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Application of electron shuttle on anaerobic digestion for carbon dioxide reduction

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Summary:

In anaerobic digestion, organic wastes are decomposed in the absence of oxygen primarily to produce biogas, which consists of methane and carbon dioxide. The methane content in biogas can be optimized by reducing carbon dioxide to obtain biomethane, which can be used as an alternative to natural gas. In this thesis, it is then of interest to study ways to enhance methane production from biogas. One of the approaches to increase the methane content is by facilitating the electron transfer mechanism, using conductive materials, between the electron-donating bacteria and electron-accepting methanogens.

The investigation was more focused on the potential of conductive materials such as Anthraquinone-2,6-Disulphonate (AQDS) and activated carbon to enhance the production of biogas and, consequently, methane. A biomethane potential (BMP) test was carried out in the laboratory under mesophilic conditions, to determine the methane production capacity of the samples inside a bioreactor that contains the conductive materials. The pressure developed inside the bioreactors was monitored regularly, and the volume of the biogas was calculated using ideal gas equations.

Our initial focus was to use AQDS as the conductive material in order to enhance methane production. However, the use of AQDS did not yield a substantial amount of methane. In addition, with the use of AQDS, excess nitrogen was produced. The possible reasons for the excess nitrogen and the much lower methane productions could be aged inoculum contaminated with nitrogenous compounds, incomplete biodegradation of the substrate (ethanol), and the impact of AQDS on the nitrogen level.

On the other hand, the use of activated carbon at 15 g/L as a conductive material had a significant positive impact on biogas and methane production, while at concentrations beyond 15 g/L, methane production decreased. However, the methane production at 60 g/L was surprisingly higher than 30 g/L and 45 g/L. Various reasons are outlined for the unexpected production of biogas and methane at 60 g/L, viz., microbially favourable conditions, higher adsorptive capacity, and a larger surface area and porosity of the activated carbon.

Furthermore, in this thesis, we have also reused the activated carbon from the first batch experiment to conduct another batch experiment. In that subsequent experiment, the reuse of activated carbon resulted in a decrease in biogas production. This could be because of a decline in the electron transfer effectiveness of the activated carbon caused by the adsorption of organic pollutants on its surface. Additionally, the study also examined biofilm formation on the surface of the activated carbon by using Scanning Electron Microscopy (SEM) to assess the attachment of microorganisms.

Preface

This master's thesis entitled "Application of Electron Shuttle on Anaerobic Digestion for Carbon Dioxide Reduction" was conducted during the spring semester of 2023 as a partial fulfilment of the requirements for master's degree in Process Technology at the University of South-Eastern Norway.

I wish to extend my deepest gratitude to my supervisor, Associate Professor Nabin Aryal, for providing continuous supervision, valuable guidance, feedbacks, and encouragement throughout the thesis writing process, which resulted in the successful completion of this thesis work. The discussion with Nabin regarding the state of the art in the biogas upgrading techniques has intrigued me to keep abreast myself in the field. And, I would also like to continue to work in this field if there is a possibility in the school’s research laboratory or with external partners.

Additionally, I express sincere appreciation to my co-supervisor, researcher Dr. Eshetu Janka Wakjera and external partner senior lecturer Pabasari Arundathi Koliyabandara, for their valuable technical support, suggestions, and feedback in the finalization of this project.

I am incredibly grateful to chief engineer Mr. Sandeep Gyawali for assisting me throughout the project by providing necessary lab equipment and helping me to conduct experiments, despite his busy schedule.

Last but not the least I would like to thank my family and the friends for their constant love, support, and motivation throughout this journey.

Porsgrunn, 15.05.2023

Manjil Bista

Contents

Nomenclature

AC	Activated Carbon
AD	Anaerobic Digestion
AQDS	Anthraquinone-2,6-Disulphonate
BMP	Biochemical Methane Potential
DIET	Direct Interspecies Electron Transfer
IET	Interspecies Electron Transfer
IFT	Interspecies Formate Transfer
IHT	Interspecies Hydrogen Transfer
GAC	Granular Activated Carbon
SEM	Scanning Electron Microscopy
TS	Total Solid
VFA	Volatile Fatty Acid
VS	Volatile Solid

1 Introduction

In recent times it is evidenced that a major threat to the environment, the energy infrastructure, and the fuel supply has been posed by emissions of greenhouse gases and the climatic change they cause. Climate change involves a range of outcomes that go beyond the mere rise in mean temperatures. These consequences include but are not limited to extreme weather incidents, alterations in animal populations and their surroundings, elevated ocean levels, and numerous other impacts [1]. Apart from environmental concerns, climate change also has an immediate impact on energy output, fuel supply, and the physical durability of existing and future energy infrastructure. Reduced fossil fuel emissions are even more important given how stressed out present energy production is due to heatwaves and droughts [2]. It is therefore time for the switch to clean energy to lessen these effects. Biogas generated from the anaerobic digestion of organic waste will aid in this, as will the generation and use of other renewable energy sources [3].

The anaerobic digestion process involves decomposing organic waste using microbes in the absence of oxygen, producing biogas mostly comprising methane and carbon dioxide. Anaerobic digestion is gaining a lot attention as a sustainable waste management technique and alternative to non-renewable resources [4]. Methane, when upgraded by reducing the amount of carbon dioxide, also known as biomethane, can be used as indirect substitute for natural gas. This increases the need to optimize the production of methane using optimization techniques like feedstock pretreatment [5], co-digestion [6], biogas upgrading [7] and so on. One such strategies researchers are exploring involves the electron transfer between electron-donating microorganisms and electron-accepting methanogens. This exchange of electrons can occur either indirectly through electron carriers like hydrogen or formate, or directly through physical or electrical connections enabled by conductive materials, conductive pili, or membrane-bound electron transport proteins. This mode of electron transfer, known as Direct Interspecies Electron Transfer (DIET), has been demonstrated to augment the efficiency of biogas production [8].

Various carbon based and non-carbon-based conductive materials such as Granular Activated Carbon (GAC), biochar, graphite, graphene, carbon cloth, magnetite, haematite etc, humic acid shows electron shuttle properties that can enhance this process, resulting in increased methane production and decreased CO_2 emissions. This study aims to explore the effectiveness of an electron shuttle especially activated carbon and AQDS in anaerobic digestion, contributing to reducing the carbon footprint of energy production and optimizing the use of organic waste as a renewable resource.

1.1 Aim

The aim of this thesis was to investigate the potential of different electron shuttles to enhance methane production in anaerobic digestion. The objective of thesis is given below:

1. To optimize the production of CH_4 using different electron shuttles.
2. To test the effect of AQDS at different concentrations in anaerobic digestion.
3. To investigate the effect of different loading rate of activated carbon on the anaerobic digestion process.
4. To examine the surface of conductive materials.

1.2 Structure of the report

The thesis consists of six chapters. The outline of the chapters is as follow:

1. The first chapter provides background information, aim and an understanding of the topic.
2. The second chapter gives a comprehensive overview of the literature on anaerobic digestion, its operational parameters, and various optimizing techniques to enhance methane production.
3. The third chapter describes the materials and methods used in the study, including experimental setup, and data analysis procedures.
4. The fourth chapter presents the results of the experiment during the study and discusses the effectiveness of electron shuttles in reducing carbon dioxide emissions.
5. The fifth chapter is the discussion, explaining and analyzing the results with proper arguments.
6. The sixth chapter provides a brief overall conclusion, findings, and recommendations for future research.

2 Literature review

This chapter gives a brief overview of the process of anerobic digestion (AD) and their operating parameters, followed by a thorough examination of the methane optimization technique. The interspecies electron transfer strategies to enhance the production of methane are well studied here.

2.1 Anaerobic digestion

Anaerobic digestion is one of the eco-friendly waste management techniques that has the potential to produce biogas, a source of renewable energy [9]. In this process organic waste such as municipal waste, food scraps, manures, are transformed into biogas and digestate, a nutrient-rich soil supplement in the absence of oxygen [10]. Biogas consists of flammable methane that makes up between 40-75% [11], with the exact percentage varying based on the substrate being digested. CO_2 makes up the remaining 25-60% of biogas. These gases can be captured and utilized as a sustainable energy source. Other components in biogas include water, oxygen, nitrogen, small amounts of H_2S, hydrocarbons, volatile organic compounds and siloxanes [12] [13].

Biogas is a renewable energy source that can be used to generate heat and electricity using gas or combustion engines. Also, it can be transformed into biomethane by removing carbon dioxide out, offering a renewable substitute to natural gas for multiple applications such injection into the gas grid or as a fuel for vehicles [14] as shown in figure 2.1. A important resource for agriculture is the nutrient-rich digestate created during the production of biogas, which contains phosphates and nitrogen [15] [16].

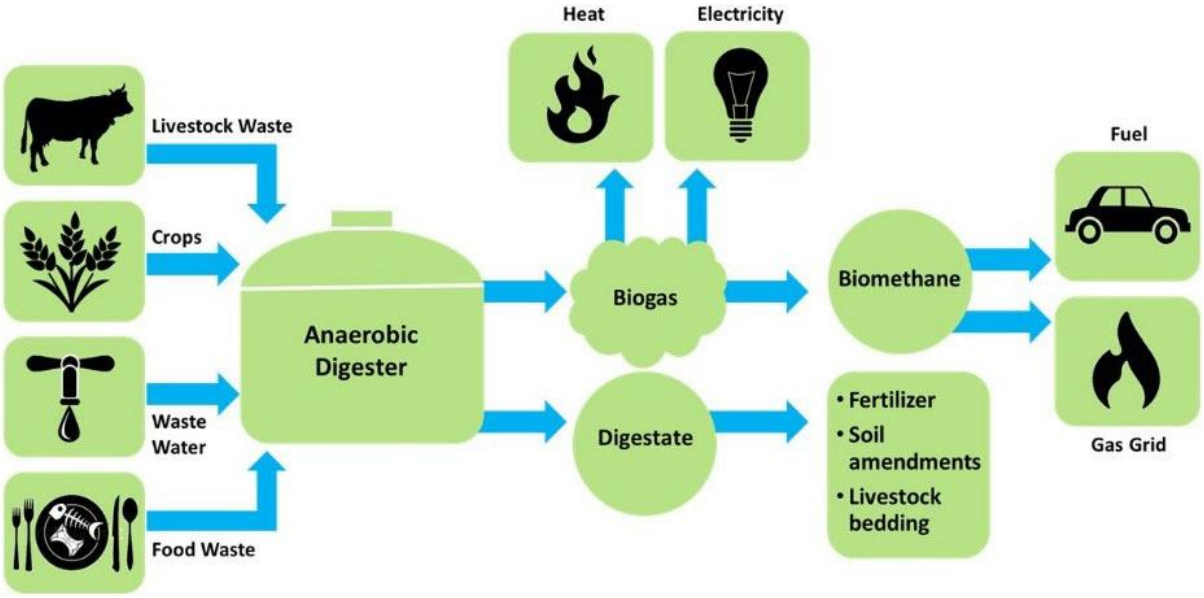


Figure 2.1: Anaerobic digestion process flow converting waste to energy and other products [17]

2.1.1 Steps of anaerobic digestion

During anaerobic digestion anaerobes facilitate the degradation of organic waste to biogas. Four distinct stages of anaerobic digestion are brought about by syntropic microbial interactions between various microorganisms simultaneously [18]. These stages as shown in figure 2.2 are briefly described below.

I. Hydrolysis:
Anaerobic digestion begins with the breakdown of complex organic material into basic monomers that microbes can use. This breakdown is accomplished through the rate-limiting stage of AD, known as hydrolysis, by hydrolytic or fermentative bacteria. In this stage carbohydrates, proteins, and lipids are converted into soluble molecules including monosaccharides, amino acids, and fatty acids [19] [20] .

II. Acidogenesis:
The soluble compounds are converted into intermediate Volatile Fatty Acids (VFA)s such as acetic acid, propionic acid, and butyric acid by acidogenic bacteria [19] [20].

III. Acetogenesis:
These intermediate VFAs must be converted to acetate so that the methanogenic bacteria can act upon them, even though the acetate produced during the acidogenesis process is readily available for this purpose. In this process, acidogenesis bacteria convert VFAs like propionic acid, and butyric acid to acetate along with the production of hydrogen and carbon dioxide [19], [20].

IV. Methanogenesis:
Methane producing bacteria converts available intermediates including acetic acid, hydrogen, and carbon dioxide into biogas. Methanogens are specialized bacteria that aid in this process. This process occurs through two pathways, with the first and major pathway being acetolactic methanogenesis, where acetate is converted into methane, and the second being hydrogenotrophic methanogenesis, where hydrogen reduces carbon dioxide to produce methane [19] [20] [21] [22].

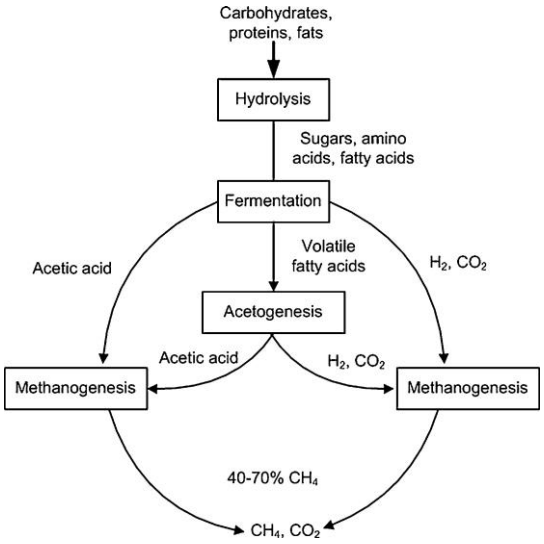


Figure 2.2: Steps of anaerobic digestion [19]

2.2 Parameters of anaerobic digestion

2.2.1 Temperature

Anaerobic digestion can be conducted in three distinct temperature levels: psychrophilic (below 20°C), mesophilic (between 20-43°C) but desirable at 35-37°C, and thermophilic (between 50-60°C) but desirable at 55°C. The temperature range has a significant impact on the microorganisms' metabolism, gas transfer rate, and settling properties of biological sludges involved in the process [23].

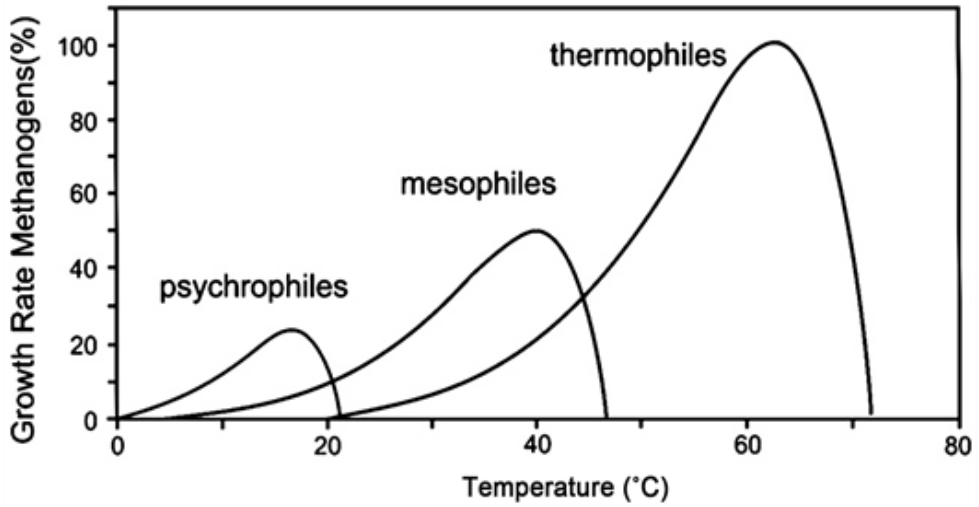


Figure 2.3: Growth rate of methanogens Vs Temperature [24]

The variation of growth rate of methanogens with temperature is shown in above figure 2.3. The methanogens grow slowly in psychrophilic conditions, grows moderately in mesophilic conditions, and rapidly in thermophilic conditions. Hence, the bacteria in the digesters operating in thermophilic conditions yield more biogas and methane and the pathogens are also destroyed faster as compared to mesophilic conditions.

2.2.2 pH

pH influences the effectiveness of anaerobic digestion and the production of methane. For the methanogens to carry out their activity the optimal pH level is in between 6.8-7.5. However, in case of hydrolysis and acidogenesis, the optimal pH ranges in between 5.5-6.5 [25]. In an anaerobic digestion the pH is affected by the alkalinity, carbonate content, VFA, and CO_2 produced. Maintaining a constant pH depends on the control of the relationship between VFA and carbonate concentration, as the pH decreases due to VFA and CO_2 accumulation in cases of digester overloading [26].

2.2.3 Volatile fatty acid (VFA)

Anaerobic digestion generates VFAs as intermediates in the acidogenesis process which serves as an indicator of the anaerobic process's effectiveness, especially the activity of acidogenic and methanogenic bacteria. Acetic acid contributes to 75% of methane synthesis,

with the other VFAs contributing to 25% [27]. VFA build-up can lower the pH and inhibit methanogens, thus leading to the decrease in the efficiency of the process. However, as the process continues, methanogens absorb VFAs, raising the pH and stabilizing the system [28].

