NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTAMENT OF ELECTRICAL AND COMPUTER ENGINEERING

AIDA VATANPARAST

BSc in Chemical Engineering

ENHANCED ANAEROBIC DIGESTION BY ADDING BIOCHAR AND SELENIUM OXIDE

MASTER THESIS IN RENEWABLE ENERGY ENGINEERING

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Advisers: Doctor Márcia Ventura
Full Professor, NOVA University Lisbon

Doctor Nuno Lapa
Full Professor, NOVA University Lisbon

Examination Committee:

Chair: Professor Pedro Pereira,
Assistant Professor, FCT-NOVA

Rapporteurs: Doctor Inês Matos,
Principal Researcher, FCT-NOVA

Adviser: Doctor Márcia Ventura
Junior Researcher, FCT-NOVA

Master in Renewable Energy Engineering

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ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my supervisors, Professor Doctor Márcia Ventura and Professor Doctor Nuno Lapa for their invaluable guidance, insightful feedback, and unwavering support throughout my research. Their extensive knowledge and experience were instrumental in the completion of this research.

Moreover, I am immensely grateful for the help and personal support provided by my supervisors and the dedicated academic staff of the Department of Chemistry at Nova University of Lisbon. Their contributions were essential in making this thesis a reality.

To Professor Doutora Inês Alexandra Morgado do Nascimento Matos for her presence and her advice in the Laboratory of Chemistry.

To Professor Doctor Pedro Miguel Ribeiro Pereira our coordinator in renewable energy engineering, for the support he provided throughout my study.

To the Valorsul for making it possible to collect the samples used in this study.

I am eternally grateful to my family and my partner, Pouria Babapour, for their unwavering love and support. Their presence in my life has made this journey infinitely more meaningful.

ABSTRACT

The continuous use of fossil fuels and the effect of greenhouse gases (GHGs) on the environment have initiated research efforts into producing alternative fuels from bioresources.

To tackle GHGs emissions in the environment, biogas produced from solid waste and residues through anaerobic digestion (AD) presents a promising alternative to fossil fuels. Recent evaluations indicate that biogas offers significant advantages in both energy efficiency and environmental sustainability. Waste control and its management are crucial to the environment.

While AD of FW is a sustainable method for producing biogas, it faces several challenges that can limit its efficiency. In recent years, significant attention has been given to the AD of organic waste, with the use of additives to enhance biogas yield and process stability. This study investigates the synergistic effects of biochar and Se as additives. By incorporating these substances, the study aims to optimize the AD process and enhance the methane production rate. This research aligns with the global transition towards renewable energy sources and sustainable waste management.

Low concentrations of Se added as sodium selenite (2.5 mg/L and 5 mg/L) boost biogas production, with 2.5 mg/L improving yields by 8% over the control. However, higher concentrations (10 mg/L) can inhibit the anaerobic digestion (AD) process. The combination of Se and biochar enhances AD performance, particularly when Se is deposited in smaller biochar particles at 10 mg/L, yielding an 18% increase over control with biochar and a 75% increase over those without. Biochar mitigates the toxic effects of higher Se levels, fostering microbial communities and promoting electron transfer. Overall, the synergy between Se and biochar can optimize biogas production enabling the use of higher Se concentrations effectively.

Keywords: Anaerobic Digestion, Food Waste, Biochar, Biogas, Selenium

CONTENTS

1. INTRODUCTION 5
1.1 Municipal Solid Waste 5
1.1.1 European Overview of Waste Management 6
1.1.2 Anaerobic Digestion 7
1.2 Food Waste as Substrate On AD 8
1.3 Additives in AD 8
1.3.1 Biochar Addition 8
1.3.2 Selenium Addition 9
1.4 Motivation 10
1.5 Goals of Experiment 10
1.6 Future Outlook 11
1.7 Summary of the Work 11
2 STATE-OF-ART 12
2.1 Basis of Anaerobic Digestion 12
2.1.1 Steps of Anaerobic Digestion 12
2.1.2 Interspecies Electron Transfer (IET) 14
2.2 Products of the Anaerobic Digestion 15
2.3 Types of Bioreactors 16
2.3.1 Single-Stage Bioreactors 16
2.3.2 Multi-Stage Bioreactors 17
2.4 Biogas Production: Feedstock and Effluent 18
2.5 Important Considerations and Parameters of Anaerobic Digestion 19
2.6 Challenges in AD Systems 24

2.7 Additives to Enhance the AD Process 26
2.7.1 Biochar 26
2.7.2 Selenium 31
2.8 Substrate/Feedstock 32
2.9 Research Question 33
3 MATERIALS AND METHODS: 34
3.1 Analytical Methods 34
3.2 The Study Characterization of the material used in this study 35
3.3 Collection and Preparation of Material 42
3.3.1 Inoculum 42
3.3.2 Substrate 43
3.4 Characterization of Biochar 45
3.4.1 Particle Size Distribution of Biochar 45
3.4.2 Biochar Leaching Test 47
3.5 Selenium 48
3.5.1 Adsorption Assay 48
3.5.2 Deposition Assay 49
3.6 Experimental Trials 50
3.6.1 Trial 1 50
3.6.2 Trial 2 51
3.7 Mathematical Modelling of Biogas and Methane Production 55
4 RESULTS AND DISCUSSION 56
4.1 Characterization of the Inoculum, Substrate, and Used Mixtures 56
4.1.1 Characterization of the Inoculum Used in the Trials 56
4.1.2 Characterization of the Substrate 56
4.1.3 Solid Content (TS, VS, and FS) and COD (CODt, and CODs) for Trial 1 57
4.1.4 Solid Content (TS, VS, and FS) and COD (CODt, and CODs) of Trial 2.. 58
4.1.5 Removal Efficiency of VS, TS, CODt, and CODs of Trial 1 and Trial 2 ... 60
4.2 Biochar Characterization 63
4.2.1 Particle Size Distribution 63
4.2.2 Biochar Leaching Test 63
4.3 Trial 1 65

4.3.1 Analysis of Quantity and Quality of Biogas 65
4.3.2 Biogas Composition of Trial 1 66
4.4 Trial 2 69
4.4.1 Analysis of Quantity and Quality of Biogas 69
4.4.2 Biogas Composition of Trial 2 72
4.5 Gompertz Mathematical Module 76
4. CONCLUSION 82
4.6 Future Challenges 82
REFERENCES 83
APPENDIX 91

LIST OF FIGURES

Figure 1. Steps of AD **13**
Figure 2. DIET mechanism between fermenting bacteria and methanogen **15**
Figure 3. Single-stage bioreactor **17**
Figure 4. Multi-stages bioreactor adapted from (Van, 2020) **18**
Figure 5. Parameters of AD **20**
Figure 6. Physical and chemical properties of biochar **27**
Figure 7. Possible benefits involved in AD with nanomaterials **28**
Figure 8. Effects of biochar in stages of AD **29**
Figure 9. Microwave furnace at 105℃ **36**
Figure 10. Muffle furnace at 550℃ **36**
Figure 11 Drying samples at 100℃ **37**
Figure 12. Dried samples for measuring solid content **37**
Figure 13. Process of determination of VS and TS **39**
Figure 14. Preparation digestion at 100℃ for measuring CODt and CODs **40**
Figure 15. Titration the solution for CODt and CODs **41**
Figure 16. Digestion at 100℃ for 75 minutes **41**
Figure 17. Inoculum from the thermophilic digester from the Valorsul **42**
Figure 18. FW mix before grinding and FW after dilution **44**
Figure 19. Process of biochar particle size distribution **45**
Figure 20. Biochar fractions **46**
Figure 21. Biochar leaching test **47**
Figure 22. Process of adsorption of Se into biochar **48**
Figure 23. Process of deposition of Se into biochar **49**
Figure 24. Measuring pH in the first and second days of the trials **51**
Figure 25. Preparation of bioreactors for putting in bath (Grant) **53**
Figure 26. Bioreactors in 50℃ bath (Grant) **53**
Figure 27. Measuring the composition of biogas by GFM **54**

LIST OF TABLES AND GRAPHS

Table 1. Composition of the food residue 43
Table 2. Biochar particle size and Se concentration in each bioreactor for trial 2 52
Table 3. Inoculum characteristics used in this study from the same AD used in other work 56
Table 4. Characteristics of substrate used in this study and the other work 57
Table 5. Characterization of the influent in each sample of Trial 1 58
Table 6. Characterization of the effluent in each sample of Trial 1 58
Table 7. Characteristics of the influent in each sample of Trial 2 59
Table 8. Characteristics of the effluent in each sample of Trial 2 60
Table 9. Concentrations of elements in the biochar leachate 64
Table 10. Kinetic constants of the Gompertz model applied to biogas volume in trial 1 and trial 2 and coefficient of determination (R^2). 78

Graph 1. Removal efficiency of VS, TS, CODs, and CODt in each sample of Trial 1 62
Graph 2. Removal efficiency of VS, TS, CODs, and CODt in each sample of Trial 2 62
Graph 3. Cumulative particle size distribution of biochar 63
Graph 4. Biogas cumulative production for trial 1 66
Graph 5. Biogas composition of each sample in trial 1 68
Graph 6. Biogas cumulative production for Trial 2 Group 1 70
Graph 7. Biogas cumulative production for Trial 2 Group 2 71
Graph 8. Biogas composition of each sample of trial 2 group 1 74
Graph 9. Biogas composition of each sample of trial 2 group 2 75
Graph 10. Adjustment of the Gompertz model to the experimental results of trials 1 and 2 of this work 81

ACRONYMS

AD	Anaerobic Digestion
GHG	Greenhouse Gas
BC	Biochar
GFM	Gas Flow Measurement
FW	Food Waste
WW	Wet Weight
DGA	Dissolved Gas Analysis
EU	European Union
EC	Electrical Conductivity
AS	Anaerobic Sludge
TGA	Thermogravimeter Weight Analysis
GPR	Biogas Production Rate
SGP	Specific Biogas Production
Se	Selenium
TS	Total Solid
VS	Volatile Solid
FS	Fixed Solid
VFA	Volatile Fatty Acid
COD	Chemical Oxygen Demand
CODt	Total Chemical Oxygen Demand
CODs	Soluble Chemical Oxygen Demand
DM	Dry Mass

SYMBOLS

C	Carbon
CH_4	Methane
CO_2	Carbon Dioxide
AC	Acetic Acid
H_2	Hydrogen
H_2O	Water
H_2S	Hydrogen Sulfide
FC	Fixed Carbon
N	Nitrogen
NH_3	Ammonia
O_2	Oxygen
S_{in}	Substrate Concentration in the Influent
S_{out}	Substrate Concentration in the Effluent
V	Volume
X	Biomass Concentration in the Reactor
X_{out}	Biomass Concentration in the Effluent

1. INTRODUCTION

Historical Background: Biogas usage dates back to ancient civilizations, with modern research beginning in the 17th century, focusing on flammable gases from organic decomposition. Alessandro Volta and Sir Humphry Davy contributed significantly to understanding methane and biogas production in the 18th and 19th centuries.

