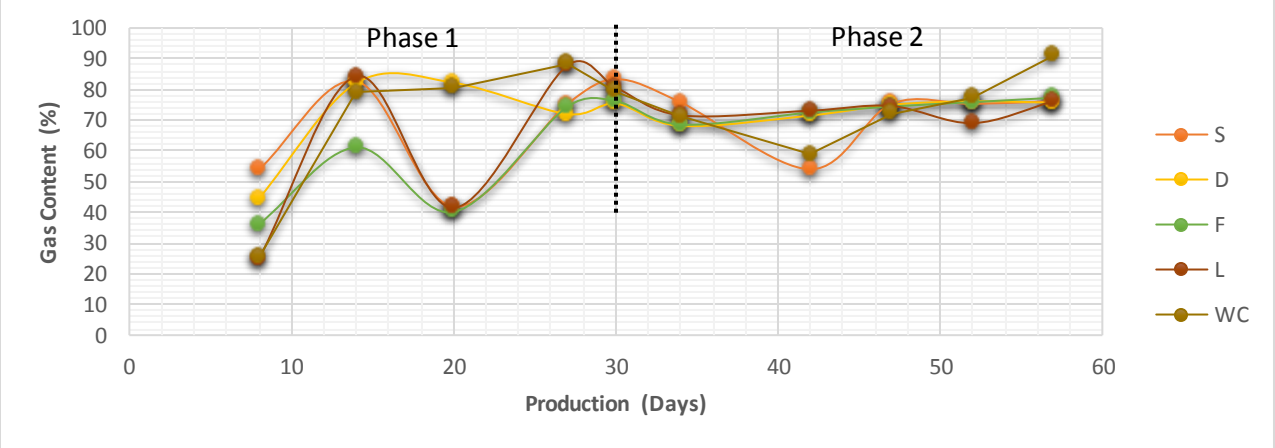

Fig. 3. 16: Gas composition of all the five samples for both phases.

After comparing all the samples based on their parameters with a reference scenario, it can not be resolved which sample had better efficiency over the others. Theoretically, it was projected to have a higher percentage from samples D (position different) and F (with filling) which had a wide area to increase the HM in the reactor. Experimentally, sample D had better performance, but not sample F in contrast with the reference scenario (***Table 6***).

The filling material in sample F became dry at a certain point which was in the upright position in the reactor. Therefore, microorganisms in the upper part did not get enough moisture to enrich. Moreover, there was uncertainty to get enough flow of H_2 and CO_2 for all microorganisms. In some research, it has been mentioned that initial methanogens work faster with substrate than those who are at the end of the reactor.

Fig. 3. 16 is exemplifying that, in phase 1, except for sample WC (nutrient influenced) all the samples were unstable, which might be due to the high concentration of the H_2 for what the reaction went through the CH_3COOH production instead of CH_4 which made the process acidic. The GC results also indicate that almost all the samples had O_2 present, which is sensitive to an anaerobic process.

As seen in ***Table 6***, sample WC had the maximum and sample L (inoculum influenced) had the minimum percentage of methane in phase 1 which was 88.36% and 24.96% respectively. In phase 2, all the samples had an average of 60% of methane where WC had a higher percentage as in phase 1. The faster-growing HM was noticeable in phase 2 which was overall a stable production to all the samples.

Sample L (inoculum different) which was not digested with necessary trace elements as nutrition to enrich hydrogenotrophic methanogens and was not sieved, had an acceptable performance in contrast with the reference scenario. Nonetheless, this sample differed in both periods initially. To get a better result, it should have been sieved one time to remove unwanted solid particles.

Moreover, the pressure drops caused another uncertainty in the experiment as most of the samples revealed a high-pressure drop scenario throughout the process. Theoretically, the pressure drop should not be less than 1.1 bar if there is no leakage in the environment and it should be stable at a certain point. In most of the samples, the pressure drops to 1 bar or less than 1 bar was noted, which might be the reason for the fluctuation of the production in the reactor.

Nonetheless, in this project, we didn't investigate the pH and VS of the samples which could be helpful to explain some other facts and factors in the performance of the experiment. In view of the concentration of VFA and the apparent percentage of CO_2, it is obvious that the sample with cattle manure inhibits the hydrogenotrophic methanogens enrichment in the environment.

However, all the uncertainties should be considered before turning a conclusion as well as discussing the economic aspects. In both phases, the maximum percentage of methane was in sample WC and sample D while both samples were with similar inoculum but different nutrition. As the aim of this project was to enrich the hydrogenotrophic methanogens for biomethane production in a more sustainable way, so sample D which was with cattle manure and higher surface area, can be the better one to bear in mind also with the economic aspect.

16. Limitations and perspectives

The utilization of CO_2 in the process with H_2 to produce biomethane now has opened a new era in the renewable sector where the enrichment of HM has already proven a potential process for biomethanation. The objective of this project was to enrich HM under different conditions, but there were some challenges that could be resolved to make it more efficient.