2.3 Optimization of methane production

Biogas, an alternative energy source, is primarily composed of methane, which can substitute natural gas following upgrading or optimization. Optimizing methane production is a prerequisite for enhancing the efficiency and sustainability of biogas production, offering valuable economic and environmental benefits. Moreover, methane optimization can aid in waste management by utilizing organic waste as a valuable energy source, while simultaneously reducing greenhouse gas emissions and mitigating climate change. Various techniques have been explored and reported for optimizing methane production in biogas as shown in figure 2.3, such as feedstock pretreatment, co-digestion, trace element supplementation, biogas upgrading, and the incorporation of conductive materials, among others [29].

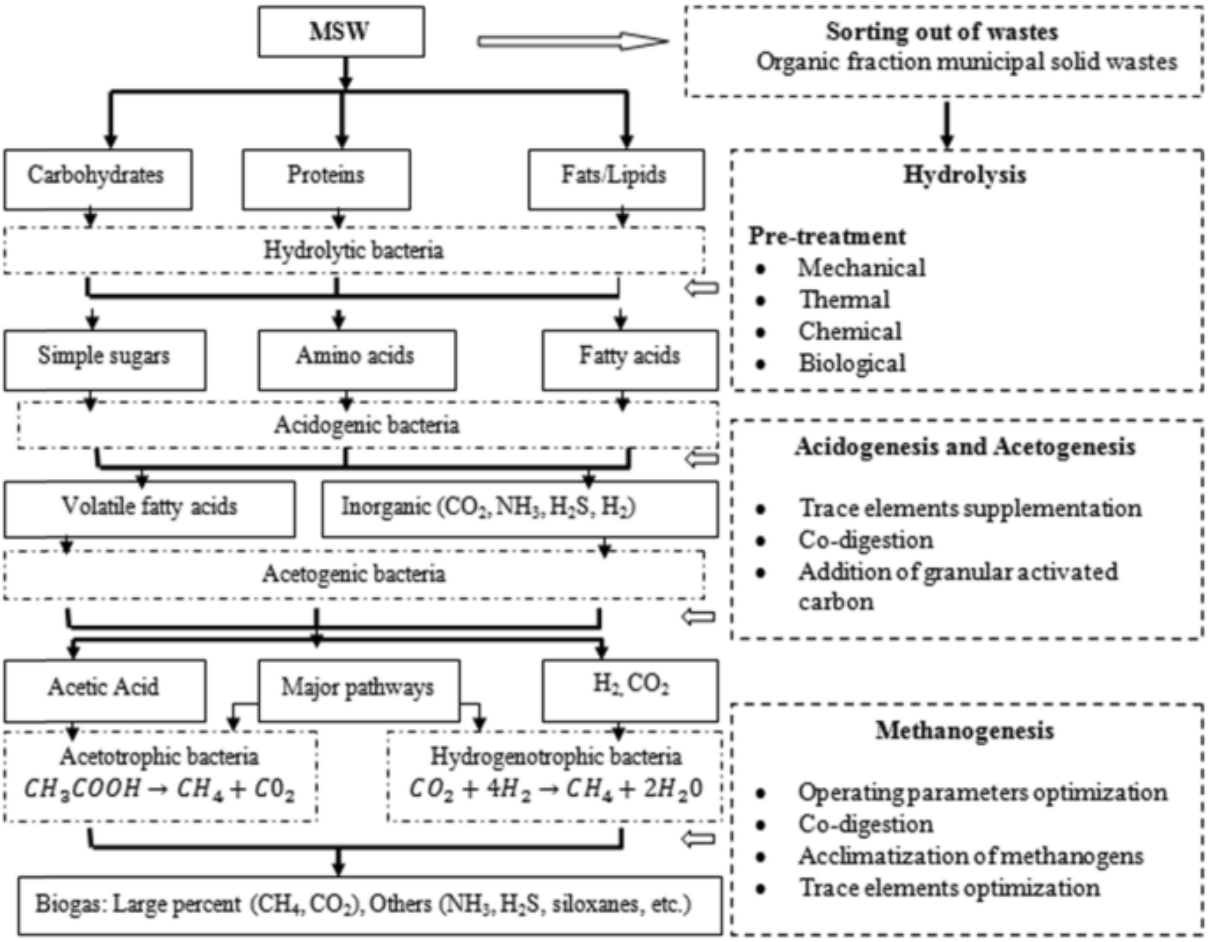


Figure 2.3: Strategies to improve metabolic stages of the anaerobic digestion process for optimizing methane production [29].

2.4 Direct interspecies electron transfer in anaerobic digestion

During anaerobic digestion, the transfer of electrons occurs between electrons donating bacteria and electron accepting bacteria. The energy generated during this process is utilized by the bacteria for their metabolic activity and the growth. The transfer of electrons was proposed to be carried through electron carriers such as hydrogen or formate which acts as an electron shuttle to maintain the syntropic interaction between bacteria and methanogens. This way of transferring electrons is known as Interspecies Electron Transfer (IET) which can either be Interspecies Hydrogen Transfer (IHT) or Interspecies Formate Transfer (IFT) depending upon electron carriers. However, it was observed that the high H_2 partial pressure and the formate concentration led to slow volatile fatty acid degradation which affects the activity of methanogenesis leading to the inhibition of the anaerobic process [30]. Thus, in recent times a mechanism of transferring electrons was proposed commonly known as direct interspecies electron transfer in which electrons are shared through either physical or electrical connection instead of electron shuttles.

2.4.1 Mechanism of DIET

To date, three distinct mechanisms have been reported for direct interspecies electron transfer [31] as illustrated in figure 2.4. Additionally, figure 2.5 depicts a schematic diagram of those mechanisms.

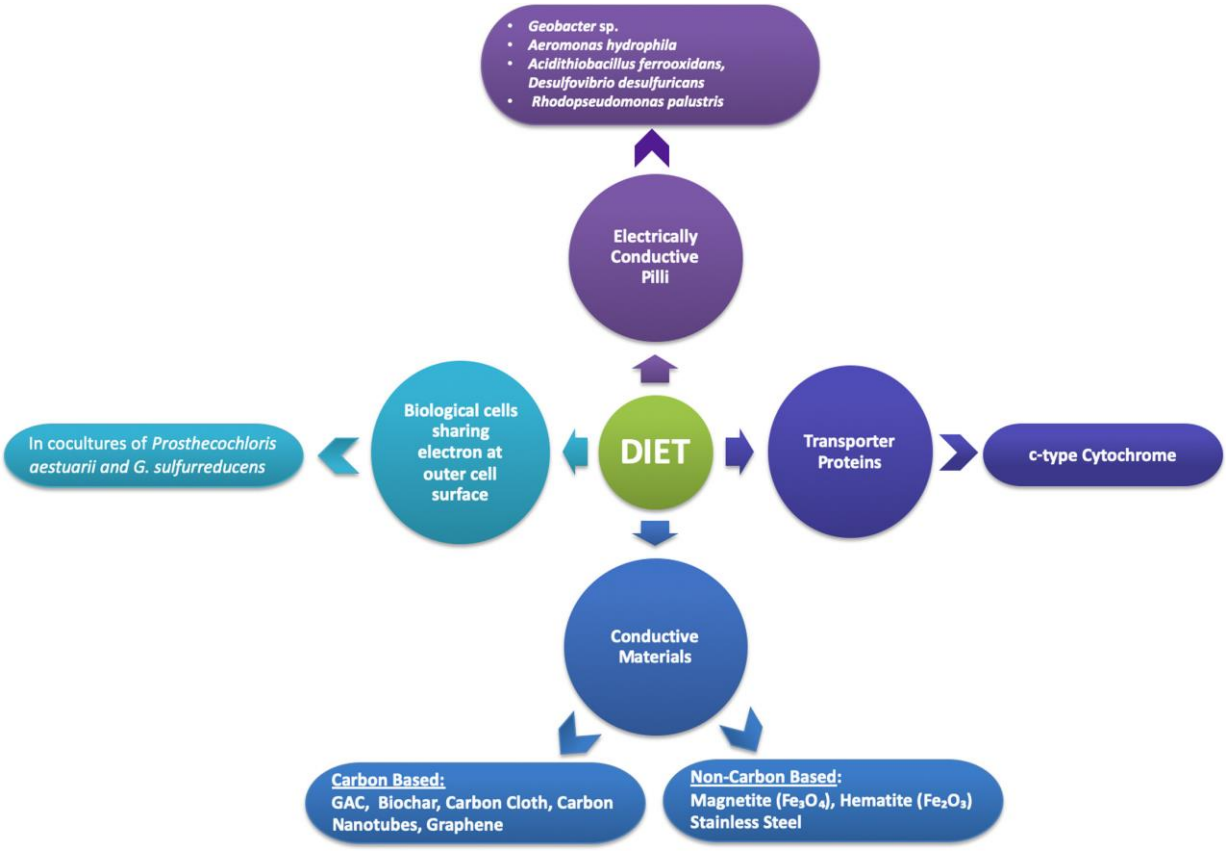


Figure 2.4: Various mechanisms of DIET [32]

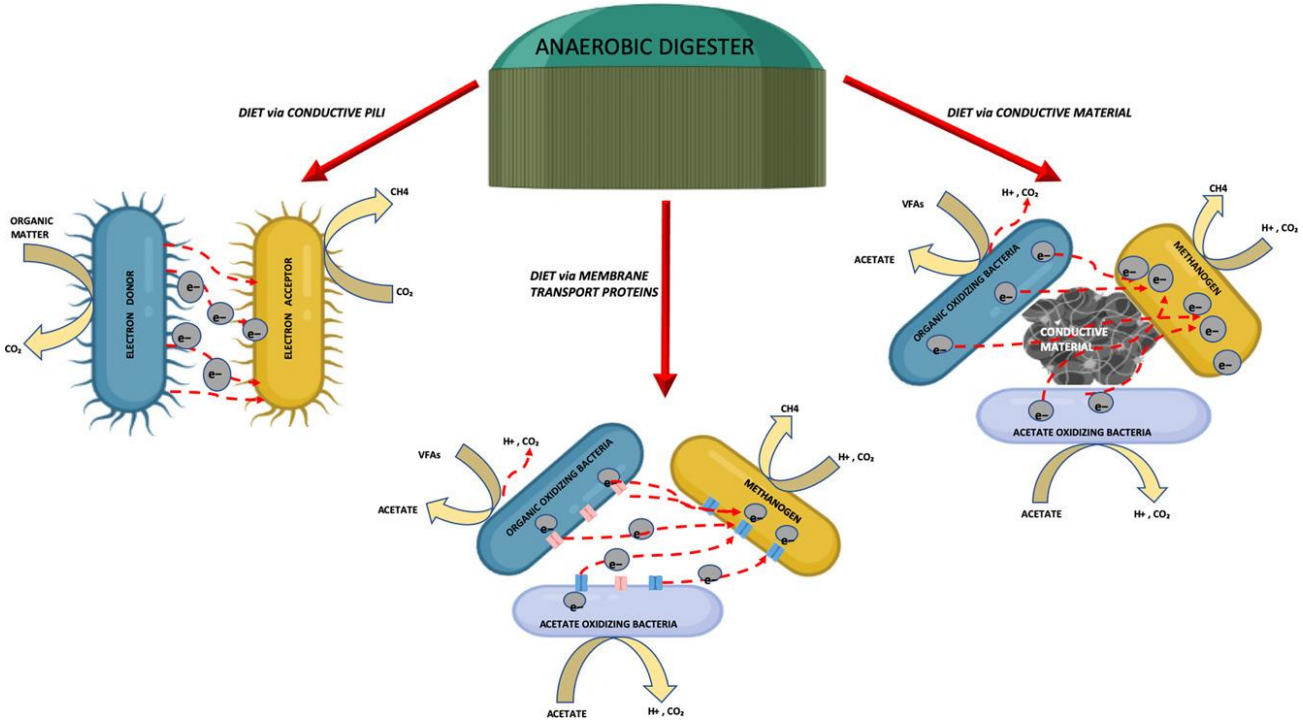


Figure 2.5: Schematic diagram showing three different DIET mechanisms between electron donor and acceptor (a): DIET via conductive pili, (b): DIET via membrane-bound electron transport proteins, (c): DIET via conductive material [32]

2.4.1.1 DIET via conductive pili

In this mechanism, the long-range direct transfer of electrons takes place through the formation of electrically conductive filaments called pili between electrons donating bacteria and accepting methanogens as shown in figure 2.5 (a) [31]. The DIET phenomenon was first described by Summers et al., in defined co-cultures of an ethanol-oxidizing bacteria, *Geobacter metallireducens*, donating an electron to a fumarate-reducing bacteria, *Geobacter sulfurreducens*, through conductive pili instead of using hydrogen or formate as intermediates. The electron transfer does not occur through hydrogen or formate due to the inability of *G. metallireducens* to utilize hydrogen [33]. Rotaru et. al recently showed that the electron transfer directly between *G. metallireducens* and acetoclastic methanogens like *Methanosaeta harudinacea* via conductive pili to produce methane [33].

2.4.1.2 DIET via membrane-bound electron transport protein

Ha et al. observed DIET via membrane-bound electron transport proteins, where electrons were directly transferred between microorganisms through specialized proteins embedded in their cell membranes [31]. Specifically, electrons released by *G. sulfurreducens* were transferred to *Prosthecochloris aestaurii* through outer surface cytochromes as in figure 2.5 (b), without the need for intermediates like hydrogen or formate [34].

2.4.1.3 DIET via conductive materials

In addition to conductive pili and cytochromes, electrons can also be directly transferred between microorganisms through conductive materials which is shown in figure 2.5 (c). In this mechanism, electrons released from the oxidation of organic matter by electron-donating bacteria are transferred to conductive materials, which function as electrical conduits. The electrons then travel through the conductive materials to the electron-accepting methanogens, which use them to reduce CO_2 and enhance methane production [31]. The use of conductive materials reduced the lag time by 10-70%. Lag time refers to the initial phase during which microorganisms acclimate to their environment by synthesizing enzymes and cell constituents that are required for their growth. A shorter lag time indicates that methanogenic organisms grow faster and that there will be higher methane production [34].

Keto et al. were the first to report the DIET phenomenon through conductive materials, which involved the formation of an electrical conduit between *Geobacter* and *Methanosaeta* species using magnetite as a conductive material. This mechanism was found to enhance methane production [35].

2.5 Electron shuttles

2.5.1 Carbon-based conductive materials

Carbon-based conductive materials, such as biochar, activated carbon (either granular or powdered), carbon cloth and graphite, have been used to enhance methane production [32]. Numerous studies have investigated the efficacy of using different concentrations of GAC for promoting DIET [32], employing diverse substrates such as glucose, ethanol, dog food, propionate, and butyrate.

Watanabe [36], Pham et al. [37], and Yang et al. [38] studied the potential of GAC to enhance the methane production in a mixed batch reactor containing activated sludge inoculum and carbon-based substrates. They found that the addition of GAC led to a 17.4% increase in methane production at a concentration of 5 g/L. The researchers noted that the large surface area of GAC facilitated the attachment of microbes and allowed for the adsorption of toxic chemicals.

Lee et al. investigated the effect of continuous mixed acetate substrate on methane production in a mixed reactor. They found that adding 1 g/L of acetate led to 1.8 times increase in methane production. The study suggested that continuous substrate feeding, and regular mixing may enhance the overall methane production process [39].

Yan et al. investigated the effect of adding glucose substrate on methane production in a batch mixed reactor. They found that adding 10 g/L of glucose led to 2.68 times increase in methane production. The researchers noted that the addition of glucose increased the microbial activity and therefore increased the methane production [40].

Liu et al. investigated the effect of adding ethanol substrate in both batch co-culture and mixed reactors on methane production. They found that adding 25 g/L of ethanol in a batch mixed reactor led to 2.5 times increase in methane production, while the addition of ethanol in a co-culture reactor resulted in a short lag phase and 25 times more methane production.

The study concluded that the type of reactor configuration and substrate concentration could significantly affect the overall methane production [41].

Dang et al. investigated the effect of adding dog food substrate on methane production in a batch mixed reactor. They found that adding 50 g/L of dog food led to 18 times increase in methane production. In a semicontinuous mixed reactor, the same concentration of dog food led to 13 times increase in methane production, indicating that the microbial community was resistant to high organic loading. The researchers suggested that using complex substrates such as dog food may improve methane production in mixed reactors [42].

Xu et al. investigated the effect of adding ethanol and glucose substrates on methane production in a batch mixed reactor. They found that adding a combination of ethanol and glucose at a concentration of 5g/L led to increased methane production and improved resistance to high organic loading. The study suggested that using a combination of substrates could enhance the overall performance of mixed reactors [43].

Liu et al. reported that methane production only occurred when granular activated carbon was present in the co-culture of *Geobacter metallireducens* and *Methanosarcina bakeri*. In this process, SEM images showed the microorganisms were found to be rigidly attached to the surface of the conductive materials, rather than being physically or electrically connected, and the transfer of electrons was mediated by the GAC, rather than through conductive pili or electron transport proteins. He also demonstrated that methane production was higher when electrons were transferred through conductive materials compared to conductive pili [44]. This was due to the higher conductivity of the conductive materials.