At the start of the new millennium, waste management has become a political priority in many countries. Current waste management practices in developed countries contribute substantially to methane emissions.

In 2015, landfills, animal waste management, and wastewater treatment were among the largest sources of methane, ranking third, fourth, and seventh respectively.

To achieve this goal, renewable energy generation must double its annual growth rate compared to the 2005-2020 period.

Additionally, concerted efforts are required to mitigate the challenges of renewable energy's intermittent nature, develop a mutually reliant decentralized energy grid, and, most importantly, integrate renewable energy into traditionally carbon-intensive industrial processes. (European Environment Agency, n.d.)

One of the main problems today is to cope with an increasing amount of primary waste in an environmentally acceptable way.

Biowastes, i.e., municipal, agricultural, or industrial organic waste, contaminated soil, etc., have traditionally been deposited in landfills or dumped into the sea or lakes without much environmental concern.

In recent times, environmental standards of waste incineration and controlled landfilling have gradually improved, and new waste sorting methods and resource and energy recovery have been developed.

Treatment of biowastes by AD processes is often the optimal way to convert organic waste into useful products such as energy (in the form of biogas) and fertilizer products.

1.1 Municipal Solid Waste

With global emphasis on environmental sustainability, particularly in Europe, waste management is a critical political priority. Municipal waste and biowaste management have evolved from simple landfilling to sophisticated processes such as AD, which reduces methane emissions and produces renewable energy.

Municipal solid waste can be defined (MSW) as household waste or other similar waste like industrial waste, particularly for its nature or composition, namely from the service sector or from commercial or industrial establishments and commercial or industrial establishments

and health care units. According to Decree-Law no. 239/97 of the Ministry of the Environment, in many cases, daily production does not exceed 1100 L per producer.

In the past, the leftover solid waste in unoccupied places resulted in various types of pollution that could put human health at risk, such as the bubonic plague in medieval times. Since the Industrial Revolution, economic and industrial developments have caused more production of waste, and as a result, controlling and managing this huge amount of waste is much more difficult than before (Trotta, 2011).

Despite the early recognition of biogas' potential as an alternative fuel, recent interest has surged due to dwindling natural gas reserves and rising greenhouse gas emissions. In the early 20th century, the value of compost byproducts boosted AD technology and fostered a biogas economy.

Using landfills is the most common way to dispose of this waste, but it is not an environmentally ideal solution. Laws like the European Union's Landfill Directive (1999/31/EC) and Waste Framework Directive (2006/12/EC) discourage landfilling because it takes up a lot of space and harms the environment. Using landfills for waste residues can pollute the soil and the groundwater and can release greenhouse gases that contribute to climate change (Ariunbaatar J. P., 2014).

1.1.1 European Overview of Waste Management

In 2008, the European Union launched the Waste Framework Directive 2008/98/EC, which revised the concepts of Directive 2006/12/EC. This directive established measures to protect the environment and human health, preventing or reducing the current impacts of waste generation and management.

It lists a hierarchy of waste management proposals, with organic recovery as one of the priority treatments. In December 2008, the Green Paper was launched to explore the potential for bio-waste management in the EU (Carrilho, 2012).

In the longer term, the European Union has established the ambitious goal of building a competitive low-carbon economy in 2050 and reaching 80% to 95% GHG emission reduction by 2050 (N. Scarlat, 2015). The Energy Roadmap 2050 investigated possible pathways for a transition toward decarbonizing the energy system and the associated impacts, challenges, and opportunities (Renewables 2020 - Global status report, 2020).

The installed capacity of renewable energy reached 1440 GW in 2012 worldwide, of which 312 GW in the European Union (EU) (EC, 2014). In the heating and cooling sector, biomass, solar, and geothermal energies account for a rising share of final heat demand, amounting to 10% worldwide and even more than 15% in the European Union. The use of biofuels has increased continuously to reach about 106 billion liters worldwide in 2012, of which 82.6 billion liters of ethanol and 23.6 billion liters of biodiesel. In the European Union, 3.7 billion liters of ethanol and 13.7 billion liters of biodiesel were used in 2012, making the EU the highest biodiesel user (European Environment Agency, n.d.) (IEA, 2012).

1.1.2 Anaerobic Digestion

AD is a biological process where organic matter decomposes in an oxygen-free environment, producing biogas (methane and carbon dioxide) and digestate, a nutrient-rich by-product. AD is essential for renewable energy production and minimizing greenhouse gas emissions. The AD reduces greenhouse gas emissions by employing locally available raw materials. The main mechanism behind AD is interspecies electron transfer, which is predominant in bio-methanation. This process can produce biogas, considered a renewable energy, and other by-products.

The biogas that is the output of the process consists of a mixture of different elements such as methane (CH_4) with a concentration of (50%–75%) and carbon dioxide (CO_2) with a concentration of (25%–50%) (IEA, 2012).

The AD has many advantages such as reducing greenhouse gas emissions (GGE), treating wastewater, producing nutrient-rich fertilizer, enhancing soil health, and the process is sustainable and environmentally friendly. The organic content present in waste to energy-rich biogas by the influence of microorganisms in the absence of oxygen.

Although AD has various advantages, it suffers from instability which is a major drawback that can significantly impact the process, regardless of the source of the inoculum or the type of substrate used.

This instability is often linked with the lack of micronutrients or trace elements. To tackle these problems different approaches have been studied. Previous studies showed that using additives can potentially increase the methane production rate (Carvalho, 2019).

The effects of different additives for recovering AD systems from failure have been studied extensively (Ko, 2018), (Kutlar FE, 2022), (M.S. Romero-Güiza a b, 2016).

1.2 Food Waste as Substrate On AD

The FW is a significant part of the organic waste that comes from the organic fraction of municipal solid waste (OFMSW), comprising up to 37% of landfill solid waste disposal (Dung Kong 1, 2012). An estimated 4 million dry metric tons per 38 years of landfilled food and processing residuals could generate 248 MWe in an energy 39 recovery process such as AD (Gary C. Matteson, 2007).

The first-generation biofuels were produced using terrestrial crops such as corn, sugarcane, sugar beets, palm oil, and wheat as a substrate during the last decades, but their cultivation and use have been shown to have negative impacts at environmental and economic levels, due to massive consumption of fresh water needed to maintain these crops and need of fertilizers to grow them properly. FW contains biodegradable solids and high-water content; thus, it serves as a perfect substrate for AD. Nevertheless, previous studies have shown that regardless of the inoculum origin, a prolonged operation of AD on FW, even at low organic loading rates, could suffer from instability due to the increased inhibition by VFA, ammonia, and sulfide (B. Demirel, 2010). This instability is often linked to the lack of micronutrients or trace elements. The effects of TE to recover anaerobic digesters from failure have been studied extensively (Liyuan Zhanga, 2015).

Supplementing TE does not only prevent or recover an inhibition; it can also enhance the AD process and yield a higher biomethane production.

Food waste's total solid (TS) content ranges from 7.62% to 23.70%. The ratio of VS/TS is more than 80% showing a high organic content for biological treatment. The pH of the FW ranges from 3.6 to 6.6, which is not friendly for AD and easily causes acidification. Carbohydrates were the main component in TS, while the lipids also occupy a certain proportion which will harm the AD.

1.3 Additives in AD

Biochar and trace elements like Se are discussed as potential additives to improve AD performance. Biochar improves microbial habitat, enhances electron transfer, buffers pH, and increases methane yield.

Selenium, in trace amounts, supports microbial processes, particularly in methanogenesis, but can be toxic at higher concentrations.

1.3.1 Biochar Addition

Biochar is a carbon-rich material in the production of biomass through thermochemical conversion called pyrolysis. Biochar is a soil additive produced from the thermal degradation of organic material in the presence of little or no amount of oxygen, a process known as pyrolysis. (Shafizadeh, 1982). Biochar has both advantages and disadvantages which are mentioned as follows:

Improving the soil quality by restoring soil degradation; improving agricultural productivity and helping soils retain water. burning biomass to produce biochar produces energy, which can be used for heat or electricity.

Reversibility: the carbon captured via biochar can be released if the soil is disturbed. recognizes bio-residues as valuable resources that can reduce the need for new materials in production chains and contribute to more sustainable material usage.

One reason biochar is gaining interest is that the European Union (EU) has proposed various directives to encourage member states to use bio-residues for sustainable development.

Directive (EU) 2018/851 of the European Parliament recognizes bio-residues as valuable resources that can reduce the need for new materials in production chains and contribute to more sustainable material use.

Sustainable methods for treating residues and converting them into usable materials, such as biomass, have been developed to maximize their benefits. When biomass is utilized correctly and systematically, it can play an important role in the circular economy. This utilization is crucial for sustainable resource production, enabling reductions in greenhouse gas emissions, mitigating pollution (such as eutrophication and acidification of water effluents), and generating green energy.

Biochar addition has been shown to improve AD performance by alleviating inhibitions, promoting microbial growth, and enhancing electron transfer.

Biochar possesses a range of physicochemical properties that can potentially improve AD performance. These properties include high porosity, large specific surface area (SA), good electrical conductivity (EC), excellent ion exchange capacity (CEC), and abundant surface functional groups. (Cai, 2016).