The following challenges had been observed through the research:

The inoculum had been used in the enrichment without centrifugation, so it is not completely clear from the GC or VFA result if there was any influence on the enrichment with or without centrifuged inoculum.

For the sample L with garden sediment, it was not sieved and degassed so we got only results from raw biomass which fluctuated in the whole period. Therefore, the finding cannot be extrapolated for this limitation.

The flow rate used for the H_2 and CO_2 was not altered to verify whether the flow rate was adequate for the methanogens or not. However, some of the VFA results indicate that the concentration of substrate was high at a certain point.

This project followed a mesophilic temperature which was 37 ºC Some researchers have recommended that the mesophilic temperature could be altered within ±3 ºC and it doesn't have an impact on the enrichment of HM. Nonetheless, in this project, this was not looked at for comparison of result.

From the GC result, it is concluded that most of the samples had O_2 which is a big challenge for an anaerobic environment. Furthermore, we used the needle to feed the microorganisms which might have an influence on the enrichment because we were not aware enough to use a separate needle for every other sample.

Except for these confines, there were several notable strengths of this project, but the full potential of the approach has not been proven because of the above limitations. Hence, this research could further be improved by modifying those.

In this project, one of the microbial assisted technologies, i.e., ex-Situ technology was investigated, and filling material was developed in two of the reactors to examine the enrichment of microorganisms which showed considerable results. A study on different filling materials with different surface areas could be in mind for further research to get comparable results.

In this fed-batch process, feeding and pressure measurement of the reactors had been done manually, so due to leakage in the reactor air from outside had an impact on the system and pressure dropped very fast sometimes. Minimizing the presence of O_2 and finding an alternative

way to feed the microorganisms could be addressed in future studies. The sample in the laying down position (sample D) showed consistent results compared to others. Therefore, extending the experiment in a lab-scale focusing on the reactor's position in the incubator might give a more valuable result of methanogen enrichment. Furthermore, it would be also interesting to adjust the temperature range and flow rate of the gas.

17. Conclusion

Biogas is one of the eco-friendliest energy resources people are looking for nowadays. The biogas upgrading system can play a vital role not only to fulfill the energy demand but also to limit greenhouse gas emissions. The raw biogas has several impurities that can be removed using commercial technologies such as water scrubbing, pressure swing adsorption, amino scrubbing method, etc. It is expected that this upgrading technology will be advanced along with regulations for its widespread applications.

The main objective of this project was to investigate the method for improving the enrichment of microorganisms. Though this was a lab-scale project, the outcome of the work is significant. The co-digestion of two different inoculums was studied focusing on the growth of hydrogenotrophic methanogens, where it managed more than 75% methane for all the samples at the end of the first phase. In general, the raw biogas contains 50-65% CH_4 and 35-50% CO_2, so the result agrees with the findings and this proposed method might be comparable.

In the second phase, the initial percentage of methane was above 50% which indicated that with increasing the number of days the growth of methanogens was also increasing. However, concentration of VFA was initially high in the sample with cattle manure. Observing the partial pressure of the substrate in those samples might give a better solution. Besides the operational parameters might be explored in the future to optimize the H_2 assisted enrichment.

However, this project is still under development, so, the knowledge gap is burdened to fulfill the target of this project. Giving more efforts to bridge the knowledge gap between pilot and large scale could make this process more efficient.

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18. Appendix

Mineral Medium

Preparation of solution A, B, C, D, E

The basic medium is prepared from the following stock solutions, (chemicals given below are concentrations in g l^{-1}, in distilled water)

(A) NH_4Cl, 100; NaCl, 10; $MgCl_2 \cdot 6H_2O$, 10; $CaCl_2 \cdot 2H_2O$, 5

(B) $K_2HPO_4 \cdot 3H_2O$, 200

(C) Resazurin 0.5

(D) Trace-metal and selenite solution: $FeCl_2 \cdot 4H_2O$, 2; H_3BO_3, 0.05; $ZnCl_2$, 0.05; $CuCl_2 \cdot 2H_2O$, 0.038; $MnCl_2 \cdot 4H_2O$, 0.05; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.05; $AlCl_3$, 0.05; $CoCl_2 \cdot 6H_2O$, 0.05; $NiCl_2 \cdot 6H_2O$, 0.092; ethylenediaminetetraacetate, 0.5; concentrated HCl, 1 ml; $Na_2SeO_3 \cdot 5H_2O$, 0.1

(E) Vitamin mixture (componets are given in mg/l): Biotin, 2; folic acid, 2; pyridoxine acid, 10; ridoflavin, 5; thiamine hydrochloride, 5; cyanocobalamine, 0.1; nicotinic acid, 5; P-aminobenzoic acid, 5; lipoic acid, 5; DL-pantothenic acid.

To 974 ml of distilled water, the following stock solutions were added A, 10 ml; B, 2 ml; C, 1 ml; D, 1 ml and E, 1 ml. The mixture is gassed with 80% N_2 – 20% CO_2. Cysteine hydrochloride, 0.5 g and $NaHCO_3$, 2.6 g, are added and the medium is dispensed to serum vials and autoclaved if necessary. Before inoculation the vials are reduced with $Na_2S \cdot 9H_2O$ to a final concentration of 0.025%.