According to Zhao et al. the use of biochar, which is a more cost-effective option than granular activated carbon, can stimulate DIET between *Geobacter metallireducens* and *Methanosarcina bakeri* in a mixed inoculum when ethanol is used as the substrate. As a result, methane production was found to be increased by 30-45% [45]. In general, carbon-based conductive materials facilitate the transfer of electrons released due the oxidation of alcohols and VFAs by *Geobacter* species, which are then accepted by *Methanosarcina* species to reduce the lag period, enhance methane production, and inhibit ammonia by promoting the growth of microorganisms that belong to these families. In addition, iron-based conductive materials such as magnetite and haematite have been recently used to enhance methane production [32]. The collective findings from these studies indicate that employing conductive materials that have been enriched with DIET-promoting bacteria can serve as a valuable approach for augmenting the performance of anaerobic digestion processes.

Previous research has extensively investigated the efficiency of using different loading rates of AC to enhance methane production. To the best of our knowledge, no research has investigated how different AC concentrations affect methane and biogas output. Hence, taking inspiration from the research discussed above, this thesis explored the effects of different AC loading rates, including 15, 30, 45, and 60 g/L, on biogas and methane production.

2.5.2 Humic acid and AQDS

AQDS has been used as a model compound to study the influence of quinones on anaerobic processes, such as microbial reduction of contaminants and electron transfer in microbial communities Quinone compounds such as humic acid, AQDS acts as the redox mediators (RMs) to improve anaerobic digestion by promoting DIET among microorganisms [46].

Ho and Ho carried out a study in thermophilic condition where he observed a slight increase in methane production rate of about 27-29% and improved degradation of volatile fatty acids when low levels of humic acid (1 and 5 g/L) were added as an exogeneous additive, and the pH was reduced [47]. The study suggested that humic acid might be involved in electron transfer during anaerobic degradation of volatile organic acids.

Xu eta al. performed a batch experiment using 100µM AQDS to study its effects on anaerobic digestion. They found that AQDS improved CH_4 production by 7.3%, maximum CH_4 production rate by 10.8%, and reduced the methanogenic lag phase by 13.8%. He discussed the mechanism by which AQDS improved the anaerobic digestion process involved an electron transfer network among *Anaerolinea* and *Methanosaeta*. These microorganisms transfer electrons to each other, and AQDS functioned as an electron shuttle to facilitate this transfer. This electron transfer network accelerated the process of converting acetate to methane and improved overall methanogenesis. Therefore, AQDS acted as a mediator of this electron transfer network and improved the anaerobic digestion process under ammonia stress [48]. However, the effects AQDS concentrations above and below 100µM was not studied, but it is possible that they could result in an even stronger improvement in organic degradation and methane production. Inspired from the above research done on AQDS, this thesis explored the effects of different concentration of AQDS (0.01, 0.5, 1, 2, and 5 g/L) on the production of methane and biogas.

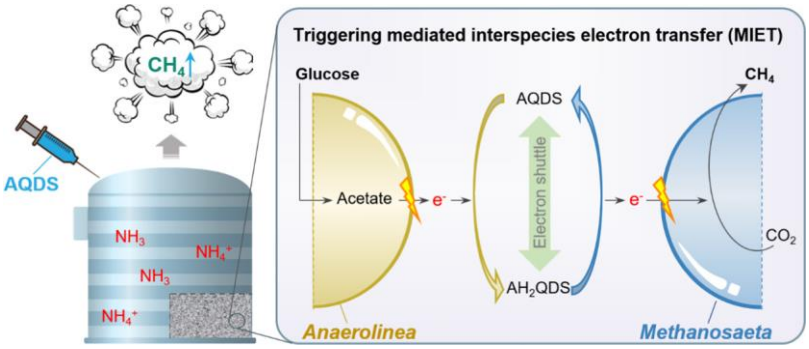


Figure 2.6: AQDS triggered mediated interspecies electron transfer (MIET) [48]

3 Method and materials

This chapter provides an overview of the materials and methods used in the study, including experimental setup, and data analysis procedures. Two conductive materials, activated carbon and AQDS, were used to enhance the methane production. The composition of each experiment involving these two conductive materials is described in this section.

3.1 Inoculum source and preparation

This thesis comprised two experiments, each utilizing a distinct inoculum source. The first experiment involving AQDS employed pig manure obtained from one-litre anaerobic digester operated at USN Vestfold campus. In the second experiment with activated carbon, inoculum was obtained from the Knarrdalstrand municipal wastewater treatment plant in Porsgrunn, Norway. To prevent the inoculum from contributing to total methane production, each inoculum was pre-incubated for approximately 6-7 days for degassing [49] before being supplied with appropriate nutrients and salt media for microbial growth as shown in table 3.1 [50]. Additionally, 0.245 mL of yeast extract has been added to the experiment to support the activity of methanogens during the production of methane

Table 3.1: Nutrients media and their concentrations

Nutrients media	Concentration (g/L)
KH_2PO_4	2.72
$Na_2HPO_4.2H_2O$	3.55
NH_4Cl	0.28
$CaCl_2 \cdot 2H_2O$	0.0076
$MgSO_4.7H_2O$	0.001
$MgCl_2 \cdot 6H_2O$	0.09

3.2 Selection of electron shuttle

3.2.1 AQDS

Anthraquinone-2,6-disulphonate (AQDS) as shown in figure 3.1 is a commercially available chemical used as an electron shuttle to mediate electron transfer between bacteria and methanogens. It was supplied by VWR, a chemical and laboratory supplier. The product characteristics data was provided by the producer [51].

Figure 3.1: Commercial AQDS

3.2.2 Activated Carbon

The experiment used DARCO® activated carbon, a granular product with a 20-40 mesh particle size, as in figure 3.2, was commercially available from Sigma-Aldrich. The product's characteristic data was provided by the manufacturer [52].

Figure 3.2: Activated carbon

3.3 Reactor setup

For small-scale bioreactors serum bottles with a total volume of 122 mL were used, with a working space of 50 mL (41%) and a headspace of 72 mL (59%) to allow for gas collection [53]. The serum bottles were flushed with nitrogen gas to displace the oxygen in the headspace before the addition of the microbial inoculum. The flushing minimizes the risk of exposing the bacteria to oxygen and is beneficial for promoting optimal microbial growth and metabolism in anaerobic bioreactors, as well as maintaining anaerobic conditions [54]. To prevent air from entering the bioreactors, blue rubber stoppers and metal caps were used. A picture of a serum bottle used as a bioreactor is displayed in figure 3.3.

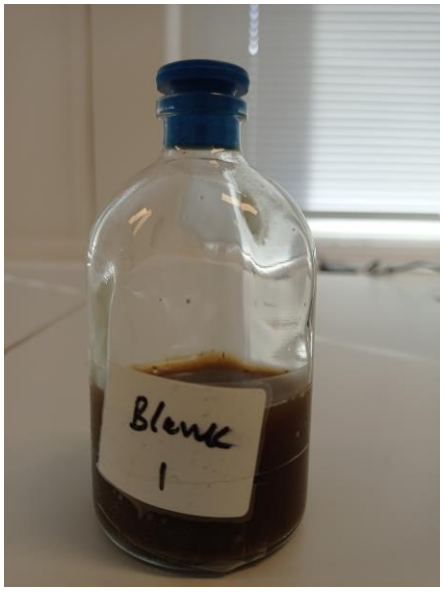


Figure 3.3: Serum bottle used as bioreactor.

3.4 Experimental setup

A biomethane potential test was conducted as part of the experiment to evaluate the samples' capacity to produce methane. The samples were evaluated in duplicates (parallels). The amount of inoculum added to each bioreactor was 50 mL, and the concentration of ethanol in each sample was held constant throughout the experiment. The sample bottles were continuously stirred with the help of magnetic stirrer placed inside an incubation chamber and the speed of stirrer was set at 150 RPM as shown in figure 3.4.

3.4.1 Experiment with AQDS as an electron shuttle

The experiment included a negative control, a positive control made up entirely of ethanol, and four samples as shown in figure 3.3 that each contained AQDS at a different loading rate 0.01, 0.5, 1, 2, and 5 g/L. Each bioreactor received a 50 mL inoculum, and each sample's ethanol content was maintained at 0.0563 mL. The composition of each sample for experiment in BMP test is shown in table 3.2.

Table 3.2: Composition of each sample in the BMP test for experiment

Name	Number of parallels	Inoculum (mL)	Ethanol ($g/L_{inoculum}$)	AQDS) (g/L)	AQDS (g) ($g/L_{inoculum}$)
Blank	2	50	-	-	-
Ethanol	2	50	0.053	-	-
AQDS +Ethanol	2	50	0.053	0.01	0.005
AQDS +Ethanol	2	50	0.053	0. 5	0.025
AQDS +Ethanol	2	50	0.053	1	0.05
AQDS +Ethanol	2	50	0.053	2	0.1
AQDS +Ethanol	2	50	0.053	5	0.25

Figure 3.4: Samples in the bioreactor

3.4.2 Experiment with AC as an electron shuttle

The first experimental trial had to be prematurely terminated due to the failure of the bioreactors to generate any internal pressure and the development of back pressure in some bioreactors that yielded unfavourable outcomes. In addition, there was not sufficient

generation of methane. Consequently, a second experiment involving different concentrations of activated carbon, 15 g/L, 30 g/L, 45 g/L, and 60 g/L, was conducted. In this experiment in addition to AC the bioreactor is fed with 50ml of inoculum and an ethanol content of 0. 192mL.The composition of each sample in the BMP test for experiment is presented in table 3.3.

Table 3.3: Composition of each sample in the BMP test for experiment

Name	Number of parallels	Inoculum (mL)	Ethanol (mL)	AC (g/L)
Blank	2	50	-	-
Ethanol	2	50	0.192	-
AC+Ethanol	2	50	0.192	15
AC+Ethanol	2	50	0.192	30
AC+Ethanol	2	50	0.192	45
AC+Ethanol	2	50	0.192	60

3.4.3 Second batch experiment for experiment 3

Following the depletion of the organic substrate in the experiment involving AC, a subsequent second batch experiment was performed to continue the investigation. In this second batch, 3gm of ethanol, corresponding to 0.192µl, was added to each sample. However, it is worth noting that the volume of inoculum was reduced by 5mL in each sample as it had been utilized to measure the pH and determine the concentration of VFAs at the conclusion of the first batch. Thus, for the second batch experiment, the volume of inoculum was duly adjusted.

3.5 Evaluation of biofilm formation

The evaluation of biofilm formation on activated carbon used as additives in bioreactors was carried out by monitoring the surface area of the particles using Hitachi SU3500 Scanning Electron Microscopy (SEM) as shown in figure 3.5. Initially, the residual activated carbon resulting from the experiment was segregated into individual petri dishes. Subsequently, activated carbon obtained from certain samples underwent a desiccation process within a fume hood. Additionally, activated carbon obtained from other samples was subjected to a salt media rinse and subsequently dried within the fume hood. Thereafter, the samples were subjected to SEM analysis for the observation of biofilm formation [55].

Figure 3.5: Hitachi SU3500 Scanning Electron Microscopy.

3.6 Measurement and calculation

3.6.1 Gas pressure

A pressure measuring device, Bourdon Dial Pressure Gauge (MAT1D10B15), with a 1 bar measurement range as shown in figure 3.6 was used daily to measure the pressure of the biogas being produced.

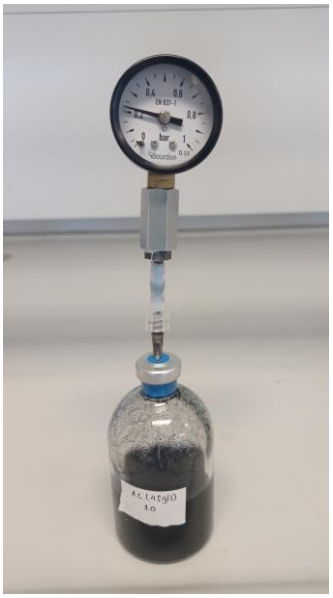


Figure 3.6: Bourdon Dial bottom entry Pressure Gauge 1bar, MAT1D10B15.

3.6.2 Biogas volume

To measure the biogas produced, the pressure inside the bioreactor was measured daily, and an equation (3.4) was used to calculate the volume of biogas [56].

$$PV = nRT \tag{3.1}$$

$$\frac{PV}{T} = nR\ (constant) \tag{3.2}$$

$$\frac{P_r V_r}{T_r} = \frac{P_a V_{biogas}}{T_a} \tag{3.3}$$

$$V_{biogas} = \frac{P_r T_a V_r}{P_a T_r} \tag{3.4}$$

V_{biogas} = volume of daily biogas production (mL)
P_r = pressure in bioreactor (kPa)
P_a = ambient pressure (kPa)
T_a = ambient temperature (K)
T_r = temperature of bioreactor (K)
V_r = headspace volume (mL)

In this experiment the ambient temperature, headspace volume, ambient pressure, and the temperature of bioreactor was set at 25 ℃, 72 mL, 100 kPa, and 35 ℃ respectively.

3.6.3 Gas composition

The gas chromatograph SRI 8610C was used to obtain the corresponding areas of each gas present in the biogas which was later used to determine the concentration of methane, carbon dioxide, oxygen, and nitrogen using equation (3.5). In this procedure, the oven runs at 80 °C with helium acting as the carrier gas at 2.1 bars of pressure and a flow rate of 20 mL/min. A 250 mL/min H_2 flow rate and a 150 °C operating temperature for the Flame Ionization Detector (FID) are required.

$$\%\ Gas = Response\ factor * corresponding\ area\ of\ gas \tag{3.5}$$

The response factor for each gas was also determined using a standard gas which contained 1% O_2, 1% N_2, 38% CO_2, and 60% CH_4 and is given as:

$$Response\ factor = Standard\ \text{Area\%/Standard Area} \tag{3.6}$$

3.6.4 pH

The Horiba pH-33 LAQUAtwin Compact pH Meter as shown in figure 3.7 was used to measure the pH within the bioreactors.

Figure 3.7: Horiba pH-33 LAQUAtwin Compact pH Meter

3.6.5 Total solid and volatile solid

The procedure for the measurement of total solid and volatile solid of the sample follows American standard method APHA 2540 B [57] .

Firstly, a thoroughly cleaned porcelain crucible was taken and dried at 105°C in an oven followed by cooling to a room temperature. Its weight (W1) was then measured using an analytical balance, Sartorius. Then, a 30mL of the thoroughly mixed sample was added to crucible and its weight is noted as W2. Following that, the crucible containing the sample was put in the oven at 105°C. The sample was then removed from the oven and placed inside the desiccator to cool the crucible to room temperature. The weight of the crucible with the sample was measured again (W3). The total solids in the sample were then calculated using an equation (3.7) and (3.8).

$$TS\ \left(\frac{g}{l}\right) = \frac{W3\ (g) - W1(g)}{V\ (l)} \tag{3.7}$$

$$TS\ \left(\frac{g}{kg}\right) = \frac{W3\ (g) - W1(g)}{W2\ (g) - W1(g)} * 1000\ \left(\frac{g}{kg}\right) \tag{3.8}$$

The dried sample obtained after placing the crucible in the oven at 105°C was then placed inside a muffle furnace at 550°C for a duration of 20 minutes, and then allowed to cool to ambient temperature in a desiccator. The crucible containing the sample was then weighed again using the same analytical balance as before, and the weight was recorded as W4. The volatile solids content in the sample was calculated using equation (3.9) and (3.10).

$$VS\ \left(\frac{g}{l}\right) = \frac{W3\ (g) - W4(g)}{V\ (l)} \tag{3.9}$$

$$TS\ \left(\frac{g}{kg}\right) = \frac{W3\ (g) - W4(g)}{W2\ (g) - W1(g)} * 1000\ \left(\frac{g}{kg}\right) \tag{3.10}$$

3.6.6 Volatile fatty acid

A volume of 2 mL of inoculum from each bioreactor was collected and diluted with distilled water in a 1:10 ratio. The diluted mixture was then subjected to centrifuge machine that separates the fluid based on densities. The resulting centrifuged mixture was subsequently filtered using GxF/Glass and wwPTFE syringe filters. To measure the volatile fatty acids, 150 µL of formic acid was added to 1.35 mL of the diluted and filtered mixture in vials. The concentration of different volatile fatty acids was identified using the THERMO Scientific TRACE™ 1300 Gas Chromatograph as shown in figure 3.8.

Figure 3.8: THERMO Scientific TRACE™ 1300 Gas Chromatograph.

3.6.7 Methane yield

The Buswell formula, given by equation (3.11), is widely employed in the computation of methane yield. This formula involves the division of the volume of methane produced by the mass of VS added, multiplied by 100 divided by the methane percentage in the biogas [58].

$$Methane\ yield\ \left(\frac{ml\ CH_4}{g\ VS}\right) = \left(\frac{volume\ of\ methane\ produced}{mass\ of\ VS\ added}\right) * \left(\frac{100}{methane\ percentage}\right) \quad (3.11)$$

Equation (3.12) is utilized to determine the volume of methane produced by considering the total gas volume and methane percentage.

$$\text{Volume of methane} = \frac{\text{Total volume of gas produced} * \text{Methane percentage}}{100} \quad (3.12)$$

Due to the presence of gases other than methane in biogas, the methane percentage division is necessary to calculate the actual methane yield from the total volume of biogas produced. As ethanol is used as the substrate in the experiment, the VS content can be determined by considering the mass of ethanol used, as ethanol is entirely volatile.