1.3.2 Selenium Addition

During the past years, there have been many studies that confirm the addition of TE can have a positive impact on AD (C.M. Ajaya, 2020), yet, there are still doubts about the kind of trace elements, their toxicity, and the amount of concentration that can be used in the system. According to (Yafan Cai, 2018), the effects of Mo, Se, and Mn on the AD of rice straw were explored. The results showed that the methane yield increased by 59.3%, 47.1%, and 48.9% in the first 10 days following the addition of Mo (0.01 mg/L), Se (0.1 mg/L) and Mn (1.0 mg/L), respectively (Yafan Cai, 2018).

Supplementing TE not only prevents inhibition of the system but also can enhance the AD process and yield higher production.

Regarding their toxicity and accumulation of VFAs (Yafan Cai, 2018) were reached to the fact that the Se, Mo, and Mn elements with higher concentrations of 1000 mg/L can be toxic. The existence of trace elements can change the structure of the bacteria therefore this transformation can influence the performance of the AD (Yafan Cai, 2018); Se is one of the most important elements due to its specific role in microbial metabolism. In the final stages of

an AD process, Se can directly support the growth and activity of methanogenic archaea which leads to higher methane production (Banks, 2012).

Adding Se in the range of 25-50 µg/L can boost biomethane production by 30-35% (Yafan Cai, 2018).

Se also acts as a cofactor for enzymes that break down complex organic matter so it can accelerate overall AD; because of the specific role in methanogenesis and enzyme activity that Se has; therefore, it can be used as a valuable addition to FW substrates.

Se addition can be beneficial in small amounts. However, excessive supplementation can be toxic to the microorganisms involved in the AD process therefore it is necessary to control the concentration of Se participating in the digestion.

Whenever the goal is to enhance the dissolution and bioavailability of a compound, maintaining its amorphous state can be beneficial. However, the amorphous state is often thermodynamically unstable. One effective way to promote and sustain the amorphous form is by loading the compound into a porous carrier. Over the past few years, both silica and carbon-based matrices have been utilized as porous carriers due to their textural properties and compositional control. The inclusion of various compounds within these matrices has proven successful in achieving guest amorphization (Yafan Cai, 2018).

1.4 Motivation

The escalating challenge of FW management demands innovative strategies to optimize AD processes. This research investigates the potential of biochar and Se to enhance biogas production rates, methane yields, and system stability. Biochar has been demonstrated to positively influence microbial community dynamics and nutrient availability, while Se may stimulate enzymatic activity and methanogenic bacterial growth. By systematically examining these factors and their interactions, this study aims to elucidate their synergistic effects on AD. These insights could contribute to the development of more efficient and sustainable waste-to-energy technologies, addressing environmental concerns and promoting renewable energy sources.

1.5 Goals of Experiment

The main goal of this study is to enhance the efficiency of the anaerobic digestion (AD) process of simulated food waste (FW) by adding micronutrient selenium (Se). Selenium was introduced using two different approaches: first, by adding directly Se oxide salt (sodium selenate), and second, by incorporating the salt into a biochar matrix to improve its bioavailability.

Since biochar is usually associated with the improvement of the AD process, the synergistic effect of Se and biochar for this particular case was also determined.

1.6 Future Outlook

The text suggests future research into optimizing AD through the addition of Se nanoparticles, aiming to enhance methane production and reduce system inefficiencies. Se nanoparticles consist of elemental Se, which is less toxic and equally bioavailable compared to ionic inorganic forms.

1.7 Summary of the Work

1) Bibliographic research.

2) Preparation of biochar materials containing Se was performed through the wet deposition of Sodium Selenate into the biochar. Two different particle sizes of biochar were used, and the concentrations of Se varied between 2.5 mg/L Se and 25 mg/L Se.

3) Anaerobic digestion tests of synthetic food waste supplemented with Se were performed under conditions of discontinuous flow (batch). The first trial was performed through supplementation of selenium salt in different concentrations (2,5 mg/L Se, 5 mg/L Se, and 10 mg/L Se). The second trial was performed using the biochar containing Se-prepared materials. Biogas composition was determined, and characterization of feedstock and effluent was performed.

4) MSc Thesis writing.

2 STATE-OF-ART

2.1 Basis of Anaerobic Digestion

AD is a biological natural process that converts organic matter into biogas and generates sustainable and renewable energy in the absence of oxygen.

Biogas typically is a composition of methane, carbon dioxide, oxygen, and traces of hydrogen sulfide. AD depends on several factors such as feedstock pH, temperature, hydraulic retention time, and carbon-to-nitrogen ratio (C/N); and is a relatively slow process. AD reduces greenhouse gas emissions by employing locally available raw materials.

2.1.1 Steps of Anaerobic Digestion

AD consists of four main stages; in the first step, the larger molecules are broken down into smaller sizes such as VFAs by the influence of enzymes and anaerobic bacteria. During the second step, the VFAs are converted to acetate and hydrogen by the action of acerogenins, thirdly the acetate, CO_2, and hydrogen are converted to methane (CH_4) by the influence of methanogens in the methanogenesis stage. These four steps are hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Angelidaki, 2003), (Jay N. Meegoda, 2018).

2.1.1.1.1 Hydrolysis

The first- and most consuming step of the AD process is hydrolysis. During hydrolysis, complex organic polymers, including carbohydrates, proteins, and fats, are degraded into simpler molecules, such as sugars, amino acids, and fatty acids, by hydrolytic bacteria (Duan, Dong, Wu, & Dai, 2012), (Gujer & Zehnder, 1983), (Jay N. Meegoda, 2018).

2.1.1.1.2 Acidogenesis

In acidogenesis, the simple molecules like sugars, fatty acids, amino acids, and glycerol that were obtained from hydrolysis are converted into short-chain VFAs which some of the substrates can be used in the next step (Angelidaki, 2003), (Duan, Dong, Wu, & Dai, 2012) (Jay N. Meegoda, 2018).

2.1.1.1.3 Acetogenesis

The VFAs and alcohols are oxidized by acetogenic bacteria into acetic acid, hydrogen, and carbon dioxide. This stage requires a syntrophic relationship between acetogenins and hydrogen-consuming bacteria, such as methanogens, to maintain a low hydrogen partial pressure that favors the thermodynamics of acetogenesis (Ángela Rodríguez-Abalde 1, 2016).

The most common chemical reactions in acidogenesis are the conversion of glucose into ethanol, propionic acid, and acetic acid:

$C_6H_{12}O_6 \leftrightarrow 2CH_3CH_2OH + 2CO_2$ glucose into ethanol

$C_6H_{12}O_6 + 2H_2 \leftrightarrow 2CH_3CH_2COOH + 2H_2O$ propionic acid

$C_6H_{12}O_6 \leftrightarrow 3CH_3COOH$ acetic acid

2.1.1.1.4 Methanogenesis

Methanogenesis is the final step of AD, where the products provided in the other AD steps (acetate, H_2, and CO_2) are consumed by strictly anaerobic methanogenic. Three main pathways in these bacteria are involved in the process: acetolactic, hydrogenotrophic, and methylotrophic: in acetolactic methanogenesis, acetate is converted into methane. This is approximately a ratio of 2/3 of CH_4 production in AD.

During methanogenesis, CH_4 and CO_2 are produced from CH_3COOH and H_2 in an oxygen-sensitive process by methanogens. The final stage of the AD process occurs in the presence of antibiotics, resulting in poorer methane production (Ferry, 2010), (Jay N. Meegoda, 2018).

$CH_3COOH \leftrightarrow CH_4 + CO_2$

$4H_2 + CO_2 \leftrightarrow CH_4 + 2H_2O$

$CH_3OH + 6H^+ \leftrightarrow 3CH_4 + 3H_2O$

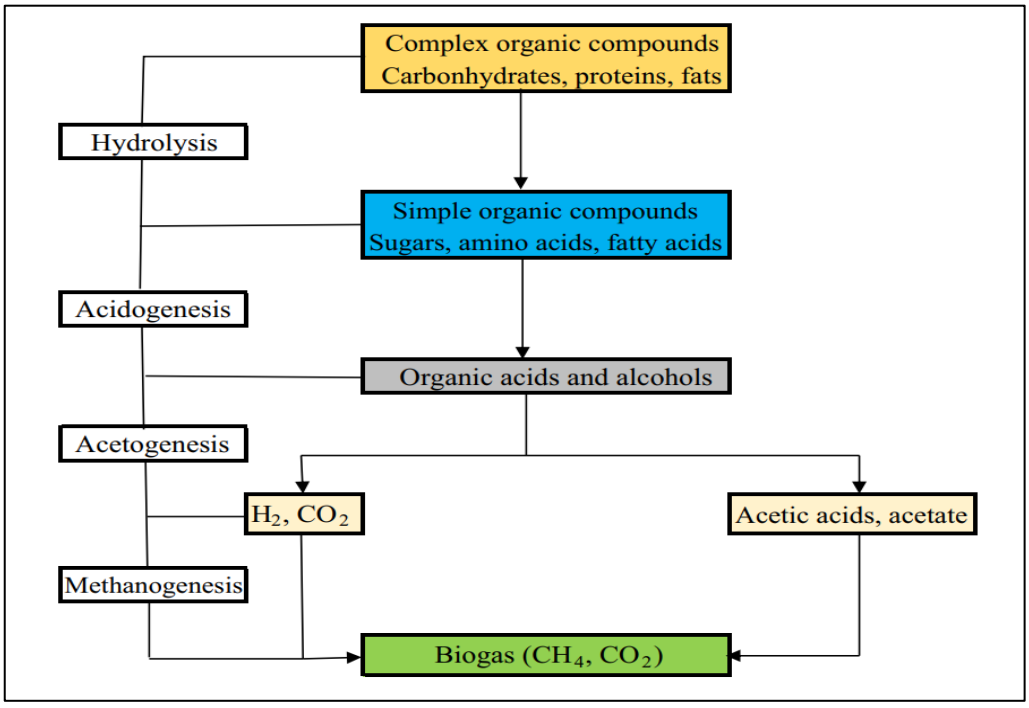


Figure 1. Steps of AD

2.1.2 Interspecies Electron Transfer (IET)

VFAs are formed at the end of the acetogenesis process and further breakdown does not occur naturally, and methanogenesis is an endothermic process.