VFA Standard Curve

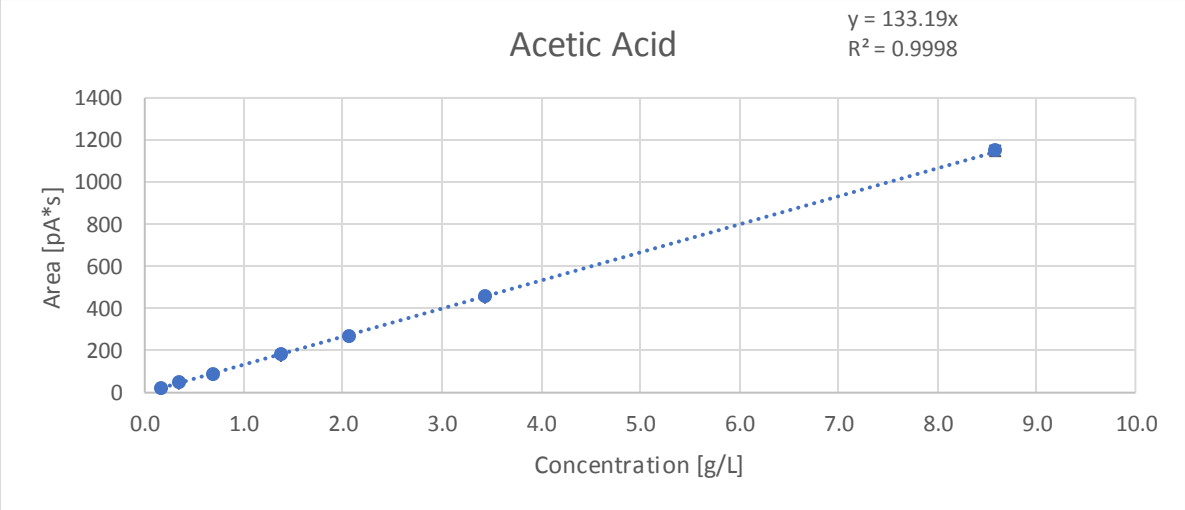

Fig 1: Standard Curve of Acetic Acid

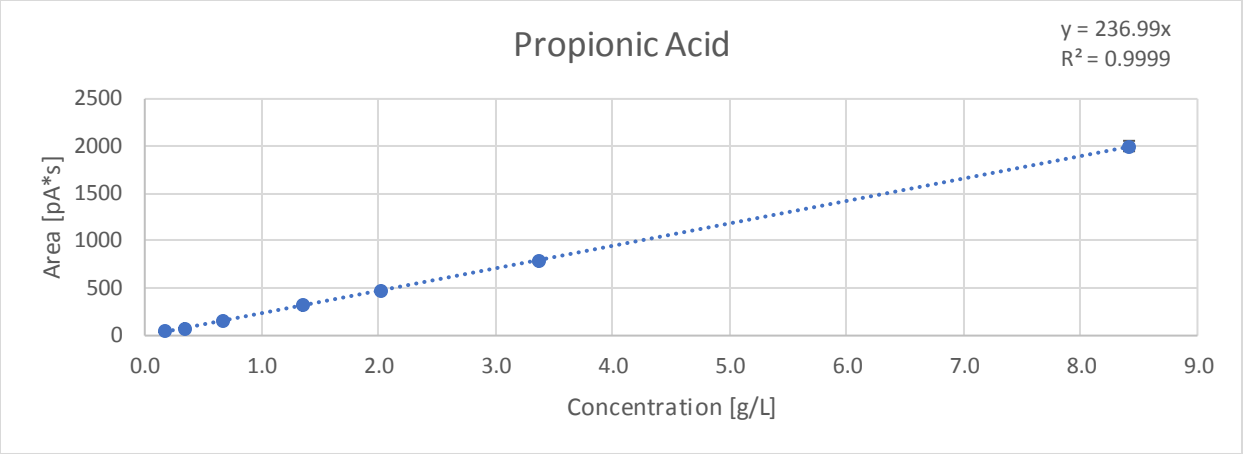

Fig 2: Standard Curve for Propionic Acid

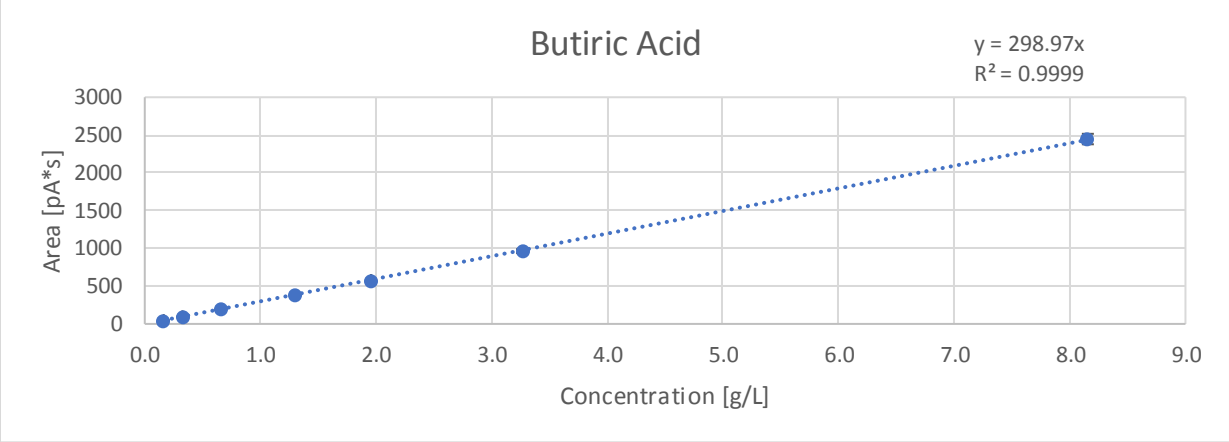

Fig 3: Standard Curve for Butiric Acid