4 Result

In this section, the results of the BMP test are presented, which was performed to evaluate the samples' ability to generate methane. The data obtained from the laboratory analysis was subjected to processing, calculation, and graph plotting using MS Excel.

4.1 VS/TS ratio of sludge and inoculum

The anaerobic sludge from the UASB reactor used in experiment 3 contained TS concentrations of 23.23 g/L or 24.83 g/kg and VS concentrations of 10.95 g/L or 11.7 g/kg. Similar to this, it was discovered that TS and VS concentrations of the inoculum were respectively 7.21 g/L and 20.62 g/L and 20.12 g/kg after the addition of nutritional media to the sludge. The VS/TS ratio for the sludge sample was 0.47, while it was 0.35 for the inoculum. In this instance, the sludge's higher VS/TS ratio indicated that it's more readily biodegradable and had a higher potential for biogas production.

4.2 VFAs of inoculum

To determine the types and concentrations of acids present prior to the addition of substrate and conductive materials, two distinct inoculums were subjected to VFA analysis. The inoculum to which AQDS was added subsequently was found to contain only heptanoic acid, with a concentration of 6.65 mg/L. Conversely, when the inoculum on which activated carbon was added subsequently was examined, two distinct acids were identified, as depicted in plot 4.1.

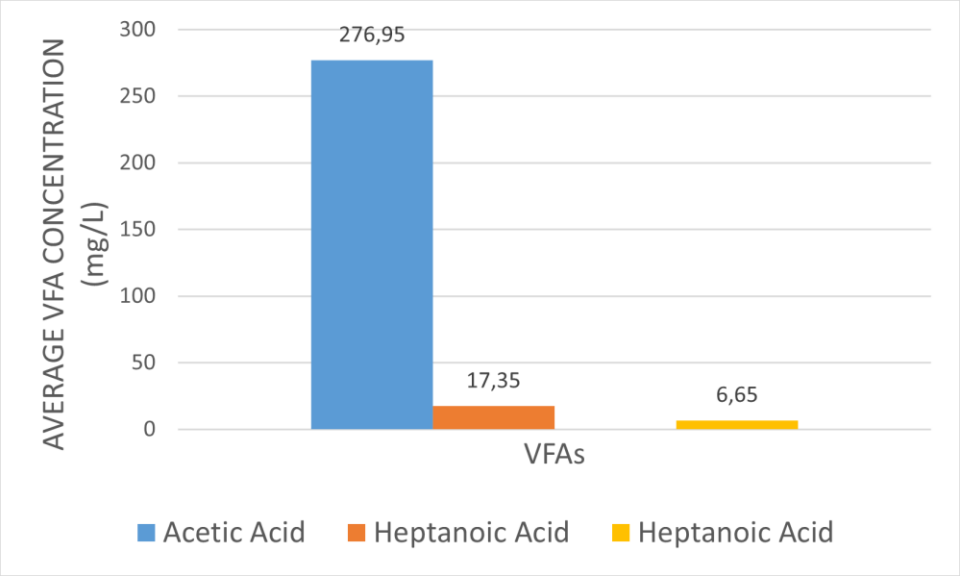


Figure 4.1: VFAs of inoculum used for the experiments

4.3 Effects of electron shuttle on Anaerobic Digestion

4.3.1 Contribution of AQDS to biogas production

The plot in figure 4.2 displays the biogas production over time for different AQDS concentrations used in experiment.

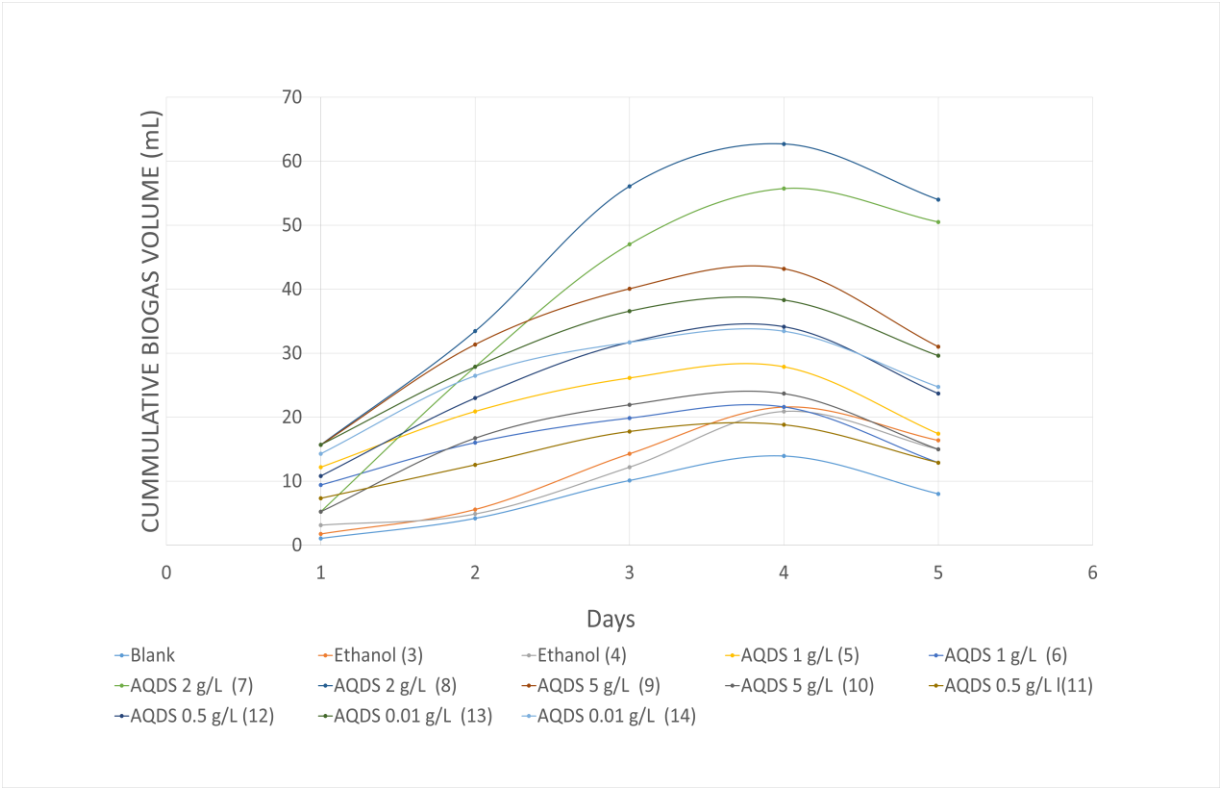


Figure 4.2: Cumulative biogas volume production using AQDS over experimental period

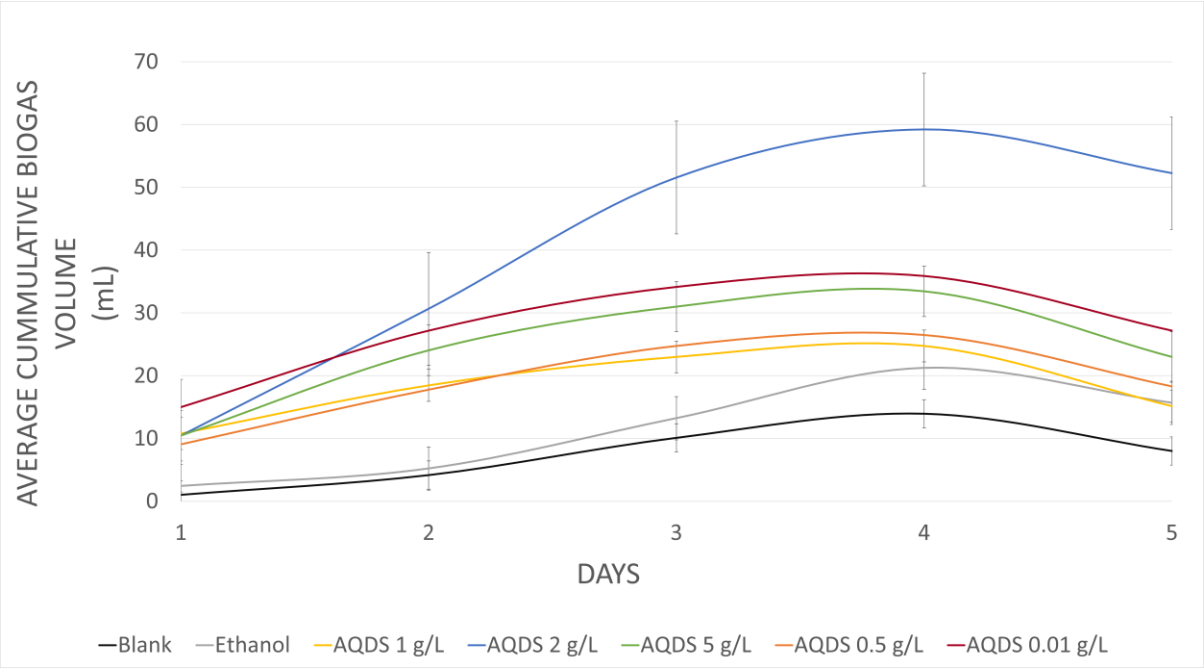


Figure 4.3: Average cumulative biogas volume production using AQDS over experimental period with error bar

Figure 4.3 illustrates the average cumulative biogas volume produced in bioreactors during experiment when different concentrations of AQDS were employed. The result depicts that the concentration of AQDS had significant positive impact on the production of biogas. The AQDS concentration at 2 g/L produces the highest cumulative biogas, followed by concentrations of 0.01 g/L, 5 g/L, and 0.5 g/L. However, to a lesser extent, AQDS at 1 g/L concentration also caused an increase in biogas.

The result suggests that the optimum concentration of AQDS could have been determined, had the experiment was conducted for longer time. As the factors such as temperature, pH changes, microbial population over the time could have affected the results.

Despite the development of pressure and biogas production in the bioreactor, the methane content was found to be too low less than 20% in each sample. The pressure developed in the reactor was attributed to the high nitrogen content, more than 65% in each sample, observed during gas chromatography. In addition, from the fifth day onwards, the reactor failed to generate any internal pressure, and in some cases, even produced a back pressure. Due to the low methane percentage in the biogas, failure to generate pressure and the development of back pressure in the bioreactor, the experiment was terminated after a week to commence a new experiment with a different inoculum and varying AQDS concentrations.

4.3.2 Contribution of Activated carbon to biogas production

The plot in figure 4.4 displays the biogas production over time for different loading concentrations of activated carbon used in experiment.

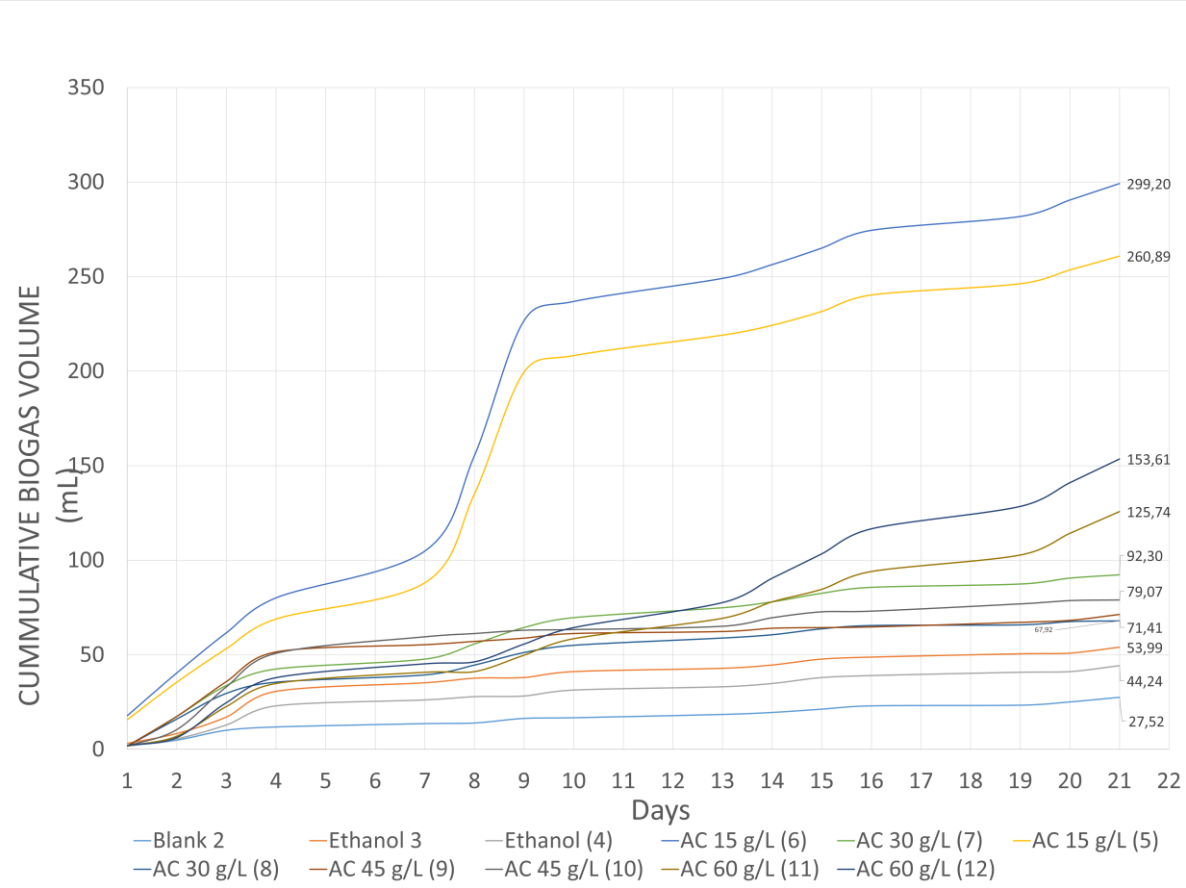


Figure 4.4: Cumulative biogas volume production by varying AC concentrations over experimental period

Figure 4.5 illustrates the average cumulative biogas volume produced in bioreactors during experiment when different concentrations of AC were employed. The plot shows that the addition of activated carbon has significant impact in the enhancement of biogas. The highest average cumulative biogas production was achieved at concentration of 15 g/L with the value of 280.05 mL which was significantly higher than the positive control, ethanol. However, with the increase in concentrations of AC beyond 15 g/L the average cumulative biogas production decreased.

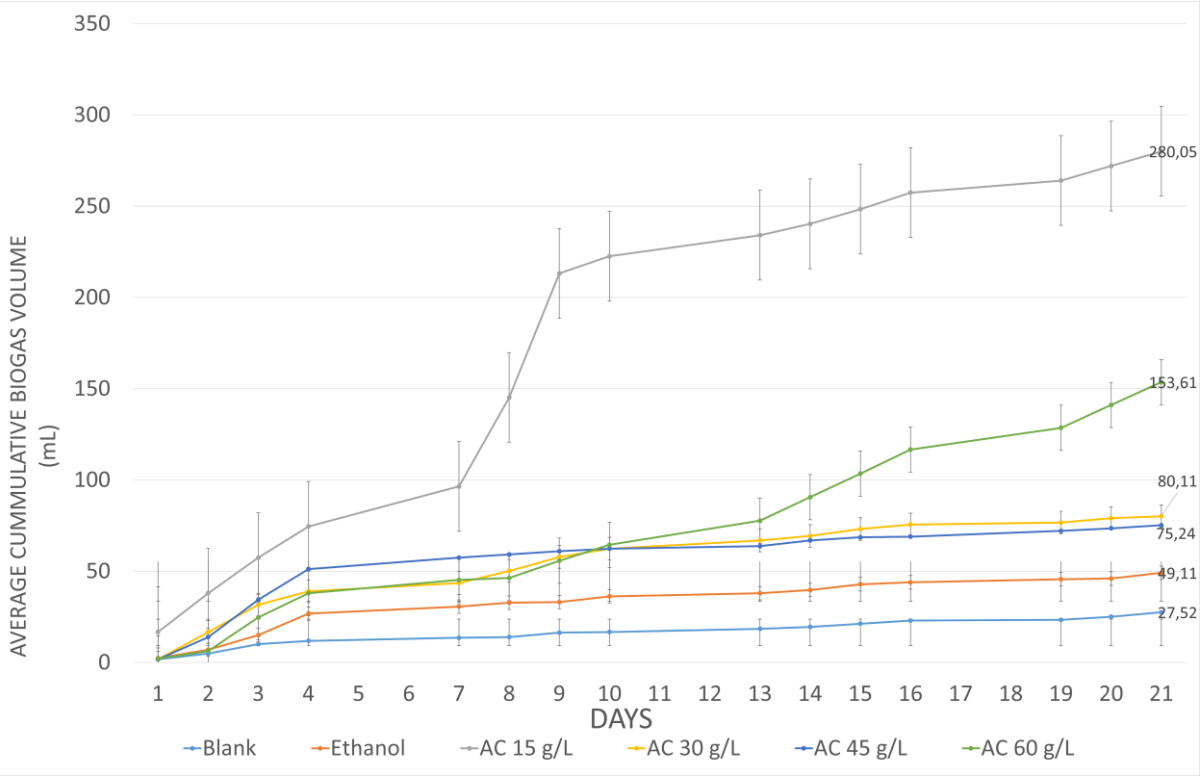


Figure 4.5: Average cumulative biogas volume production by varying AC concentrations over experimental period

4.3.3 Contribution of Activated carbon to methane production

Figure 4.6 shows the average methane percentage of different samples at the end of the first batch experiment. It's observed that the addition of activated carbon at 15 g/L produce the highest methane percentage in the biogas followed by AC at 30 g/L, 45 g/L, and 60 g/L. Additionally, relative to ethanol AC at 15 g/L had slightly higher methane percentage approximately 3.6%. However, with the increase in the concentrations of AC, the methane percentage decrease as compared to ethanol as suggested by the reductions of 1.3% at AC 30 g/L, 9.25% at AC 45 g/L, and 11.3% at AC 60 g/L, respectively.