Hence, there exists a cooperative symbiosis between fermentative bacteria and the methanogen known as syntrophic methanogenesis.

IET is the main driving force of the syntrophic methanogenic process, and it contributes to the utilization of energy during AD (Appels L. B., 2008).

IET occurs in three modes: (i) Interspecies hydrogen transfer: Hydrogen acts as an electron carrier between VFAs and CO_2, (ii) Interspecies format transfer acts as an electron carrier. (iii) Direct interspecies electron transfer (DIET) is an alternate pathway to transfer electrons between oxidizing bacteria and the methanogen during an AD.

In the AD process methanogenesis is considered a very sensitive and important step, in general, the microorganisms that perform this step are extremely sensitive to variations in the environmental conditions of the reactor (such as pH, temperature, and even the environment), which only can happen when a symbiotic relationship between methanogenic, acidogenic, and acetogenic microorganisms is reached.

Hydrogenotrophic methanogens will consume the H_2, and the CO_2 produced by acidogenic and acetogenic bacteria, maintaining the H_2 partial pressure in the proper threshold that allows acetogenic bacteria to thrive (2-10 atm), hence a symbiotic relationship hydrogen transfer between methanogens, acidogenesis, and acetogenins must exist in the process to reach the optimum outcome of AD process (Duan, Dong, Wu, & Dai, 2012).

IET using hydrogen and format as electron carriers is the predominant pathway for the exchange of electrons between fermenter bacteria and methanogenic.

Among the different modes of IET, DIET is the most sought-after area of research. Interspecies hydrogen transfer and interspecies format transfer are termed indirect interspecies electron transfer (IIET).

For methane production, DIET is preferred to IIET, for the following reasons: firstly, lower energy consumption, secondly, no requirement of redox mediators for electron exchange and thirdly no accumulation of toxic VFAs if any stagnation occurs in the process; the fourth reason is that there is no requirement for complex enzymatic steps to produce, consume and diffuse redox mediators, providing thermodynamic advantages. The corresponding studies on DIET are described in this article (Zullo, 2016), (Chen Y, 2008), (Linville, Shen, Wu, & Urgun-Demirtas, 2015).

2.1.2.1 DIET Process through Abiotic Conductive Materials

There exist three different types of DIET mechanisms: (i) DIET via conductive pili, electrons released during oxidation by fermenter bacteria are subjected to a methanogen through conductive pili (conductive nanowire) for further degradation; (ii) DIET via membrane-bound electron transport proteins, the cytochrome of mitochondria is responsible for electron transfer; (iii) DIET via abiotic conductive materials, electrically conductive materials build a bridge between fermenting bacteria and methanogen, enhancing methanogenic activity.

The performance of methanogenic bacteria can be enhanced through DIET, as it occurs at a faster rate and is found to be more efficient than IET through hydrogen and format. DIET process through abiotic conductive materials DIET via abiotic conductive material is the cell-to-cell electron pathway between acidogenic bacteria and a methanogen which stimulates methanogenic activity and thereby increases methane production.

Currently, iron- and carbon-based conductive materials in bulk size, microscale, and nanoscale have been exhaustively used by researchers to study the stimulating effect caused by DIET through conductive materials. The influence of this mechanism on biogas production and methane yield has also been studied in parallel by researchers (C.M. Ajaya, 2020), (Khalid, 2011).

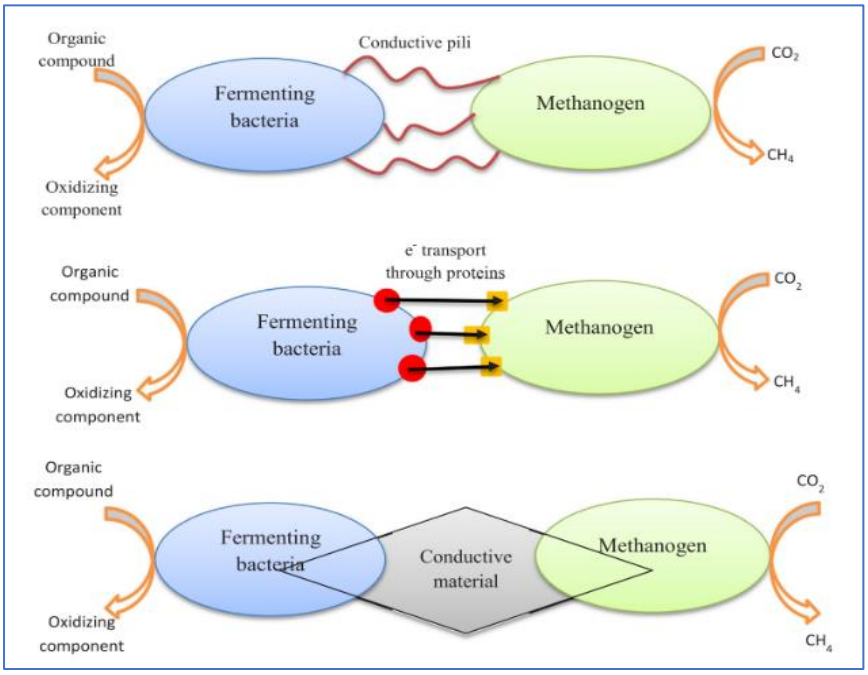


Figure 2. DIET mechanism between fermenting bacteria and methanogen

2.2 Products of the Anaerobic Digestion

The AD mainly consists of two products biogas and digestate biogas is the desired product which consists of methane, carbon dioxide, hydrogen sulfide, water vapor, and some small elements of other gases. The other bioproduct from the AD process is digestate which is the leftover of the process but can be used in different areas such as bedding for livestock, soil amendment, and fertilizers.

- **Biogas:** A mixture of methane, carbon dioxide, and trace gases, mainly used as a renewable energy source.

- **Digestate:** The leftover solid and liquid material from the process, that is rich in nutrients and used as fertilizer or soil amendment.
- **Water:** A byproduct of microbial reactions, which can be treated and reused.

2.3 Types of Bioreactors

AD plant's design depends on the feedstock. Plants can perform wet AD if the number of TS is less than 16%, semi-dry AD for values between 16% and 22%, and dry AD for TS values between 22% and 40%. Dry AD is typically used to process municipal solid waste, whereas wet AD is widely used for manure. AD processes have different optimum conditions, some plants therefore separate the processes to maximize methane yields (Ward, 2008).

In the AD operation, three types of reactors can be used (batch, continuous, and semi-continuous) and the choice is according to the substrate being used (Amaya, 2012).

The design and operation of the anaerobic reactor are characterized by two parameters including retention time (RT) and organic loading rate (OLR); the RT is defined as the average time that the substrate is maintained in a reactor and quantified by the equation: RT = reactor volume/daily flow.

The retention time (RT) in a reactor must be sufficient to allow for the complete digestion of one or more steps. In wet reactors, substrate materials are often separated into liquid and solid components, resulting in distinct hydraulic retention time (HRT) and solid retention time (SRT) (Van, 2020), (Abbasi, 2012).

2.3.1 Single-Stage Bioreactors

In a batch bioreactor, there are no continuous inputs or outputs. All substrate and inoculum are placed in the bioreactor at the same time and the bioreactor is sealed if all points of the bioreactors have the same composition.

Batch reactors are the ones with the simpler composition: it is composed of a single-phase reactor that is operated intermittently. The digester is filled once, and the substrate will be degraded without any addition or withdrawal to the reactor (discontinuous flux).

Generally, the production of biogas will increase until it reaches maximum production at about half of the residence time and decrease after that until it stops. An adaptation period of the microorganisms (lag phase) at the beginning of the operation is also observed. Then, the digestate can be fully removed and replaced with a fresh substrate or a percentage can be left to be used as feedstock for the next digestion (Van, 2020).

Batch AD has an advantage from a microbiological point of view since the microorganisms are not continuously exposed to a new substrate, allowing them to break down most of the organic matter, and they will not get washed out of the reactor.

Compared with other systems, these are considered low-cost, but it can also be harder to achieve an even degradation with high rates (especially if the substrate is rich in dry solids).

Single-stage reactors are reactors that all four steps function at the same reactor. This kind of reactor can be used for various AD applications and also is easier to develop (Van, 2020); however, these kinds of reactors can suffer from a "short-circuit" effect where the HRT is not optimal which results in lower methane yields (Ward, 2008).

In a single-stage reactor, conditions should be optimized for methanogens as they are the most sensitive microorganisms in the process.

Reactor conditions should therefore range from 15 to 30 for the substrate C to N ratio, a pH from 6,8 to 7,4, and depending on whether the AD has proceeded at mesophilic, thermophilic, or psychrophilic conditions, an HRT of respectively 30, 20, or 50 days.

The use of single-stage reactors would be more suited for uniform feedstocks and generally require a smaller capital and less Utilized Agricultural Area (UAA) (Van, 2020).

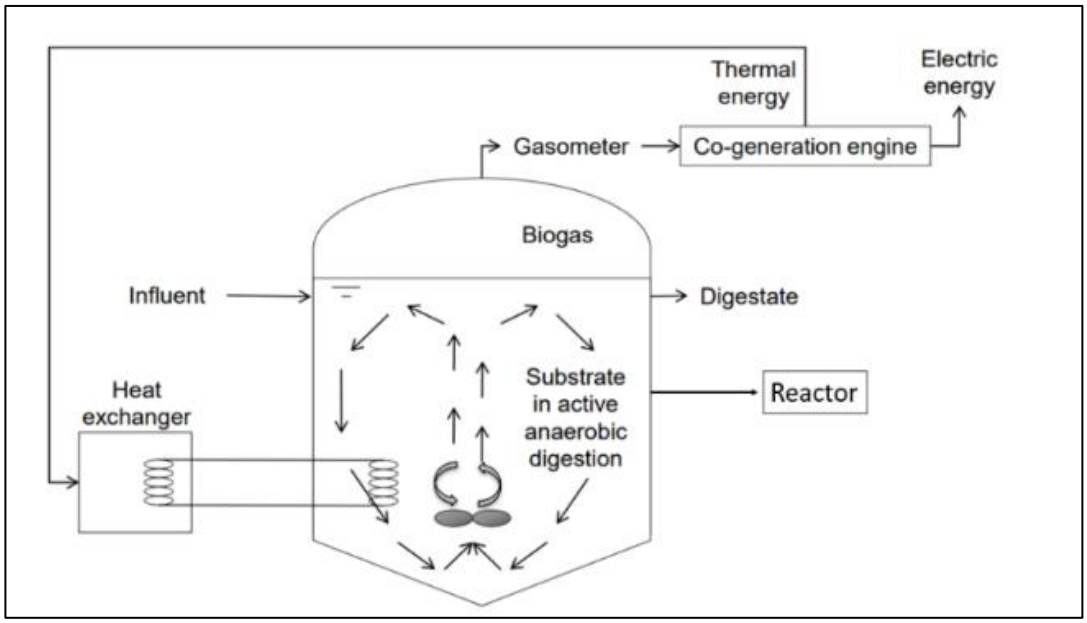


Figure 3. Single-stage bioreactor

2.3.2 Multi-Stage Bioreactors

The continuous and semi-continuous reactors are called multi-stage bioreactors. In a continuous bioreactor, the substrate is continuously pumped into the substrate, a flow of matter within the reactor throughout the day, generating continuous biogas production These plants can be used in the AD of all types of feedstocks in the multi-stage reactor, and the hydrolysis and acidogenesis steps are separated from acetogenesis and methanogenesis, therefore the methane yield production will be increased.