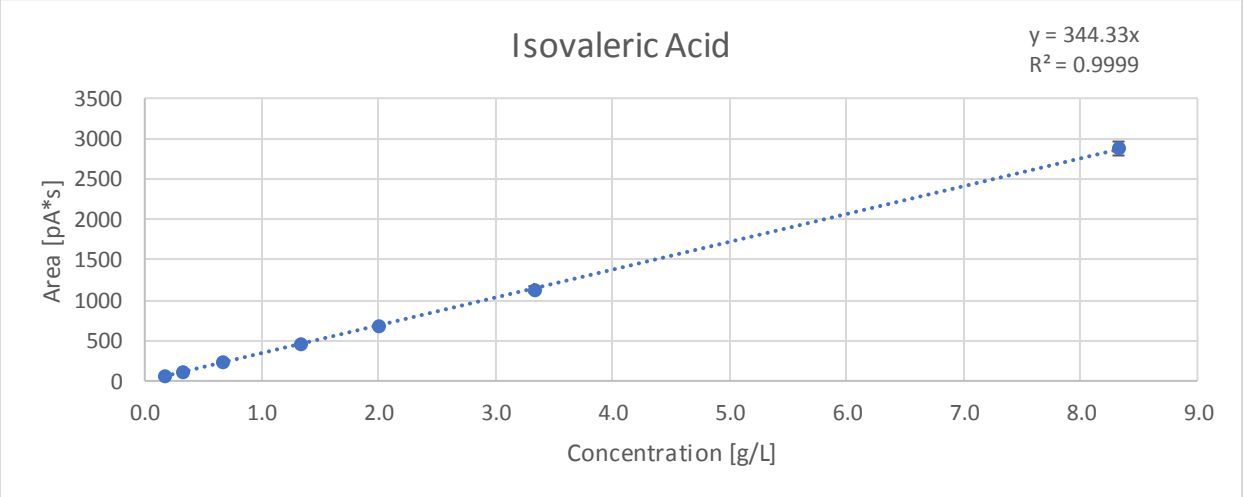

Fig 4: Standard Curve for Isovaleric Acid

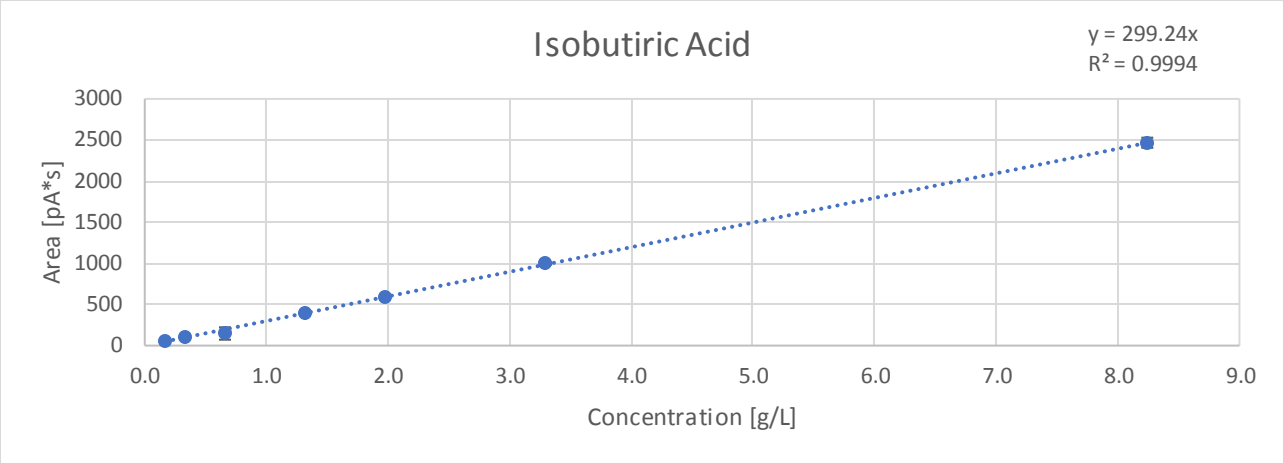

Fig 5: Standard curve for Isobutyric Acid

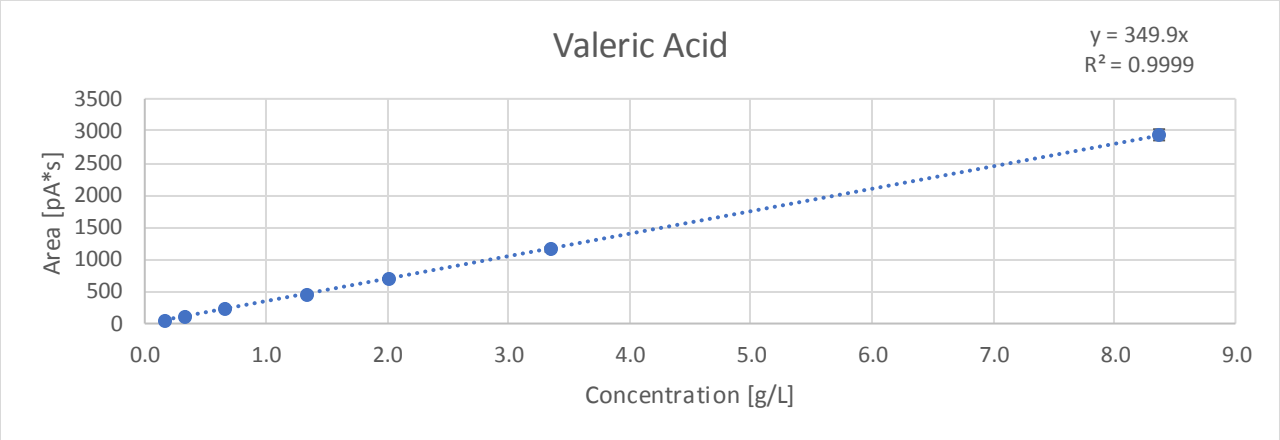

Fig 6: Standard Curve for Valeric Acid