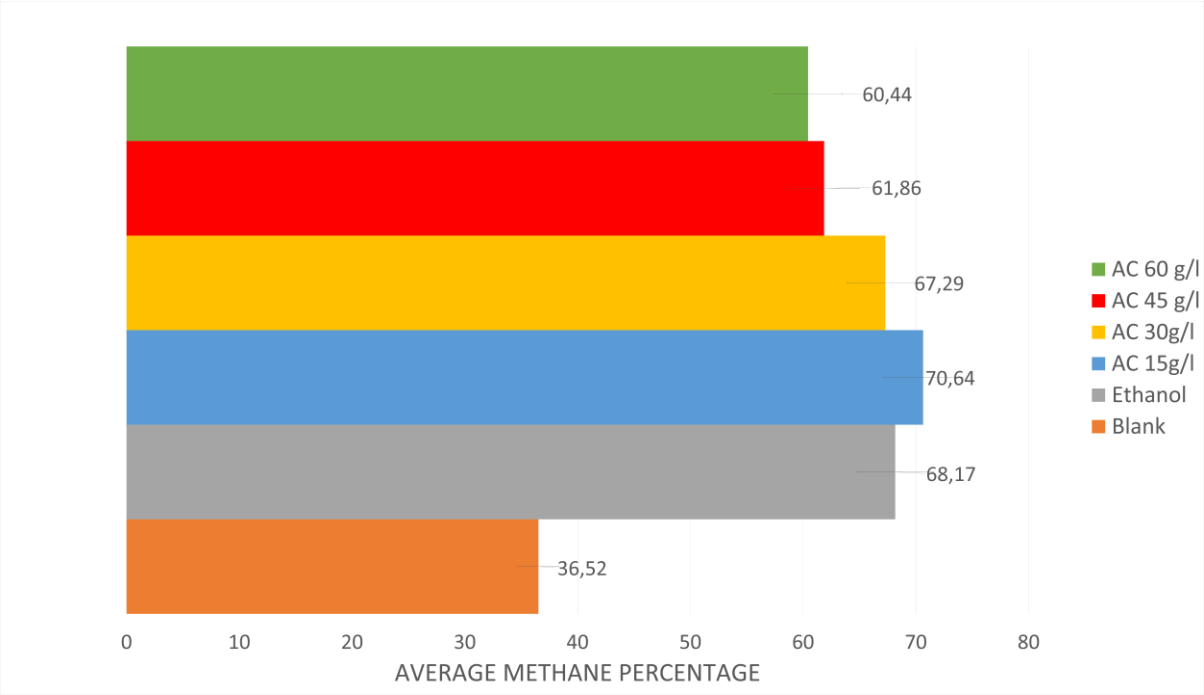


Figure 4.6: Average CH_4 percentage in the first batch with error bars

The figure 4.7 shows the average methane percentage produced over an experimental period from different samples with an increasing trend observed for all samples except the blank. This may be due to the time taken by microorganisms to adapt to the substrate and start producing methane. However, there are some fluctuations in methane production AC 15 g/L, which show a peak in methane production on day 19. This could be due to the depletion of the substrate.

For Ethanol, the highest methane production was observed on day 8 with a value of 79.62%. Similarly, among different loading concentration of AC used, the highest methane production was observed for the AC 15 g/L on day 19 with a value of 79.2%. For AC 30 g/L and AC 45 g/L, the highest methane production was observed on day 8 with values of 76.6% and 71.54% respectively. For AC 60 g/L, the highest methane production was observed on day 14 with a value of 68.8%.

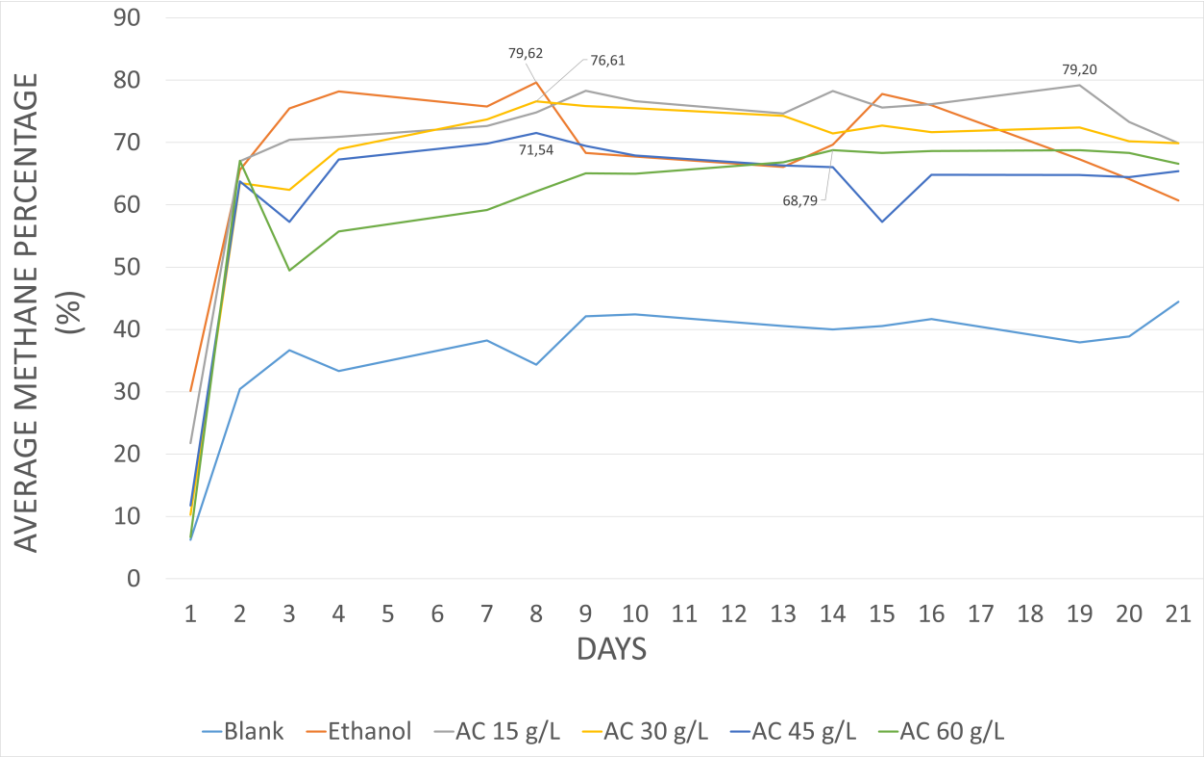


Figure 4.7: Average CH_4 percentage in the first batch over experimental period

The graph presented in figure 4.8 displays the relationship between the average cumulative methane production and time. The data presented in the graph revealed a positive correlation between the addition of activated carbon and methane production. All concentrations of activated carbon showed higher cumulative methane production compared to the positive control. Notably, the highest methane production volume was observed with an activated carbon concentration of 15 g/L, reaching 198.98 mL by day 21. This was followed by activated carbon concentrations of 60 g/L (97.82 mL), 30 g/L (54.47 mL), and 45 g/L (47.07 mL). The trend in methane production showed an initial increase in the first few days showing the adaptation period of microorganisms to the presence of AC, followed by a plateau or a slight decrease before the end of the experimental period suggesting the potential depletion of substrate.

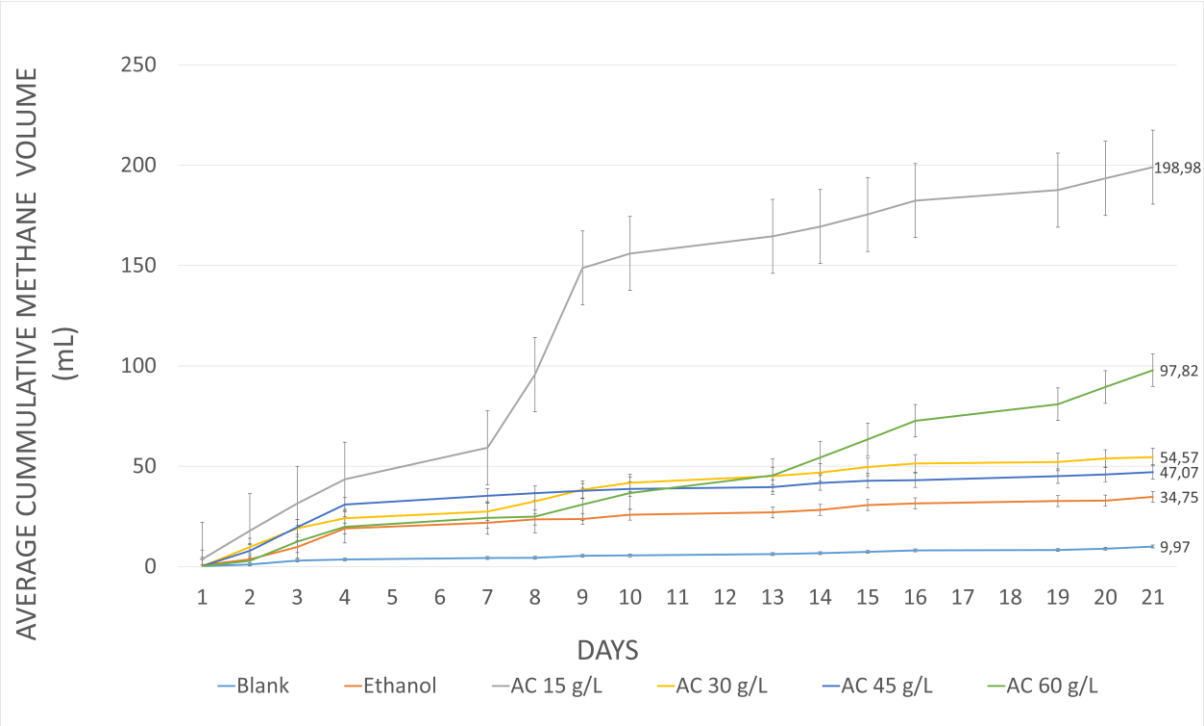


Figure 4.8: Average cumulative CH_4 volume in the first batch

Figure 4.9 displays the graph showing the average methane yield of each sample after removing the blank over the experimental period. From the plot, it's observed that the addition of activated carbon to the substrate can significantly enhance the methane yield during anaerobic digestion compared to the positive control ethanol. The optimal activated carbon concentration was found to be 15 g/L, resulting in the highest methane yield of 93.95 mL CH4/g VS, representing a 5.7-fold increase compared to the positive control. Furthermore, it's also observed that with further increase in the concentration of AC, the methane yield decreased as indicated by the value of the methane yield for 30 g/L of activated carbon, 26.6 mL CH_4/g VS, which is about 3.5 times lower than the methane yield for 15 g/L of activated carbon.

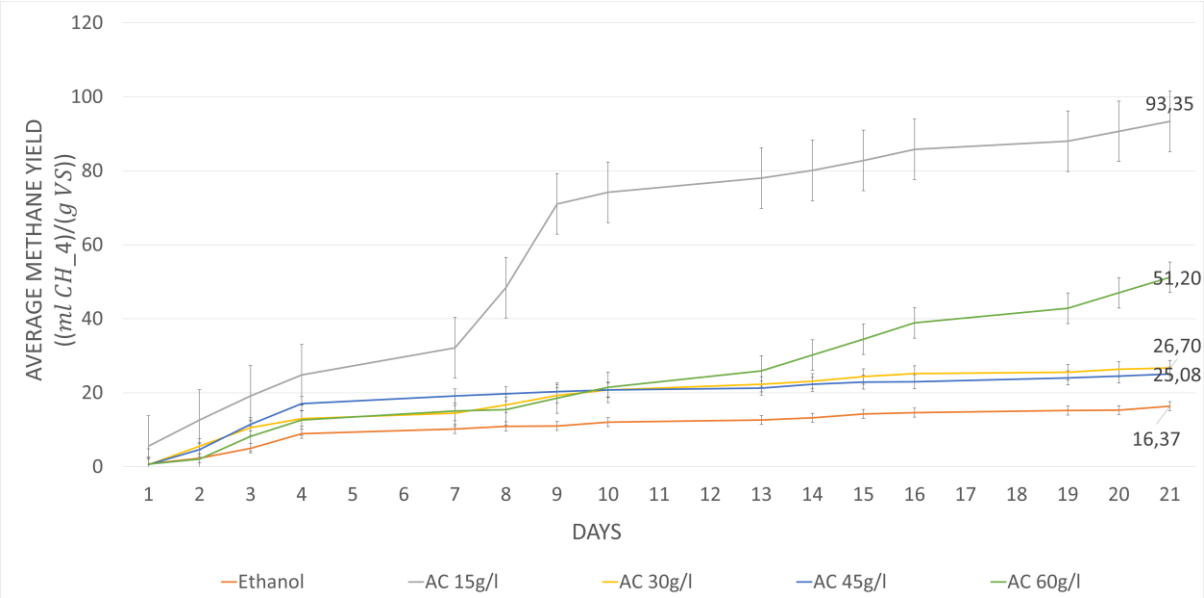


Figure 4.9: Methane yield of each sample after removing blank in the first batch

4.3.4 Volatile fatty acid

Figure 4.10 presents the total average concentration of volatile fatty acids (VFAs) for each sample at the conclusion of the first batch experiment. The blank sample, devoid of ethanol or substrate, exhibited the lowest VFAs concentration of 36 mg/L, indicating minimal organic acid production in the absence of a fermentable substrate. Conversely, the ethanol sample demonstrated a relatively higher VFAs concentration, suggesting successful fermentation of the provided substrate. The activated carbon at 15 g/L exhibited highest VFA concentration followed by AC concentrations of 60 g/L, 30 g/L and 45 g/L.

The results highlight the intricate relationship between AC concentration and VFAs generation, indicating that optimal AC concentrations promote higher VFAs production during anaerobic digestion.