The advantage of the multi-stage reactors is buffering is typically used to control the pH, remove any material that wouldn't be hydrolyzed, and adjust the C/N ratio also this reactor can function with lower HRT ranging from 1 to 12 days.

In the case of these bioreactors, HRT increases with increasing volume, i.e. the conversion of organic matter is a function of the volume of the bioreactor (Amaya, 2012), (Chen Y, 2008), (Ren, 2018).

They generally require wider UAA, are more complex to build, and are more expensive (Van, 2020). Microorganisms, at their maximum level of growth, to 15 optimize the efficiency of degradation Reactor operational conditions and design considerations.

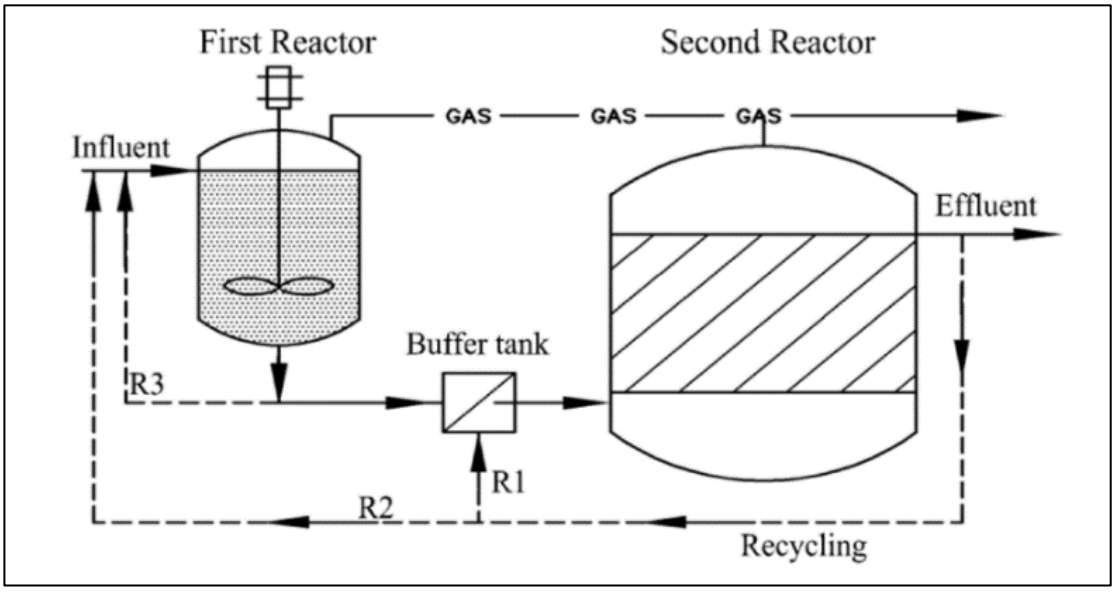


Figure 4. Multi-stages bioreactor adapted from (Van, 2020)

2.4 Biogas Production: Feedstock and Effluent

In the AD system, the materials entering the biodigester are feedstock and the output of the digestion is effluent.

Feedstocks are the starting materials and substrates that work as a fuel to the digestor here in this study the feedstocks are FW, Se, and incorporated Se with biochar.

Effluent is the material or the product of the AD process. For the AD system, these products are biogas and byproducts.

The most important effluent that is the desired product is biogas, which is a mixture of gases, mostly methane CH_4, and carbon dioxide CO_2. Understanding the characteristics of both the feedstock and effluent is crucial for optimizing biogas production efficiency.

In this case, the liquid and solid remaining after digestion can be used as fertilizer (in agriculture) and compost (Angelidaki, 2003).

2.5 Important Considerations and Parameters of Anaerobic Digestion

The rate of biogas production depends on several conditions or parameters that include hydraulic retention time, temperature, C/N ratio, organic loading rate, partial pressure, pH, nature of the substrate, microbes balance, and Oxygen exposure to anaerobic (A.N. Matheri S. N., 2017).

As indicated earlier, AD goes through a series of metabolic reactions catalyzed by diverse microbial communities, each with different physiological requirements for its growth.

These diverse communities work as a cohort and metabolize organic matter. It is therefore important to have a reactor design that provides optimal conditions for the growth of diverse microbial communities. Some of these considerations are discussed in the following section (Sagor Kumar Pramanik a, 2019).

During the AD process, parameters e.g., VFA, pH, COD removal, and kinetic parameters e.g., hydrolysis rate, lag phase, and methane production potential are the important indicators for illustrating AD process performance.

- Biodegradability of Industrial Effluents
- Toxic Compounds
- Temperature
- pH
- Nutrients and Carbon
- Organic Loading Rate
- Types of Treatment System

Figure 5. Parameters of AD

2.5.1.1 Temperature

In the AD process, there are three ranges of temperature in which microorganisms operate differently in the degradation of organic solids; 12-20℃ as the psychrophilic condition, 20-45℃ as the mesophilic condition, and 45-60℃ as the thermophilic condition (A.N. Matheri S. N., 2017); during only two ranges of temperature (mesophilic and thermophilic conditions), the process of anaerobic fermentation and methane-forming bacteria perform well, therefore these two ranges of temperature are the crucial temperature for AD.

Anaerobic bacteria grow well in mesophilic and thermophilic conditions, whereas methanogenic bacteria are sensitive to ambient conditions (temperature) and their growth rate is higher in thermophilic conditions (A.N. Matheri S. N., 2017).

Mixing is required to prevent sedimentation of dense matter, to release gas bubbles, and to ensure the efficient transfer of substrate for microorganism activity (A.N. Matheri S. N., 2017).

2.5.1.2 Retention Time

Also known as hydraulic retention time (HRT) or solid retention time (SRT) however, they are a bit different from each other.

The hydraulic retention time represents how long the substrate stays inside of the digester before being taken out as digestate (James A. Ippolito, 2012). Solid retention time is the average time the activated organic solid spends in the digester, and it depends on the rate of microorganism growth and substrate concentration (Dinku Yigzaw Mebratie, 2024).

Retention time depends on operating temperature, volume of digester, and degradability of the used substrates in the AD process.

2.5.1.3 Nutrients Concentration

In the AD of organic material, there are some nutrients exist such as nitrogen (N_2), carbon (C), phosphorous (P), sulfide (S), hydrogen (H), oxygen (O_2), calcium, and magnesium, which are required by microorganisms to grow and accelerate the process, also some macronutrients such as cobalt (Co), nickel (Ni), molybdenum (Mo) and selenium (Se), are vital for the survival of microorganisms. Carbon from carbohydrates produces energy and nitrogen from proteins contributes to the growth of microorganisms, which are responsible for AD to be processed. Microorganisms consume carbon approximately 30 times faster than nitrogen, therefore the optimum ratio of C/N for AD must vary from 15 to 30 (A.N. Matheri S. N., 2017), with phosphorous 2% of feedstock ideally for maximum biological activity. (Harilal S, 2012) has reported that the optimum C/N ratio for Biogas production is between 25-30:1 for cow dung. (Sinervo, 2017) reported that the C/N ratio can be between 15 and 25 as the recommended interval. However, not all researchers reported the same range, but it seems like 15-30:1 can be the best range for biogas production.

A higher or lower ratio will negatively affect the process and the quality of biogas plant production (methane and fertilizer) (A.N. Matheri S. N., 2017). The increase of carbon leads to the formation of high CO_2 content and low pH while, the high nitrogen content increases the rate of ammonia and raises the pH within the digester (Harilal S, 2012).

Some organic materials contain all the necessary nutrients that microorganisms require, however, others do not have enough nutrients for microorganisms to break down, therefore, the AD system requires some additional nutrients or uses co-digestion to fulfill these requirements. Co-digestion is the use of two or more different substrates to improve the nutrient content required for AD and the one needed for microorganism growth, retaining nutrients in produced digestate, it has been a big issue for its applications such as in agriculture because of the loss of nutrients during the AD process. Therefore, the use of some additional materials has been applied to reduce the losses. However, some of those additives have presented can cause environmental problems and increase the operational cost of the biogas plant. Currently, biochar has been approved as one of the cheapest and most environmentally friendly additives that can minimize these losses and retain nutrients during the AD process after the use of digestate (Michael O Fagbohungbe 1, 2017).

2.5.1.4 The pH

A pH between 7 to 14 indicates an alkaline solution. The pH value depends on the ratio of acidity alkalinity and carbon dioxide in the digester. The AD process mostly for the methanogenesis phase performs well in pH values ranging from 6.5 to 7.8 which is a neutral or mildly alkaline environment (A.N. Matheri S. N., 2017). While the fermentation process is not sensitive to pH and works in a wide range from 4 to 8 (A.N. Matheri S. N., 2017), the rate of methanogenesis can reduce the acidic or alkaline environment mechanism of fermentation and can affect the pH value by producing free hydrogen ions in the solution. The accumulation

of ammonia leads to an increase in pH value while the accumulation of VFAs leads to a decrease in pH value (Sinervo, 2017), and causes possible failure of the AD process. To maintain the stability of pH in the digester, buffer solutions such as bicarbonate and ammonium bicarbonate in the liquid phase and carbon dioxide in the gas phase can be added.