VFA Result from Experiment

First Phase (Cultivation)

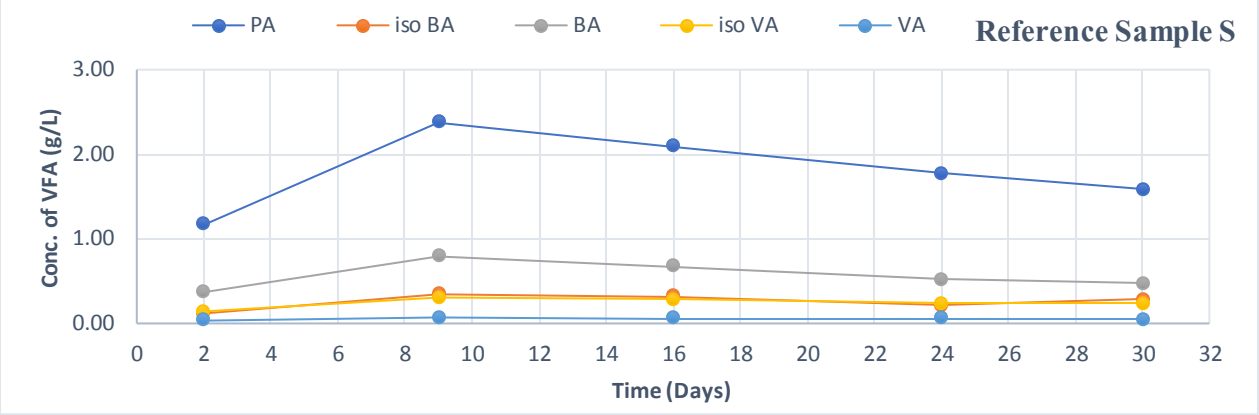

Fig 7: Overview of VFA in the Reference Sample S

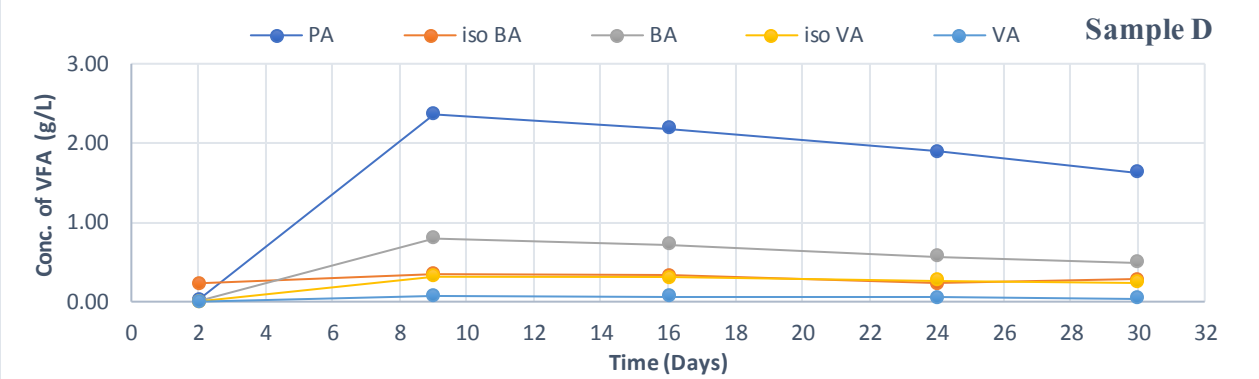

Fig 8: Overview of VFA in Sample D which was an upright position in the incubator