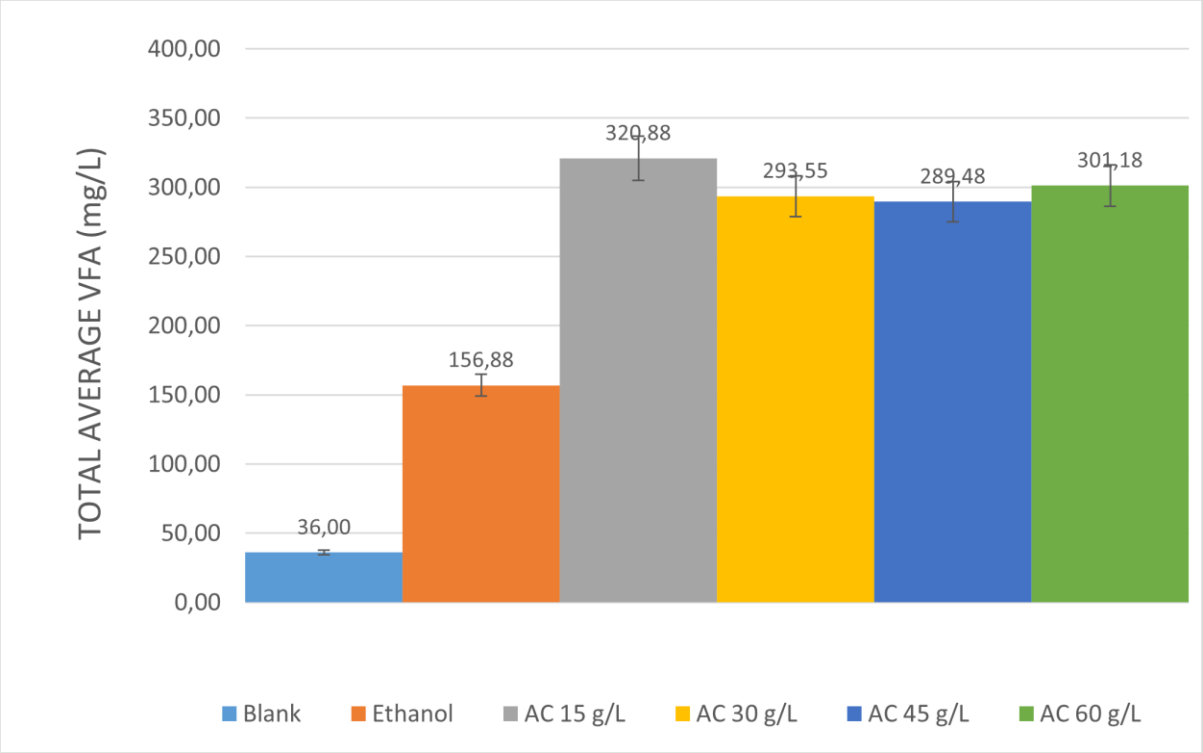
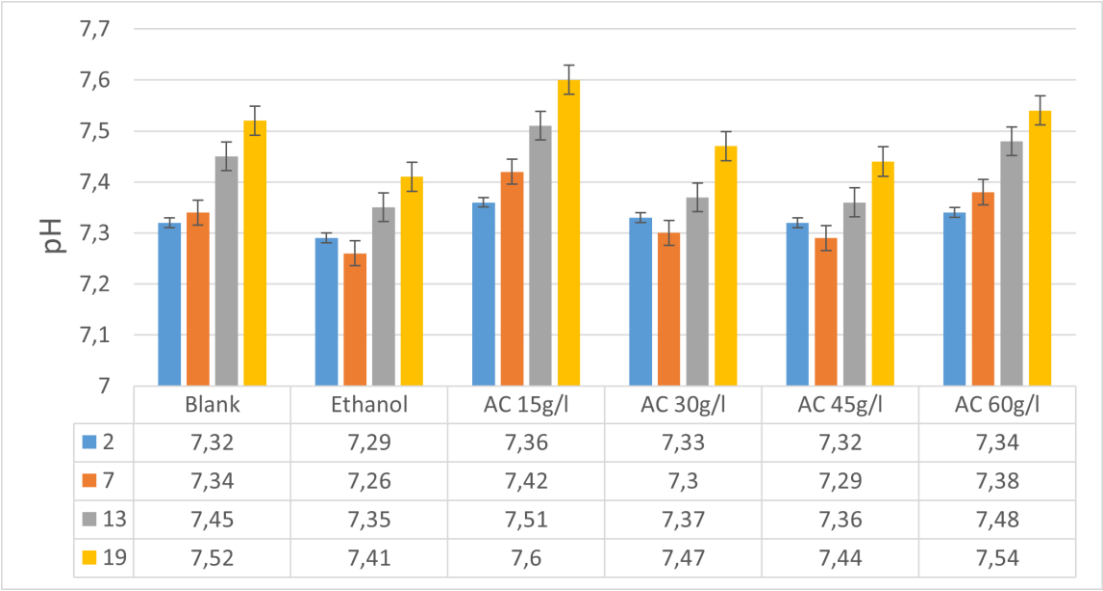


Figure 4.10: Total volatile fatty acids concentration of the samples at the end of the first batch

4.3.5 pH variation

Figure 4.11 illustrates the pH of each sample measured on the 2nd, 7th, 13th, and 19th day of the first batch experiment. It can be seen that pH values of the batch increased over time. Each sample's pH was the same at the beginning of the experiment. It's found that the AC at 15 g/L has the greatest pH values, followed by the AC at 60 g/L when pH was measured at the end of experiment.



	Blank	Ethanol	AC 15g/l	AC 30g/l	AC 45g/l	AC 60g/l
2	7,32	7,29	7,36	7,33	7,32	7,34
7	7,34	7,26	7,42	7,3	7,29	7,38
13	7,45	7,35	7,51	7,37	7,36	7,48
19	7,52	7,41	7,6	7,47	7,44	7,54

Figure 4.11: pH variations over the time with their error bars for the first batch experiment.

4.4 Reusing the electron shuttle for second feeding

4.4.1 Contribution of Activated carbon to methane production

Reusing the conductive material in the second batch experiment resulted in an increase in methane percentage compared to the first experiment. Figure 4.14 shows that with the increase in the concentrations of activated carbon, the methane percentage of the sample also increased. The same trend as of first batch experiment is observed that the addition of activated carbon at 15 g/L produces the highest methane percentage in the biogas followed by AC at 30 g/L, 45 g/L, and 60 g/L.

In comparison to positive control, ethanol, AC at 15 g/L has slightly increased methane percentage of approximately 1.03%. As we go beyond 15g/l, the methane percentage reduces as compared to ethanol with the decreased of 5.2% at AC 30 g/L, 10.8% at AC 45 g/L, and 11.6% at AC 60 g/L, respectively.

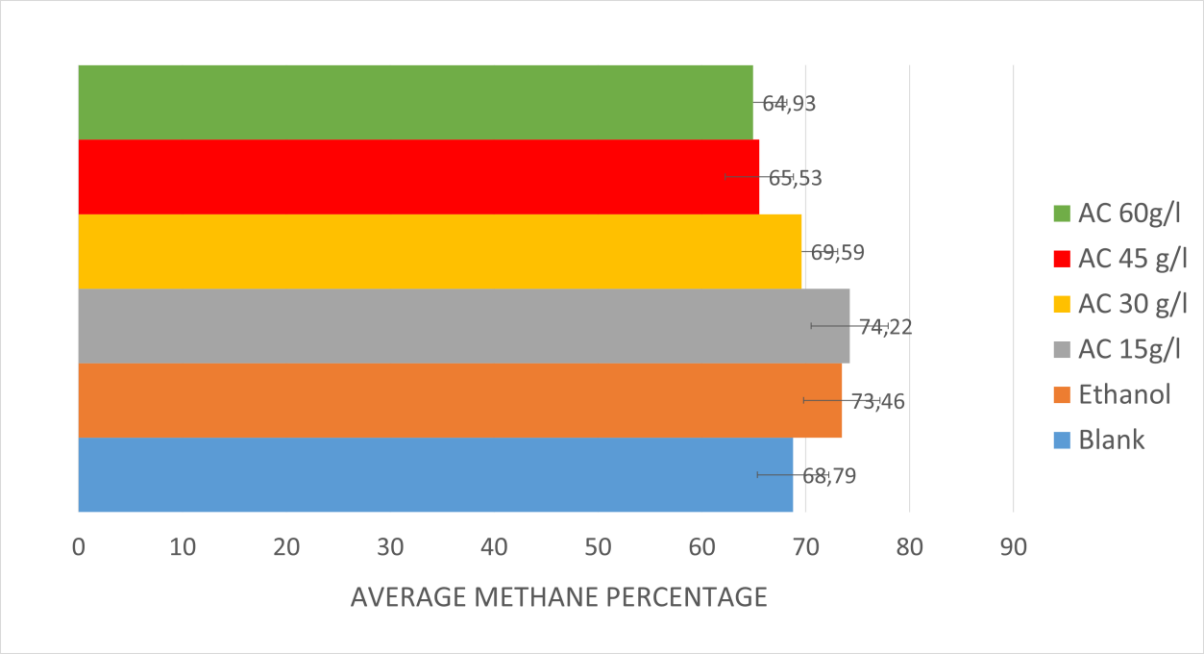


Figure 4.14: Average CH_4 percentage in the second batch with error bars

The reuse of conductive material from the first batch experiment in the second batch resulted in a decrease in the cumulative volume of methane compared to the initial experiment, as observed in figure 4.15. From the plot, it's found that the addition of activated carbon had a positive impact on the methane production, as indicated by the higher cumulative methane production at all concentrations of AC compared to the positive control. By the end of day 19, the 15 g/L AC concentration exhibited the highest methane production volume of 132.36 mL, followed by 60 g/L (69.90 mL), 30 g/L (45.29 mL), and 45 g/L (42.04 mL).

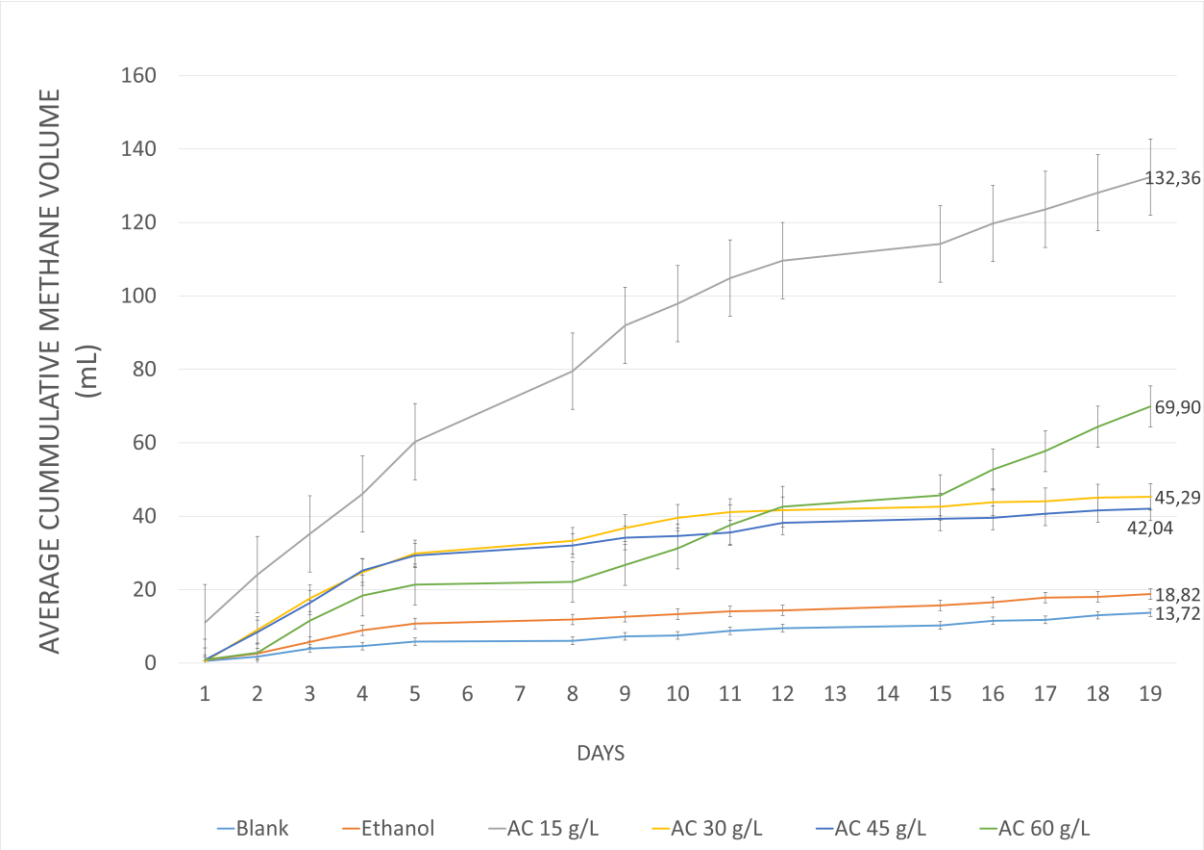


Figure 4.15: Average cumulative CH_4 volume in the second batch

When conducting a second feeding using reused conductive materials, there was a decrease in methane yield compared to the first batch experiment. Figure 4.16 illustrates the average methane yield for each sample over the experimental period. Methane yield from the digestion of the substrate with activated carbon was notably higher than that from ethanol. The highest yield was observed with a 15 g/L activated carbon concentration, resulting in a methane yield of 59.91 mL CH_4/g VS. This yield represented a 7.06-fold increase compared to the positive control without activated carbon. Increasing the concentration of activated carbon beyond 15 g/L led to a decrease in methane yield.

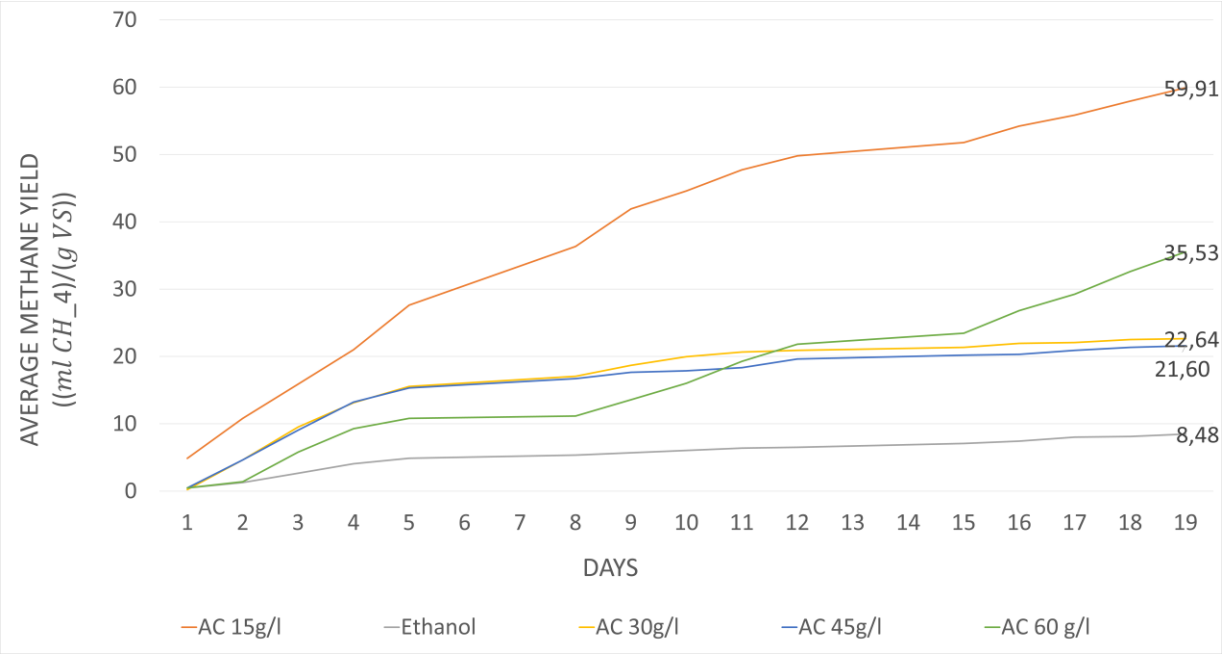


Figure 4.16: Average methane yield of each sample after removing blank in the second batch

4.4.2 Volatile fatty acid

Figure 4.17 shows results of the average concentration of VFAs of each sample obtained at the end of second batch experiment. It is found that the blank had lowest VFAs concentration of 36 mg/L, suggesting that there was minimal production of organic acid in the absence of substrate. On the other hand, the sample with ethanol had a relatively higher VFAs concentration, indicating successful fermentation of the substrate provided. Among the different concentrations of activated carbon (AC) used, the highest VFAs concentration was observed in the sample with an AC concentration of 15 g/L, followed by samples with AC concentrations of 60 g/L, 30 g/L, and 45 g/L, in that order.

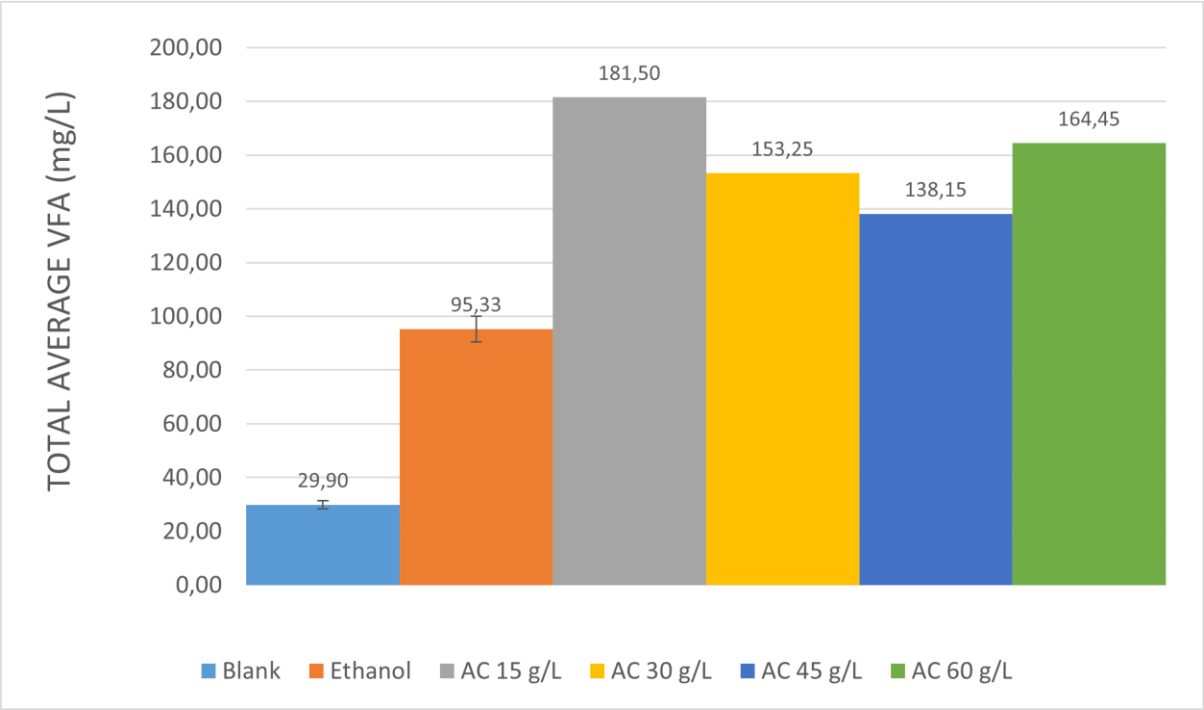


Figure 4.17: Total volatile fatty acids concentration of the samples at the end of the second batch

4.4.3 Contribution of Activated carbon to biogas production

The plot in figure 4.12 displays the biogas production over time for different loading concentrations of activated carbon for the second batch experiment used in experiment 3.