2.5.1.5 Inhibitory Factors in the Digester

There may be some inhibitory substances such as ammonia, light metal ions, sulfide, heavy metals, and organics that come from wastes as feedstocks and or formed degradation processes (Eddie F. Gómez). A higher pH value (basic environment) shows that the existence of free ammonia is higher than the ammonium ion (NH_4+), while in a lower pH environment, the ammonium ion is higher than free ammonia also depends on temperature where free ammonia is high at a high temperature. The inhibition leads to instability within a reactor, a decrease in biogas yield, and a complete failure in the AD process (Harilal S, 2012).

2.5.1.6 Inhibition of Volatile Fatty Acids (VFAs) and Organic Acid

Inhibition can occur when the pH value decreases within a reactor and it comes from the too-intensive feeding of the bioreactor, which leads to the depletion in the buffering capacity due to the rise of VFAs in solution. Accumulation of VFAs contributes to hydrolysis and can inhibit the process at a very high level. In addition, Sulfur compounds also can inhibit the AD process as a high number of sulfides are toxic to many groups of bacteria. The same is true for some light and heavy metal ions at a higher concentration can become toxic to microorganisms within a digester (Sinervo, 2017). The concentration of volatile acid measured through acetic acid's concentrations in the fermentation process greater than 2000 parts per million will inhibit the action of the methanogenic microorganisms (Harilal S, 2012). In addition, it has been reported that the optimization of AD is important and is required sometimes depending on the type of substrate and its nutrients, for example, FW which may not meet the requirements nutrients for biogas production. Therefore, some authors have proposed the use of co-digestion of two or more substrates to improve anaerobic production means biogas or methane yield (Holm-Nielsen, 2009), (Amha YM, 2018).

In general, FW contains a larger amount of easily degradable organic material and indigenous bacteria; however, its low C: N ratio usually delays the AD process because of the excessive accumulation of intermediate products like VFAs (Harilal S, 2012).

2.5.1.7 Substrate Solid Content

Organic materials contain carbohydrates, proteins, fats, cellulose, or hemicellulose which are the main components of the AD process.

Sometimes, substrates require pretreatment to enhance the production of AD. Pretreatment breaks down the complex organic structure into simpler molecules that are more susceptible to bacteria degradation; besides, pretreatment facilitates the heterogeneity of substrates and can promote the final production of AD.

The degradation rate depends on substrate contents and type of substrate; besides, the high biodegradability of FW leads to the fast accumulation of organic acids, which inhibits the

methanogenesis phase. Based on the total solid content within the digestion, digestion can be classified into two groups: dry digestion with 25-45% and wet digestion with 2-15% of total solid content. For that, wet digestion is used for substrates with high moisture content, and it is easy to manage compared to dry digestion which is used for substrates with high solid matter content in terms of process parameters, such as pH, temperature, and TS (Cai, 2016) (Sunyoto, 2016).

The total solid content within feedstock is the major parameter for biogas and methane production yield from the AD process (Yi, Dong, Jin, & X, 2014).

2.5.1.8 Toxicity

Several compounds are toxic to the microorganisms present in AD. Methanogens are the most sensitive to toxins. However, the process can acclimatize and tolerate higher concentrations of toxic agents after adaptation.

The most common inhibitor in AD is considered ammonia (Angelidaki, 2003). Studies have shown that an increase in the concentration of ammonia in the bioreactor decreases the rate of glucose degradation, showing that the accumulation of ammonia had an inhibitory effect on the glycolytic pathway through which hydrolyzed glucose from carbohydrates was degraded (Abbasi, 2012), (A.N. Matheri S. N., 2017).

However, the methane produced from acetate did not vary with the increase in ammonia, and it can be concluded that acetolactic bacteria are more resistant to the concentration of ammonia. Some authors indicate that the ammonia concentration is 55 mg N/L. However, (Angelidaki, 2003) states that AD can adapt to tolerate an ammonia concentration of 800 mg N/L. On the other hand, (Khalid, 2011) states that concentrations between 1670-3720 mg N/L can lead to a 10% loss in methanogenic activity, while 4090-5550 mg N/L decreases CH_4 production by 50% CH_4. (Sun Y. G., 2014), recorded a 10 to 20% reduction in biogas production under mesophilic conditions when the ammonia concentration reached 7000 mg N/L. Information on the limits varies between authors and it is not a parameter with clear results. Therefore, the level of ammonia inhibition strongly depends on the degree of acclimatization of the inoculum, which can tolerate concentrations much higher than those previously reported. Inhibition by sulfate can occur in two ways: firstly, through competition for substrates by the sulfate-reducing bacteria and secondly by the toxicity of sulfides to the different groups of microorganisms. Heavy metals (Ni, Cu, Zn, Pu, and others) are also inhibitors of the AD. Many enzymes depend on a minimal amount of these metals for their activity. However, when present in high concentrations, they cause an inhibitory or toxic effect on microorganisms. The chemical binding of heavy metals to enzymes and subsequent disruption of the enzyme's structure is the main cause of this toxic effect (Appels L. B., 2008). Long-chain fatty acids are toxic to AD and no adaptation to their toxicity has been observed. However, the presence of particulate material can increase the resistance of the too-long-chain fatty acids, since they are absorbed into the particulate material and therefore are not active as inhibitors (Angelidaki, 2003).

2.5.1.9 Agitation

The purpose of agitation is to maintain the temperature and uniformity of the mixture, reducing foaming inside the digester and evenly distributing the microorganisms and nutrients.

Agitation brings benefits to the AD but consumes a large amount of useful energy from the biogas produced. It is estimated that the agitator consumes between 29% and 54% of the energy needed in a biogas production unit. Therefore, a good mixing strategy can significantly reduce the need for energy in the system (Wang, 2018). Also, agitation in AD can be continuous, intermittent, or non-existent. Some authors indicate that intermittent agitation increases biogas production by 2.5% to 14.6% compared to continuous mixing. This is because the oxidative bacteria and methanogenic bacteria live in close communities, using H_2 and format as electron carriers, so in intermittent mixing, there is enough time for electrons to be transported from one community to the other, increasing biogas production (Wang, 2018).

2.6 Challenges in AD Systems

Although there were many research and studies have been working on the AD process, still several challenges are restricting the production of methane, and instability of the system, because of the high C/N of crop straw, AD often becomes unstable due to the accumulation of VFAs, which can be alleviated via co-digestion with livestock manure the quality of the biogas, etc (T. Thamsiriroj 1, 2012). Also, the chemical and physical properties of substrates, the effluent quality needs some additional processes which are size reduction to hasten degradation, complexity in balancing fermentation and methanogenic microorganisms, and low organic loading rate (OLR) (3.5 gVS/L) are yet to be solved (Chen Y, 2008).

Furthermore, small variations in the AD process can have an adverse effect, especially at the sensitive stage. Common instability in AD is caused by high OLR, pH shock, and other inhibition effects brought by the accumulation of VFAs and even those that are initially present in the feedstock such as heavy metals (HM).

The conversion of VFAs to methane by methanogens is relatively slow. Accumulation of toxic inhibitors also causes instability. This toxin can be from the substrates themselves which will disturb the syntrophic functions of the bacteria and even retard microbial growth that can lead to instability as manifested by decreased pH, rapid VFA accumulation, and low CH_4 production (Dechrugsa S, 2013), (Wojciech Artichowicz, 2021).

The slow growth rate of methanogens can cause a longer hydraulic retention time (HRT). To attain stability in the AD system, there should be a healthy environment for the microorganisms to survive to fulfill their respective functions (Kutlar FE, 2022).

Low process efficiency of AD, four different types of bacteria break down organic materials in four stages. Each type of bacteria requires specific process conditions (such as temperature, pH, and C/N ratio) to function properly (Ángela Rodríguez-Abalde 1, 2016). Due to

the syntrophic relations among bacteria at different stages, disruption in one stage produces cascading effects in other stages (Raquel Barrena, 2022).

This is particularly because methanogenesis is the most sensitive stage in the AD process such that even a small deviation of operating conditions from the threshold level can result in inhibition (Li L, 2018).

Additionally, since substrates for AD are rich in Nitrogen (N) and Carbon (C), the system is prone to excessive accumulation of organic Ammonia (NH_3) and Ammonium (NH_4+) produced during protein breakdown and VFAs which are considered major inhibitors to methanogens. VFAs and NH_3, at a safe level, can serve as essential nutrients to support the growth of microorganisms. Some literature reported ammonium levels to be safe at 1200 mg or between 1700–1800 mg. Ammonia inhibition is common in the nitrogenous substrate, animal manure, and slaughter by-products (Fagbohungbe, 2017).

It is noteworthy to emphasize the appropriate microbial community for efficient AD at minimal or reduced inhibition effects (Chen Y, 2008).

Syntrophic relations of bacteria are defined by their ability to transfer electrons at a stable and fast rate to survive considering optimum parameters are met such as pH organic loading rate (OLR), and temperature among others. Their survival and growth are well proven to promote better AD processes as indicated by improved CH_4 production and lag time.

Interestingly, direct interspecies electron transfer (DIET) was found as a new pathway for electron transfer between bacteria and archaea, facilitated by carbon materials such as biochar as electron shuttles and was claimed to be more efficient than another mode like the interspecies electron transfer (IET) facilitated through H_2 (Amha YM, 2018).

The quality of AD performance is affected by the syntrophic bacteria and archaea and the efficiency of electron transfer. Thus, a syntrophic environment allowing fast acclimatization of microbial growth through DIET for subsequent faster methane production must be further explored.

AD is a promising method of treating organic waste. However, when the protein content of the organic waste is high, a lag phase that interferes with the efficiency of biogas production occurs. study is intended to determine the conditions for reducing lag-phase and improving the efficiency of biogas production biogas production performance may differ drastically depending on the proximate composition of the input matter in terms of six components, i.e., carbohydrate, protein, lipid, moisture, crude fiber, and ash. One cause of the problem based on the proximate composition of the input matter is biogas production inhibition caused by an occurrence of the lag phase during the AD (Ko, 2018), (Javkhlan Ariunbaatar, 2016).