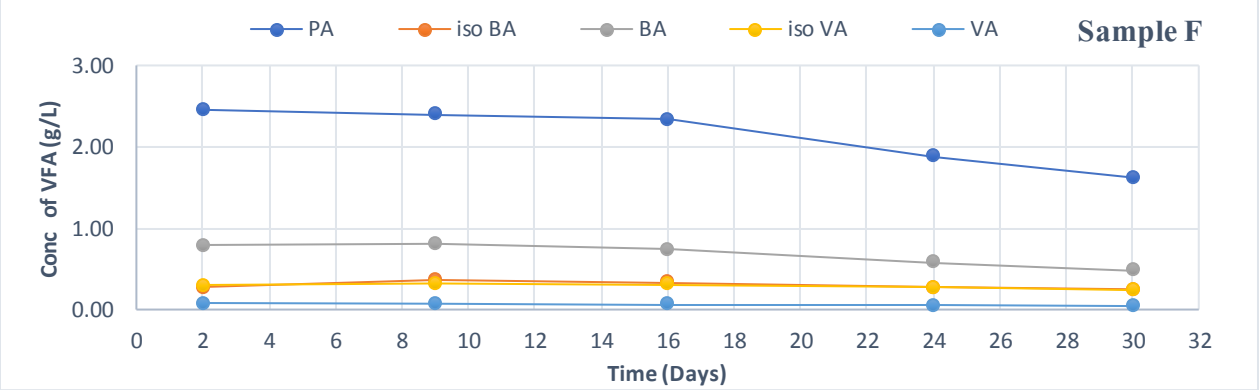

Fig 9: Overview of VFA in Sample F with filling material

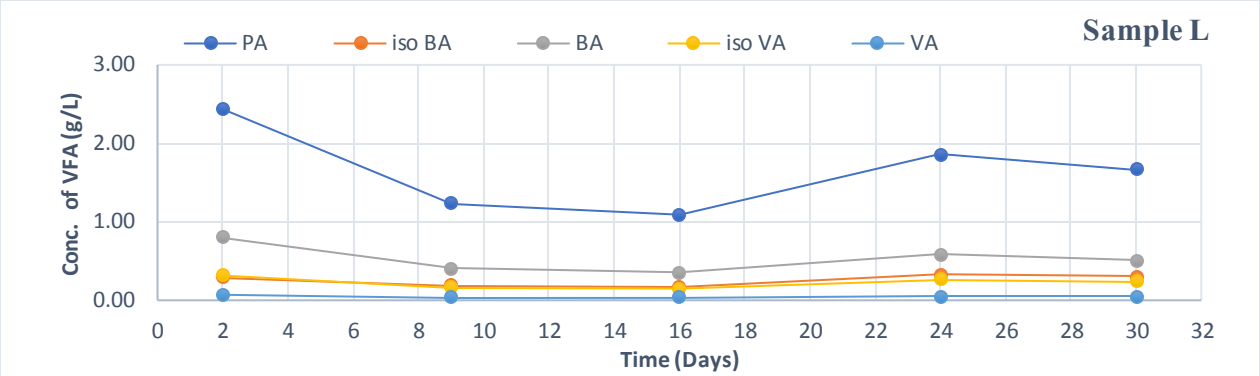

Fig 10: Overview of VFA in the Sample L with Garden Sediment Inoculum

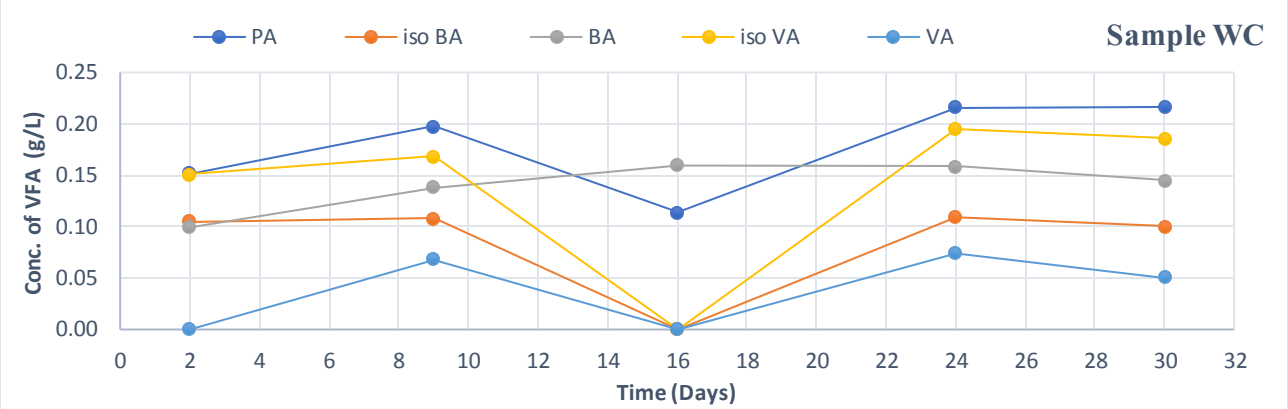