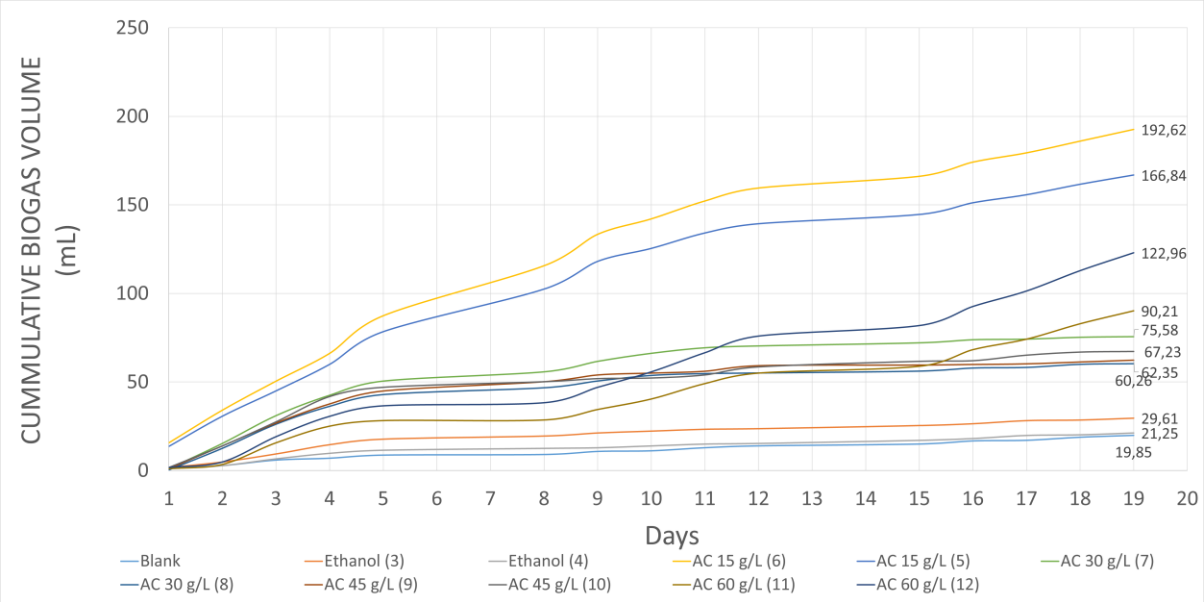


Figure 4.12: Cumulative biogas volume production by varying AC concentrations over experimental period

In the second batch experiment, it was observed that the reuse of conductive material resulted in a decrease in the biogas volume compared to the first batch experiment. Figure 4.13 illustrates the outcomes, where different concentrations of AC (activated carbon) were

utilized. The plot demonstrates that the utilization of 15 g/L of AC still resulted in the highest average cumulative biogas production of 179.73 mL, which was significantly greater as compared to positive control ethanol. However, it's also observed that beyond 15 g/L, the average cumulative biogas production decreased as supported by the value obtained using AC at 30 g/L, 45g/L, and 60 g/L.

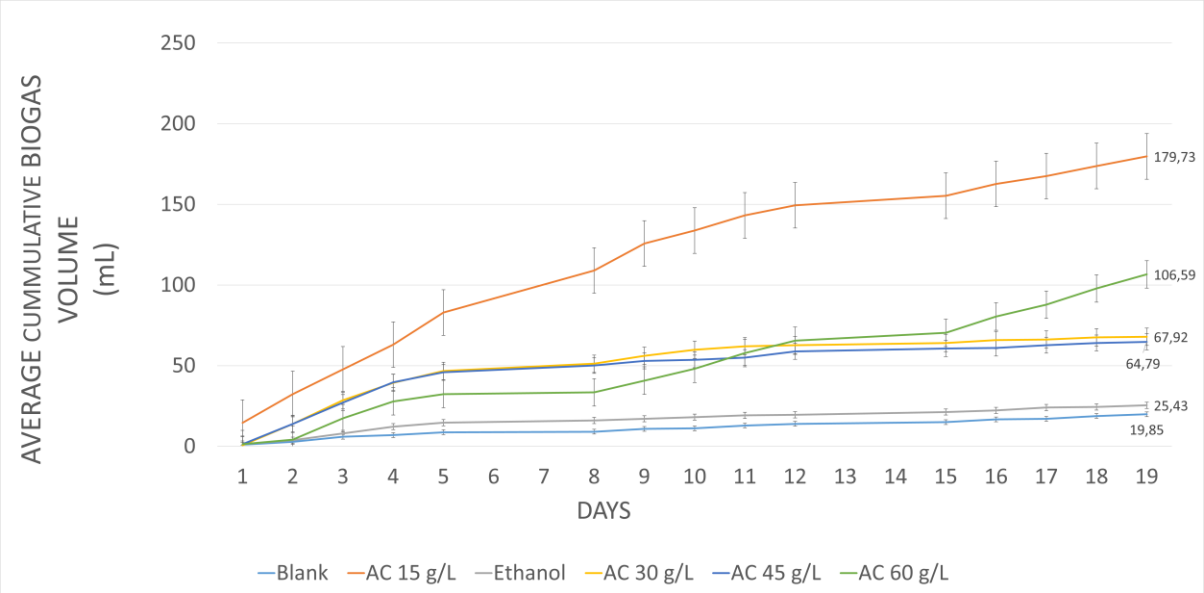
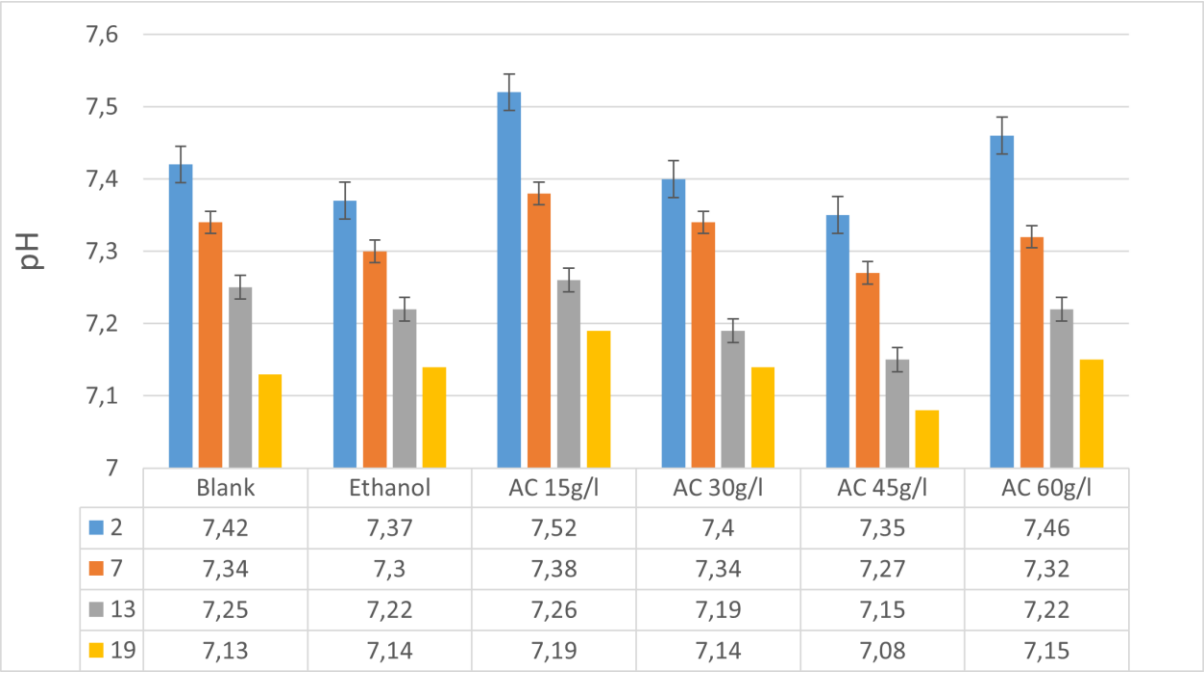


Figure 4.13: Average cumulative biogas volume production by varying AC concentrations over experimental period with error bars

4.4.4 pH variation

In Figure 4.18, the pH values demonstrated a general reduction following the second batch experiment. The activated carbon at 15 g/L and 60 g/L indicated the highest pH levels (7.52 and 7.46, respectively) at the start of the batch. Similarly, these samples exhibited higher pH levels, 7.19 and 7.14 respectively compared to the other samples of activated carbon at the end of the batch. All samples displayed a decline in pH levels to less than 7.2. These observations provide an insight into the pH variations during the experiment.

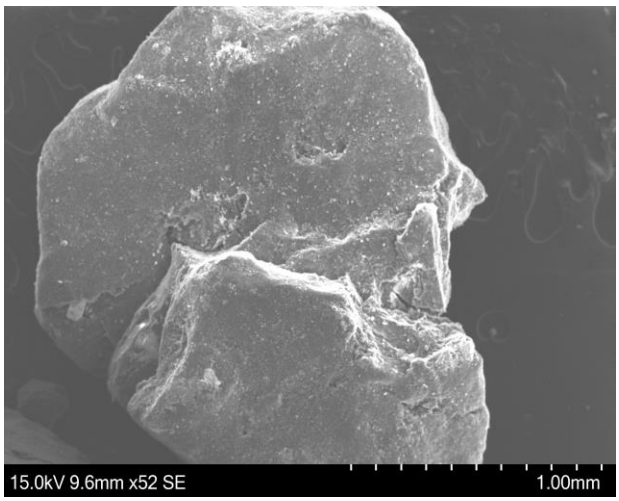


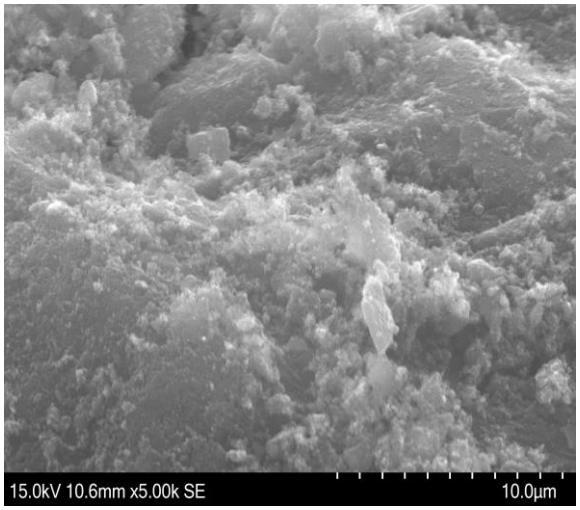
	Blank	Ethanol	AC 15g/l	AC 30g/l	AC 45g/l	AC 60g/l
2	7,42	7,37	7,52	7,4	7,35	7,46
7	7,34	7,3	7,38	7,34	7,27	7,32
13	7,25	7,22	7,26	7,19	7,15	7,22
19	7,13	7,14	7,19	7,14	7,08	7,15

Figure 4.18: pH variations over the time with their error bars for the second batch experiment

4.5 Biofilm formation

Following the completion of the BMP test, SEM was used to scan the biocarbon particles and observe the microorganisms attached to their surface. Figure 4.19 shows the various surfaces of positive control activated carbon using different dimensions and magnifications.





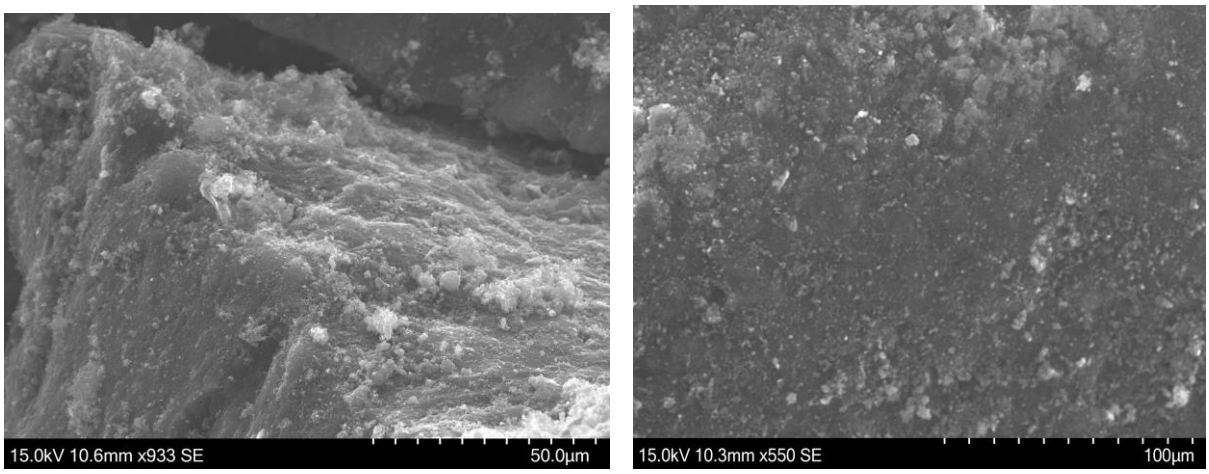


Figure 4.19: Different surfaces of the activated carbon particles from positive control AC sample in dimensions of 1mm, 10µm, 50µm, and 100µm and magnification of 52x, 5000x, 933x, and 530x respectively.

Figure 4.20 shows some fibre-like compound on the surface of the activated carbon particles, but no bacteria were detected.

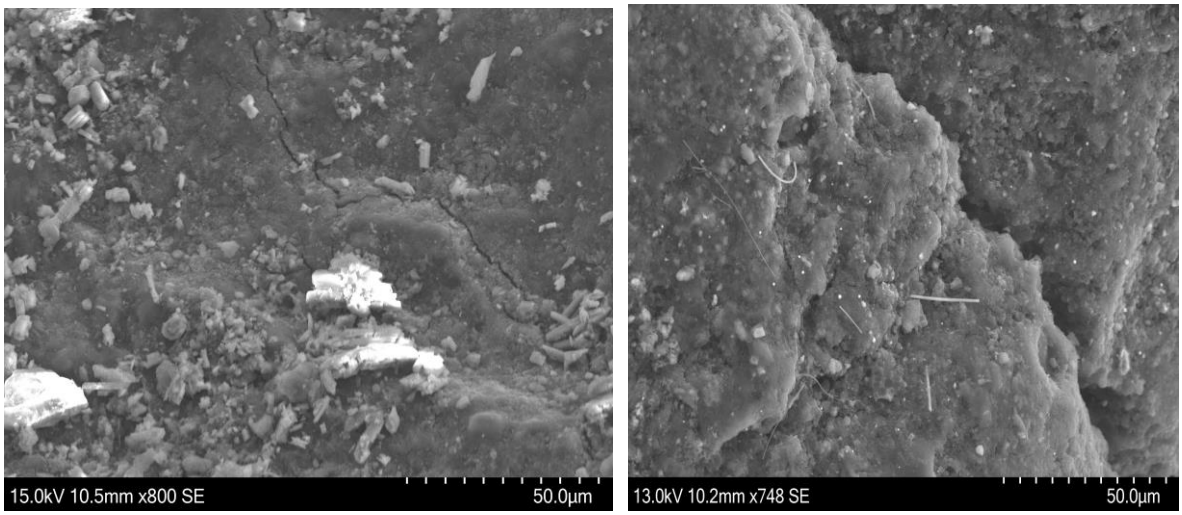


Figure 4.20: Different surfaces of the activated carbon particles from AC of 15 and 30 g/L sample in dimensions of 50µm and magnification of 800x and 748x respectively.

The bacteria were observed on the surface of activated carbon particles with a concentration of 60 g/L as indicated by the blue circles in figure 4.21. Additionally, other unknown compounds were also observed to be attached to the surface of the particles.