The lag phase appears through the inhibition of the fermentation process. One of the most common inhibitors of the fermentative bacterial population is the main product of the fermentation process, i.e., VFAs when carbohydrate is the main substrate. Protein-based by-products are hydrolyzed by anaerobic bacteria and converted into short-chain fatty acids, ammonia (NH_3–N), and small quantities of hydrogen and sulfur compounds. VFAs and NH_3–N generated during this stage are inhibitors of AD when protein is the main substrate. Lipid-based by-products (triglycerides), which account for most of the lipid content in organic wastes, are hydrolyzed to form glycerol and long-chain fatty acids (LCFAs) during AD.

LCFAs are toxic to methanogenic bacteria and, therefore, interfere with biogas production (CBF, 2020).

The inhibition of biogas production varies depending on the conditions of the inoculum, the substrate, the acclimation requirements, and the operation period. Under high organic loading and low buffering capacity conditions in the continuous reactors, VFAs accumulation may occur; if the excess VFAs cannot be reduced, the methanogen will be inhibited, and the reactor process will fail (Adrie V, 2000).

This problem can be solved by determining the suitable amount of organic loading and diluting the same to decrease the VFAs levels. However, simply modifying the organic loading rate without considering the optimal VFA levels might influence AD performance (A.N. Matheri S. N., 2017)

Furthermore, it will be difficult to maintain the stable operation condition of the continuous reactor which is suitable for the organic loading change. Therefore, an evaluation of AD performance through the biochemical methane potential (BMP) test should be conducted before the correction of organic loading for the continuous reactor systems (I, 2019), (A.N. Matheri S. N., 2017), (Chen Y, 2008).

2.7 Additives to Enhance the AD Process

Additives can be used in the digester to improve material conversion and biogas production rate. The primary role of additives is to support microbial growth, adsorption of inhibitory products, nutrient supplementation, and enhancing buffering capacity (Arif, 2018). Various conductive materials such as sand, molecular sieve, zeolite, charcoal, etc., are used to improve syntrophic activity while providing a habitat for microbial growth (Yang, 2004).

They can also adsorb inhibitory products like NH_3, and H_2S resulting in more efficient conversion. If any substrate lacks specific nutrients necessary for the digestion process, micro- and macro-nutrient supplements are added to the digestion. It stimulates biogas production while maintaining process stability (Romero-Güiza, 2016).

There are two groups of metals important for AD: light and heavy metals. Iron, Nickel, Selenium, and Cobalt have proven to be the most important heavy metals responsible for the stable AD of FW. Their excess can disrupt the function and structure of enzymes leading to inhibition of AD. However, inhibition caused by heavy metals is not usually a concern in AD of FW, since they are usually not sufficiently present in FW (Mirmohamadsadeghi S, 2019).

2.7.1 Biochar

Biochar, a typical carbon-rich solid product, was thermochemically generated from waste biomass including woody wastes, agricultural residues, waste-activated sludge, etc (Tripathi, 2016). The waste or remains of the organic matter are heated to be decomposed and remove almost all the oxygen and hydrogen-containing molecules. The process is called

pyrolysis and is carried out in the absence of oxygen to prevent the material from burning. Specifically, those biomass substances could be effectively converted to value-added products like biochar, syngas, and bio-oil, during the pyrolysis process in an oxygen-free environment under the temperature of 350–650 °C (Zhou, 2013). Biochar has various considerable functions, however the most common is its use as a soil fertilizer. In recent years, its application as an additive to AD has been studied, as an additive to the AD process to enhance efficiency and to stabilize the application. In this study the potential use of biochar which in this case specifically use the Ecochar that was produced by Ibero Massa Florestal will be discussed. Previous analysis suggests that biochar can be a promising additive to enhance AD.

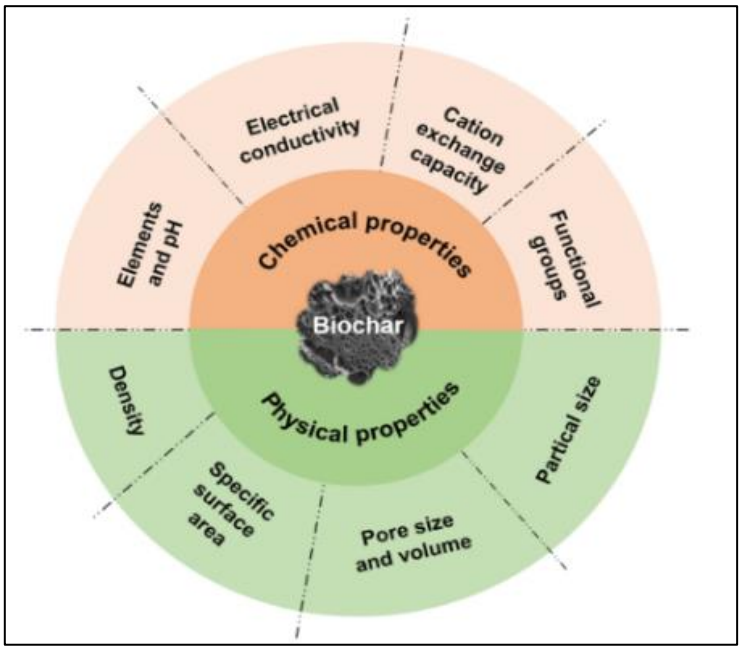


Figure 6. Physical and chemical properties of biochar

Biochar usage as an additive in AD to enhance the efficiency of the processes has drawn attention in recent years (Fagbohungbe, 2017). The techniques to maximize biogas production by AD are explicitly introduced to achieve potential reactor operations (C. Sieblist, 2011).

An essential consideration for combining AD with biochar is formed from waste biomass by thermochemical conversion.

The latest enhancement work shows the potential biochar results of augmentation of certain chemicals such as biofuels and charcoal, biodiesel, butanol, carboxylic acids, and hydrocarbons. However, more detailed, and certain interpretations of biochar and AD relations still need more research and study.

Previous research and articles show on the systematic analysis of AD efficacy with the use of biochar that the maximum methane generation using biochar with enhanced AD efficiency ranges from 650 to 900 mL/day, with organic removal efficiencies varying from 70% to 75% (Kutlar FE, 2022), (CBF, 2020).

Biochar properties provide reasonable conditions for pH adjusting, disinfectant, complementary sourcing, electron carrier servers, and the expression of physicochemical weights on an organism through various bacterial communities (Qiu, 2019).

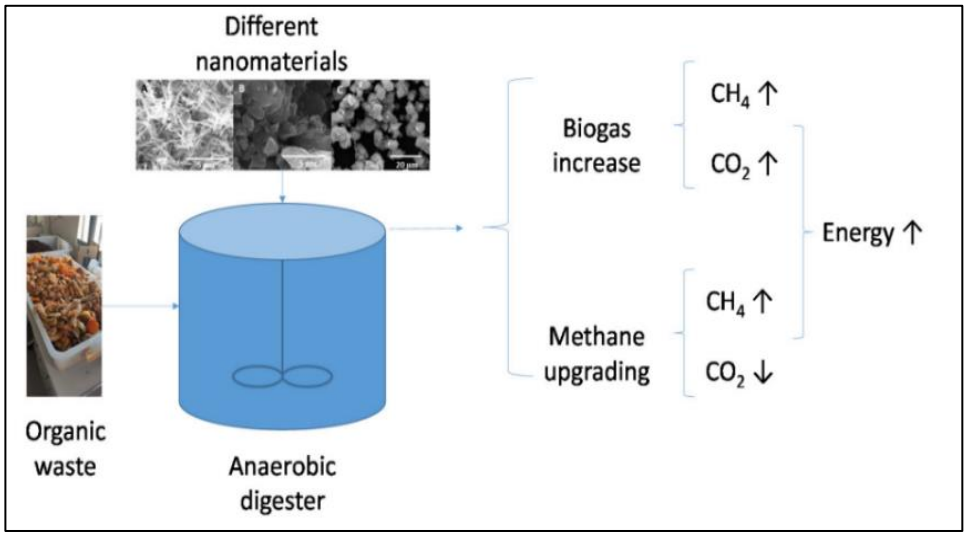


Figure 7. Possible benefits involved in AD with nanomaterials

2.7.1.1 Characteristics of Biochar

- **Porosity:** Biochar has a high porosity, which provides a large surface area for microbial attachment and nutrient exchange. The porosity of biochar is influenced by the feedstock used and the pyrolysis temperature (Tan X. L., 2017), (Tripathi, 2016).

- **Specific surface area (SA):** SA measures the surface area per unit mass of biochar. Biochar has a high SA, which increases the adsorption capacity of biochar for various substances, including organic compounds, inorganic ions, and microorganisms (Tripathi, 2016), (Tan X. L., 2017).

- **Electrical conductivity (EC):** Biochar has a good EC, which can facilitate electron transfer between microorganisms and other components in the AD system. The EC of biochar is influenced by the feedstock used and the pyrolysis temperature (Tan X. L., 2017),(Tripathi, 2016).

- **Ion exchange capacity (CEC):** CEC measures biochar's ability to bind ions. Biochar has an excellent CEC, which can remove inorganic ions from the AD system and prevent inhibition (Tan X. L., 2017),(Tripathi, 2016).

- **Surface functional groups:** Biochar contains a variety of surface functional groups, including carboxyl (-COOH), hydroxyl (-OH), and carbonyl (-C=O) groups. These

functional groups can interact with various substances, including organic compounds, inorganic ions, and microorganisms (Tan X. L., 2017), (Tripathi, 2016).

2.7.1.2 Mechanisms of Biochar-Enhanced AD

Various studies have demonstrated the promising potential of biochar in addition to enhance methane production efficiency in AD processes. For instance, Lu et al. observed a significant increase in methane production rate (up to 47.1%) when biochar was added to AD systems under conditions of high ammonium and acid concentrations. production rate with biochar addition, but the extent of methane production improvement was found to be dependent on biochar particle size. Similar improvements in methane production were observed in solid-state fermentation of municipal solid waste, where methane yield increased by 3–5%.