Fig 11: Overview of VFA in the Sample WC with Mineral Medium as a Nutrition

Second Phase (Re- Cultivation)

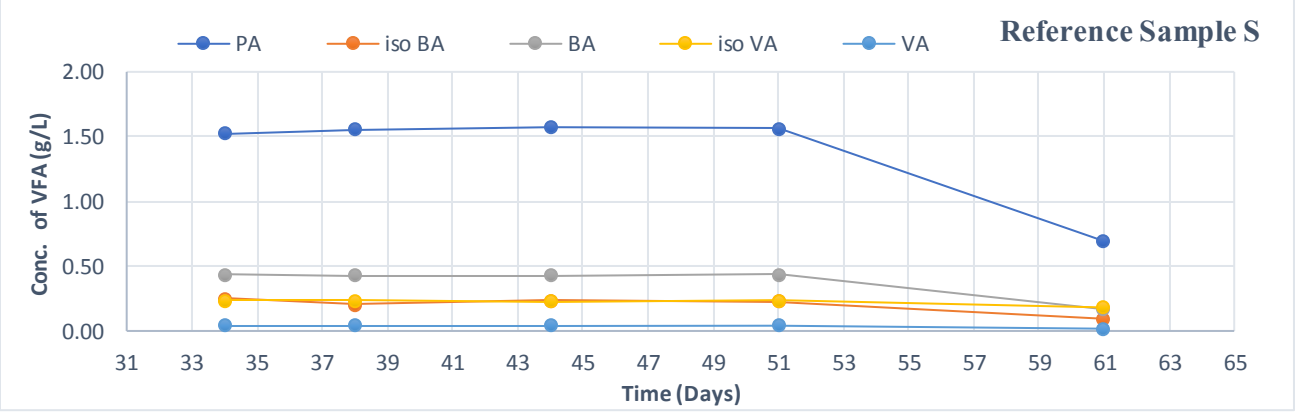

Fig 12: Overview of VFA in the Reference Sample S

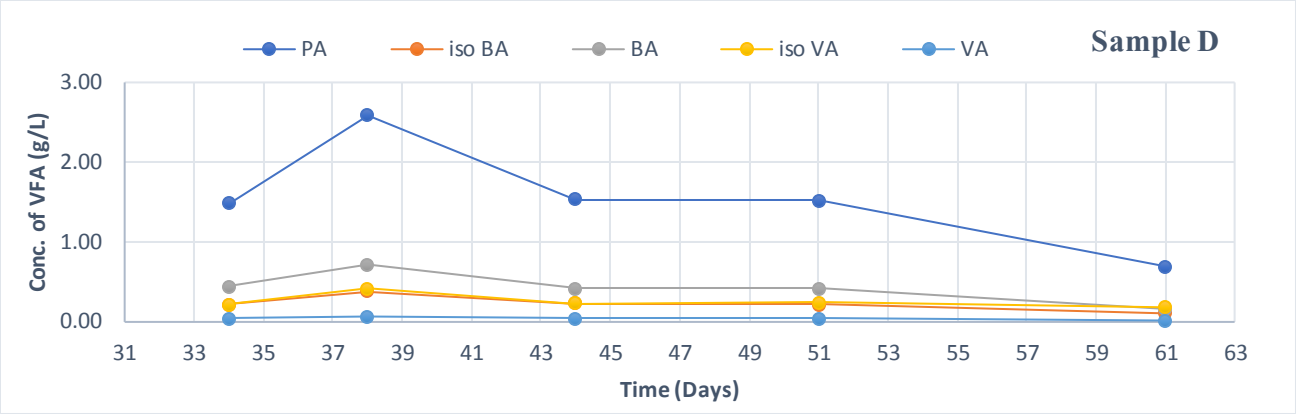

Fig 13: Overview of VFA in Sample D which was an horizontal position in the incubator