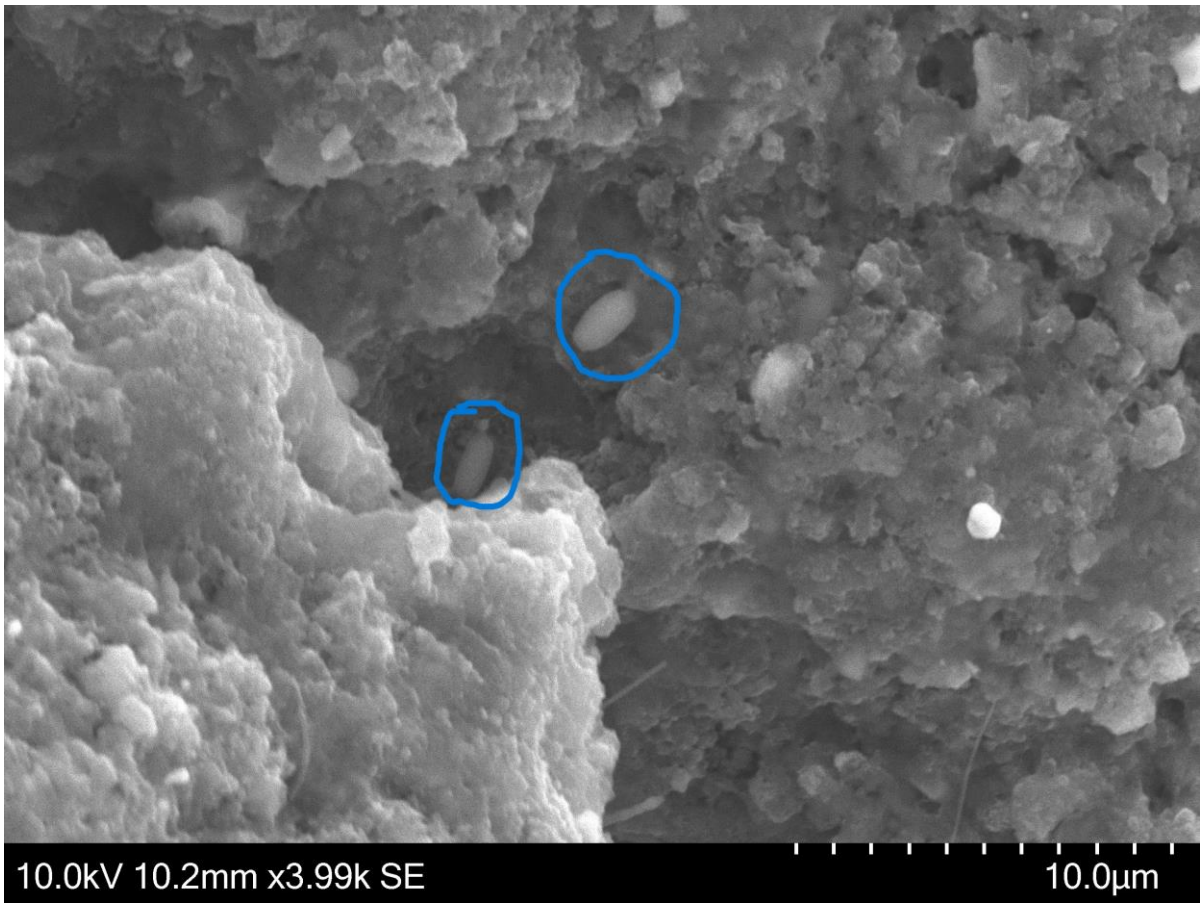


Figure 4.21: Different surfaces of the activated carbon particles from AC of 60 g/L sample in dimensions of 10µm and magnification of 3990x.

5 Discussion

5.1 Analysing AQDS Experiment Failures

Although AQDS-related experiments resulted in the production of biogas, the gas chromatography analysis revealed an unexpectedly low methane content, and an immense quantity of nitrogen were detected, suggesting that the pressure within the bioreactor was not exclusively due to methane and CO_2. While the experiments were not unsuccessful in terms of biogas volume, the insufficient methane concentration rendered them. This situation prompted further analysis to determine what factors may have contributed to the termination of both experiments.

5.1.1 Factors contributing to the excess nitrogen and lower methane production

Multiple factors could have contributed to the elevated nitrogen percentage in the BMP test results. Here are few of the factors that might have come into play.

The inoculum used for the experiment involving AQDS was an old inoculum, and it may have been contaminated with nitrogenous compound such as ammonium, nitrite, or nitrate. These contaminants can contribute to the elevated levels of nitrogen detected during gas chromatography of biogas samples [59]. Also, in one of the bioreactors, the colour of the sample changed as shown in Figure 5.1, which may be due to the contaminants or toxicants present in the inoculum. To eliminate this possibility, a blank control was prepared under identical experimental conditions without the addition of substrate or conductive material. However, the detection of nitrogen in the blank control led to the conclusion that the inoculum may have been contaminated and was one of the contributing factors to the excessive nitrogen production.

Figure 5.1: Color changed using AQDS

Another potential factor that may contribute to an excess of nitrogen is the incomplete biodegradation of the organic substrate, specifically ethanol. When ethanol is not fully digested during the process, residual ethanol may accumulate and elevate the nitrogen content in the sample. The incomplete digestion process can limit microbial activity, resulting in lower methane production, which may have been caused by inhibitory compounds in the sample or a lack of necessary nutrients. Furthermore, the provision of only salt media during experiment may have limit the availability of essential nutrients required to support optimal microbial growth and activity during the BMP test. This might have led to decreased methane production.

The addition of AQDS to the BMP test may have had an impact on the nitrogen level. Previous studies have shown that AQDS can enhance microbial activity and reduce the lag phase during anaerobic digestion, resulting in accelerated decomposition of organic matter and the potential emergence of nitrogenous compounds. However, the mechanism by which the AQDS affects methane production is not clear up to date. Furthermore, the research work carried out in [48], on which our thesis is based-on, lacked reasonable explanations regarding the effect on the nitrogen levels using AQDS. We believe, with the use of AQDS, there may be the possibility of some kind of impact on the nitrogen content of the sample. And further research on this will provide clear insight into the effect of AQDS on nitrogen levels.

5.2 Analysing the results of effects of activated carbon

5.2.1 Effects of activated carbon on biogas and methane volume

The results of this thesis clearly demonstrate that the addition of varying concentrations of activated carbon to the substrate has a notable positive impact on biogas and methane production in the bioreactor. The presence of activated carbon created a favourable environment for microbial growth and substrate utilization, leading to enhanced gas production [60]. The highest cumulative biogas and methane productions were observed with an activated carbon concentration of 15 g/L, which exceeded the production achieved with ethanol as the positive control. However, it is noteworthy that as the concentration of activated carbon increased beyond 15 g/L, a decrease in biogas and methane volume was observed. This suggests the existence of an optimal concentration of activated carbon that maximizes both methane and biogas production, with higher concentrations potentially exhibiting inhibitory effects on the microbial activity responsible for gas production. Interestingly, there were discrepancies in the trend for activated carbon concentrations of 60 g/L, as the cumulative volumes of biogas and methane production were somewhat greater compared to the other concentrations of 30 g/L and 45 g/L.

The considerable enhancement in biogas and methane production can be attributed to several underlying factors. Notably, the high surface area and porous structure of activated carbon might have play a crucial role by enabling the attachment of microorganisms and creating an environment conducive to microbial activity [39]. Microbes involved in anaerobic digestion form biofilms on the activated carbon surface, fostering the degradation of organic matter and subsequent biogas generation [42]. Moreover, the presence of activated carbon provides physical support for microbial consortia, thereby facilitating the establishment of diverse

microbial communities characterized by enhanced metabolic capabilities for biogas production.

Moreover, the adsorptive properties exhibited by activated carbon may have exerted a crucial influence on the enhancement of biogas production. Activated carbon possesses the capability to adsorb volatile fatty acids and other inhibitory compounds present in the substrate [61], which can impede the biogas production process. By adsorbing these inhibitory substances, activated carbon mitigates their detrimental effects, thereby promoting the growth and activity of methanogenic microorganisms and ultimately enhancing biogas production. This adsorption capacity of activated carbon enables the capture of potential inhibitors and facilitates improved substrate utilization, leading to an overall increase in biogas yield.

However, with increasing concentrations of activated carbon exceeding 15 g/L, a notable decline in biogas and methane production was observed. This variation in the trend can be attributed to several underlying factors. As the concentration of activated carbon increases, there is a possibility of particle agglomeration, resulting in a reduction in the available surface area for microbial attachment and activity [62]. This agglomeration phenomenon can impede the accessibility of the substrate to microorganisms, leading to a decrease in both biogas and methane volume. Moreover, the higher concentrations of activated carbon may be associated with increased adsorption capacity, which could potentially adsorb and retain valuable nutrients from the inoculum. As a result, the nutrients necessary for the microorganisms for their growth and metabolic activity is limited. Thus, the limited availability of nutrients for the methanogens can be attributed to the decreased methane and biogas volume observed at higher concentrations.

5.2.2 Discrepancies in the trend for activated carbon concentrations at 60 g/L

In contrast to the observed trend for higher concentrations of activated carbon beyond 15 g/L, it was initially anticipated that the biogas and methane volume would decrease at a concentration of 60 g/L. However, contrary to expectations, the cumulative volume of methane and biogas produced at 60 g/L of activated carbon was unexpectedly higher compared to concentrations of 30 g/L and 45 g/L, exhibiting nearly double the gas production. This intriguing finding indicates the existence of influential factors that may have contributed to this notable difference in gas production at this specific concentration.

One of the explanations for these unexpected productions could be the microbially-favoured conditions created by the activated carbon at 60 g/L. These conditions could have enhanced the growth and the metabolic activities of some specific microbial species which were spotted during the SEM test, consequently, leading to an increase in biogas production. For instance, these spotted microbial species could have developed specific enzymes such as hydrogenase, formate dehydrogenase, and acetyl-CoA synthetase [63] that are capable of efficiently degrading the organic matter. Similarly, the addition of GAC stimulates the biosynthesis of essential microbial nutrients which enhanced the production of methane in the reactor as shown by the study [64]. In the case of 60 g/L activated carbon, it is conceivable that these microbial species have acquired specific adaptations that promote their growth and stimulate enhanced methane production. To gain deeper insights into the microbial community and their functional traits related to biogas production at the concentration of 60 g/L of activated carbon, further investigations such as microbial profiling [65] would provide valuable information.

Furthermore, the adsorptive capacity of activated carbon likely played a significant role in the observed increase in biogas and methane volume at a concentration of 60 g/L. At this

concentration, activated carbon may have exhibited selective adsorption by retaining volatile fatty acids (VFAs) and other inhibitory substances, while simultaneously adsorbing valuable nutrients necessary for microbial growth and activity. This selective retention of inhibitory compounds, coupled with the adsorption of essential nutrients, could have created an optimal microenvironment that facilitated the metabolic activity of methanogenic microorganisms, ultimately leading to the enhanced production of biogas and methane. The adsorptive properties of activated carbon at 60 g/L likely provided a favourable balance between inhibitory substance removal and nutrient availability, allowing for improved microbial performance and gas production. Consequently, the cumulative volume of biogas and methane was significantly increased when employing activated carbon at this concentration.

The activated carbon concentration of 60 g/L may have exhibited an improved surface area and porosity, which in turn facilitates the attachment, growth, and metabolic activity of methanogenic microorganisms. The increased surface area offers additional sites for microbial colonization, consequently promoting the formation of biofilms as shown by the presence of bacteria at this concentration. These biofilms provide an optimal microenvironment to foster the growth and metabolic activity of bacteria, thereby enhancing the production of biogas and methane.

5.3 Reuse of conductive materials in second batch

In the second batch experiment, the conductive materials utilized in the first experiment were subjected to re-use. A decrease in biogas and methane volume was observed in the second experiment when compared to the first. Nevertheless, the highest values for average cumulative biogas, methane volume, methane percentage, and methane yield were still obtained with an activated carbon concentration of 15 g/L. This outcome suggests that despite the reuse of the activated carbon, methanogens could still employ it to facilitate electron transfer between electron-donating bacteria and methanogens, albeit to a lesser extent.

The observed increase in methane percentage in the second experiment may be attributed to the utilization of the remaining conductive material by methanogens as an electrical conduit. This may have potentially facilitated a better utilization of the substrate. This indicates that although there was a decrease in methane and biogas volume in the second experiment, a higher percentage of methane was observed, suggesting that the microorganisms may have efficiently converted the available substrate into methane.

The results indicate that despite the reuse of conductive materials, AC still retains some capacity to facilitate the electron transfer process and enhance methane production, albeit to a lesser extent. However, the decrease in biogas and methane volume suggests that reusing the conductive materials may not be economically viable due to the potential loss of effectiveness.

5.4 Biofilm development

Upon SEM analysis of the conductive materials, the absence of biofilm development was observed. The possible reasons for this could be the use of a non-active inoculum or the inoculum that was used in this thesis may contain contaminants or inhibitory substances [66]. The non-active or contaminated inoculum could lack viable microorganisms, thus hindering the growth and attachment of microorganisms on the surface of AC, ultimately resulting in the non-formation of a biofilm. Additionally, the microorganism requires nutrients for their

metabolic activity and their growth to form biofilm. The contaminated inoculum may not provide the sufficient nutrient to methanogen to create biofilm [67].

6 Conclusion

In conclusion, this thesis investigated the potential of different electron shuttles to enhance the production of biogas and methane in anaerobic digestion. While the initial focus was on AQDS, the results were not favourable, and the study was then pivoted to the use of activated carbon as a conductive material at different loading rates. The BMP experiments showed that activated carbon had a significant positive impact on biogas and methane production, with the highest cumulative production observed at 15 g/L. However, concentrations above this level exhibited inhibitory effects on microbial growth and activity responsible for methane production. Notably, there were discrepancies in the trend for activated carbon concentrations of 60 g/L, which showed somewhat greater cumulative volumes of biogas and methane production compared to other higher concentrations i.e., 30 g/L and 45 g/L. Apart from this, only a single batch process was considered during experiment. We believe that the discrepancy in the 60 g/L can be nullified if several batches of experiments were run to verify the trend observed for 60 g/L statistically.

The study further suggests that the economic viability of reusing conductive materials may be limited, given the decline in their effectiveness over time, as indicated by a reduction in biogas and methane production. This decrease in performance may be attributed to the higher susceptibility of conductive materials to adsorb organic pollutants, which could impede the attachment of microbial cells to the material surface. Consequently, introducing fresh co-substrate into the bioreactor may be necessary to enhance the efficacy of conductive materials for their reuse.

The results obtained from the utilization of AQDS as electron shuttles initially suggested a potential for enhancing biogas production, but the methane percentage obtained ultimately rendered them unsuccessful. The study delved into an analysis of the factors responsible for the failure of AQDS, identifying the use of aged inoculum that could have been contaminated, incomplete biodegradation of organic substrate, and the lack of investigation on the effects of nitrogen on AQDS. These findings shed light on the limitations of AQDS as an electron shuttle and underscore the need for further research to obtain a comprehensive understanding of the effects of AQDS on nitrogen levels in the context of the BMP test that can optimize the anaerobic digestion process.

The absence of biofilm development on activated carbon, as observed through SEM analysis, is likely due to the use of non-active or contaminated inoculum and insufficient nutrient availability for the growth and metabolic activity of microorganisms responsible for biofilm formation. These findings emphasize the significance of proper inoculum preparation and the importance of adequate nutrient supply in anaerobic digestion processes.

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Appendices

Appendix A Task Description

University of South-Eastern Norway

Faculty of Technology, Natural Sciences and Maritime Sciences, Campus Porsgrunn

FMH606 Master's Thesis

Title: Application of electron shuttle on anaerobic digestion for carbon dioxide reduction.

USN supervisors: Nabin Aryal and Eshetu Janka Wakjera

External partner: Senior lecturer Pabasari Arundathi Koliyabandara (University of Sri Jayewardenepura SriLanka)

Task background:

Anaerobic digestion (AD) is one of the versatile technologies to produce biofuel in the form of methane (CH_4). However, the presence of a significant amount of carbon dioxide (CO_2) limits the application of biogas, such as transportation fuel. Therefore, the in-situ CO_2 reduction approach is one of the best alternatives for the optimization of CH_4 production. Recently, various electron shuttle compounds, such as conductive particles and humic substances, have been tested to optimize CO_2 reduction. Additionally, shuttle compounds enhanced the removal of critical compounds such as antibiotics, ammonium etc. The electron shuttle compounds involved in the electron transfer phenomenon from the neighboring environment stimulate CO_2 reduction. Thus, the electron shuttle has a positive impact on AD. In this proposed Master thesis, electron shuttle anthraquinone-2,6-disulfonate (AQDS) and activated carbon will be tested in different concentrations to evaluate the CO_2 reduction rate on AD. AQDS has shown the potential electron mediator to perform various redox reactions such as (bio) electrochemical processes. AQDS is a representative analogue of humic acid and has the capacity to mediate the extracellular electron transfer for CO_2 reduction in AD without any adverse impact. Furthermore, activated carbon has shown the stable operation of anaerobic digestion for biogas production.

1. Literature review on application of electron shuttle on AD process.
2. Application of electron shuttle in different loading rates.
3. Perform biomethane potential test for methane production on AD process.
4. Critical analysis on mechanism involvement in electron transfer.
5. Data analysis and interpretation to understand the AD process.
6. Experimental set-up: An experiment will mimic AD on a 100 mL serum bottle. The anaerobic sludge from the digester will be used as a microbial source, and a carbon source will be provided with different concentrations of commercially available AQDS and activated carbon to evaluate the CO_2 reduction. In addition, essential process parameters such as gas composition, pH, and gas pressure will be periodically tested to assess the CO_2 reduction rate.

Student category: Process Technology student Manjil Bista

Is the task suitable for online students (not present at the campus)? No

Practical arrangements:

The necessary accessories and instruments will be provided. The supervisor will provide theexperiment set-up and operational training at USN, Porsgrunn Campus.

Supervision:

As a general rule, the student is entitled to 15-20 hours of supervision. This includes the necessary time for the supervisor to prepare for supervision meetings (reading material tobe discussed, etc.).

Signatures:

Supervisor (date and signature): Nabin Aryal (01.02.2023 & nabin aryal

Student (write clearly in all capitalized letters): (MANJIL BISTA)

Student (date and signature): 30.01.2023 and Manjil