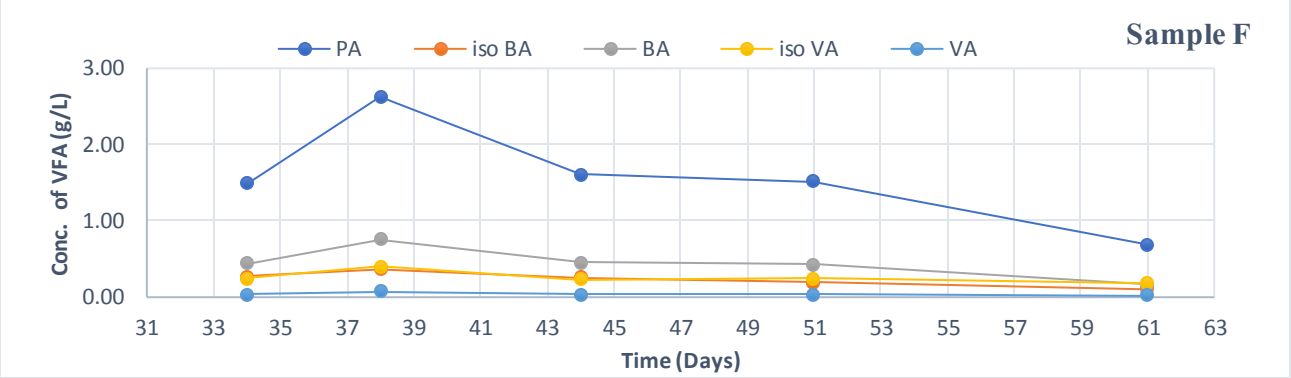

Fig 14: Overview of VFA in Sample F with filling material

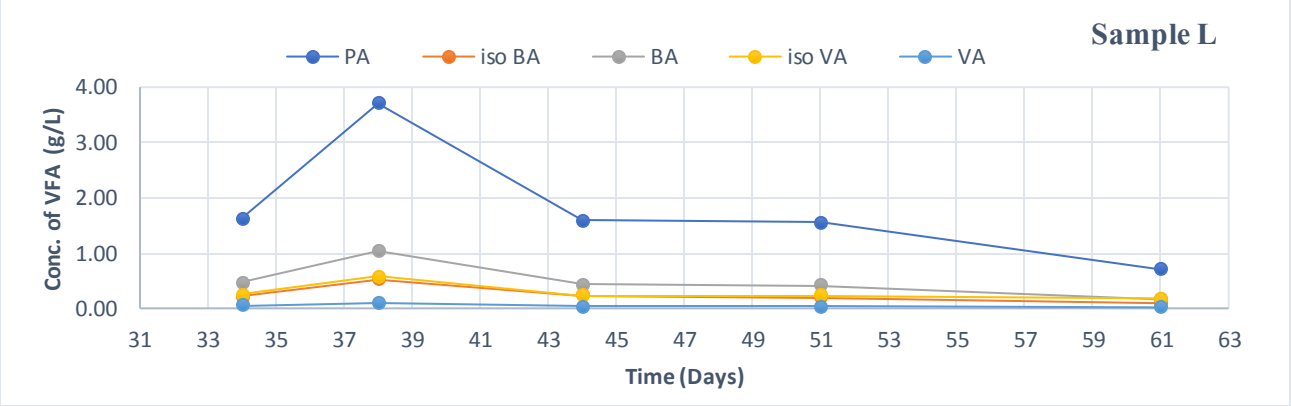

Fig 15: Overview of VFA in the Sample L with Garden Sediment Inoculum

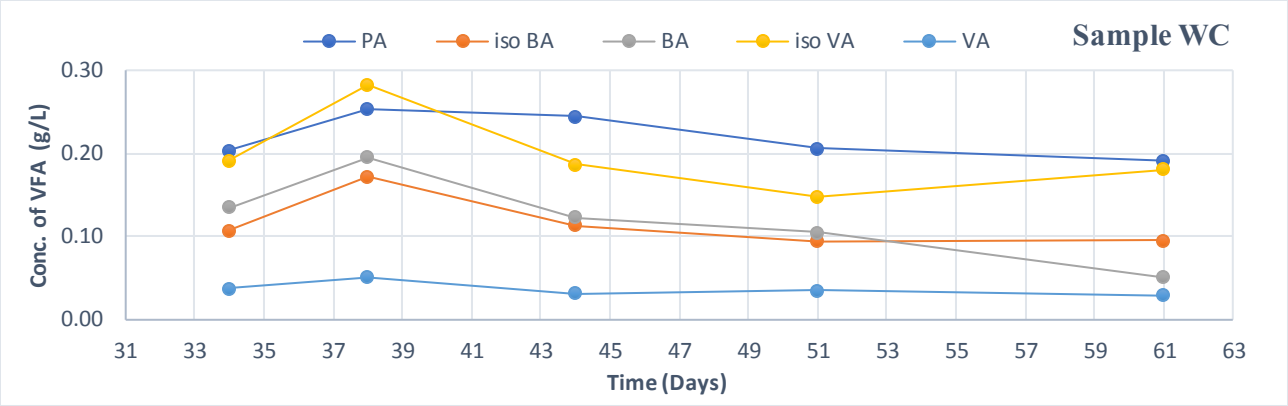

Fig 16: Overview of VFA in the Sample WC with Mineral Medium as a Nutrition