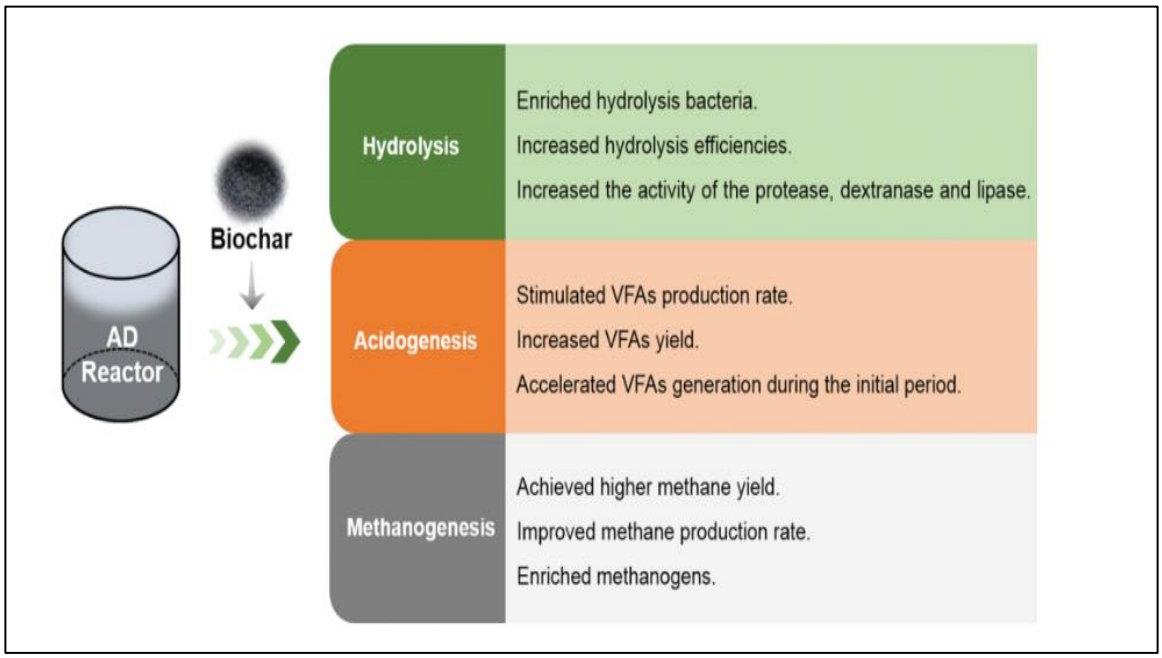


Figure 8. Effects of biochar in stages of AD

2.7.1.3 Advantages and Drawbacks of Biochar Effects to AD

2.7.1.3.1 Biochar enhancement has several advantages for AD

1. pH buffering: Biochar can buffer the pH of the AD system, which can prevent pH shock and promote microbial growth. pH buffering is a process that resists changes in the pH of a solution. It can maintain a relatively constant

concentration of hydrogen ions (H+), which determines the solution's acidity or alkalinity. Therefore, stability can be achieved during the process, which is one of the disadvantages of biogas production, and environmental protection because buffering is crucial in natural ecosystems for balancing acid rain and maintaining healthy aquatic environments.

2. Functional group adsorption: Biochar can adsorb inhibitory substances, such as ammonia, VFAs, and heavy metals, from the AD system.

3. Inhibition alleviation: Biochar can alleviate inhibition caused by toxic substances, such as ammonia and heavy metals, by removing these substances from the system or by interacting with them to reduce their toxicity. however, this mitigation is limited by the low surface area and pore volume of the biochar therefore for high concentrations of nitrogen it cannot work very well. The surface inputs contain high amounts of nitrogen, but it would be more of a biochemical effect due to its limited adsorption capacity due to its low surface area and pore volume. The application of adsorbents such as biochar creates an alternative route to remove and reduce the inhibitory effect during AD. This is because there is evidence that biochar can adsorb heavy metals and other organic compounds such as pesticides, fur compounds such as pesticides, furfural, and limonene. 10 g/L of biochar can remove up to 100% of the removing up to 100% of the copper and lead present in the bioreactor (Le Zhang a, 2019).

4. Direct interspecies electron transfer (DIET): Biochar can act as a bridge for electron transfer between microorganisms, which can enhance the overall rate of methane production (Cai, 2016), (Félix, 2018).

5. Improved methane production: Biochar can improve methane production by alleviating inhibitions, promoting microbial growth, and enhancing electron transfer (Cai, 2016), (Félix, 2018).

6. Reduced process instability: Biochar can reduce process instability by buffering pH and removing inhibitory substances. The main reason that the stability of the system is important is because of the long-term continuous conversion of biowaste. It was reported that biochar has the superior potential to promote AD process stability in terms of Ammonia-N alleviation (Lü, 2016).

7. Enhanced nutrient removal: Biochar can enhance nutrient removal by adsorbing nutrients and increasing nutrient availability for microorganisms (Cai, 2016).

2.7.1.3.2 Drawbacks of biochar amendment

1. Cost: Biochar can be expensive to produce, which can limit its widespread use.

2. Impact on microbial community structure: Biochar can alter the microbial community structure in the AD system, which can have both (Félix, 2018).

In addition, The conductive property of biochar may also contribute to the selective enrichment of microbes. (Yu, 2015) found that biochar functioned as an electron acceptor for microbial extracellular respiration and growth, suggesting that biochar acts as an electron acceptor for a diversity of electroactive microorganisms may be a general feature.

2.7.2 Selenium

Based on (Deppenmeier, 2002), Se is not essential for the acetolactic methanogenesis process. However, Se can be extensively involved in the hydrogenotrophic methanogenic process.

Some authors have reported that, after the addition of Se, the degradation of VFAs significantly improved in the AD of FW with a high ammoniacal nitrogen concentration (Charles J Banks 1, 2012).

Supplementing Se (VI) can reduce the acetic and propionic acid concentrations in the batch bioreactor, thus enhancing biomethane production by more than 30% (Yafan Cai, 2018). It indicates that Se is involved in common hydrogenases (Facchin, 2013).

Se provides co-enzymes necessary for propionate oxidation and syntrophic hydrogenotrophic methanogenesis (Banks, 2012).

Based on the studies and research the effect of Se on mesophilic AD in different addition concentrations can enhance the performance of biogas production. The use of metals/metalloids as electron acceptors is an archaic mechanism of energy generation. In the case of Se, its two oxyanions (selenate, SeO_4, and selenite, SeO_3) act as terminal electron acceptors, receiving electrons mainly from organic compounds but also from H_2 or SO (John F. Stolz1, 1999).

It can be used in the reduction of both acetic and propionic acid concentrations during the AD process.

Using trace elements as an additive has different properties such as (1) micronutrients for various enzymatic reactions as co-factors; (2) promoters of microbial aggregation, which leads to enhanced activity of the anaerobic microbes in case of syntropy; (3) agents binding carriers-proteins and nutrients such as phosphates; (4) help to overcome sulfide toxicity through metal sulfide precipitation; (5) at higher concentrations TE can become toxicants to the microbial biomass (Takashima, 1989).

During the study that took place in AD of pig manure, the low Se concentration increased methane yield and methane production rate in the biogas, however, the high concentration of Se can hurt the system and cause the methane yield production to decrease because of the enzyme activity, yet this inhibited drawback can be recovered by adding more organic matter. It is proven that the performance of AD can be significantly improved with low concentrations of Se, especially during the early stage of digestion (Charles J Banks 1, 2012).

When the additive concentration was excessive, the toxic effects of trace elements on the system were obvious and VFAs showed serious accumulation. IC50 values for Se, Mn, and

Mo were 79.9, 773.9, and 792.3 mg/L, respectively. Archaea was more sensitive to trace elements than bacteria (M.S. Romero-Güiza a b, 2016).

The study investigated the effects of Se addition on the performance and microbial dynamics of mesophilic AD of pig manure. At low Se concentrations (0.1 mg/L), methane yield and production rate increased significantly, by 121% and 113%, respectively. Conversely, high Se concentrations (30-120 mg/L) led to a substantial decrease in methane yield and production rate (23.1-100%), attributed to the inhibition of hydrolytic enzymes and F420 activity during the initial stages of AD. However, the inhibited methane production could be fully recovered by subsequently supplementing the system with additional organic matter to reduce selenite. Furthermore, selenite addition altered the relative abundance of hydrolytic and acetogenic bacteria, while increasing the prevalence of acetolactic methanogens.

These findings suggest that the inhibitory effects of Se on methane production are primarily due to competition for hydrogen and acetate between selenite-reducing microorganisms and methane-producing organisms, rather than direct inhibition of the latter (Adrie V, 2000).

Se addition has been proven to change the types of bacteria present in the digester. The study of (M.S. Romero-Güiza a b, 2016) suggests that Se-reducing bacteria compete with methane-producing bacteria for resources, but Se does not directly harm methane-producing bacteria.

As reported by (Lo HM, 2017), Se concentrations below 0.04 mg/L can play multiple roles in methanogenesis, including its involvement in carbon monoxide dehydrogenase, hydrogenases, format dehydrogenase, and formyl-methanofuran dehydrogenase. The benefits of this addition can be observed from different aspects: an increase in biogas production, methane enrichment in biogas, elimination of foaming problems, a more stable and robust operation, absence of inhibition problems, etc.

2.8 Substrate/Feedstock

The various ranges of feedstock can be used in AD, and the performance of different feedstocks can have different influences on the performance of AD and the environment. The substrate contains the nutritional requirements of the microorganisms in the inoculum, such as vitamins and trace elements with high content of water and low quantities of inorganic matter and fibrous materials are the most suitable for AD, on the other hand, the substrates that the substrate that is rich in mainly one component can hurt the reactor activity if operational parameters are not optimized such as VFAs accumulation when the substrate is rich in fats or carbohydrates and ammonia accumulation when it's protein-rich.

In Europe, biogas is mainly produced from agricultural residues, manure, FW, and energy crops. To be able to compare the different feedstock on the performance of AD, a laboratory test called Biochemical Methane Potential (BMP) tests the maximum methane yield possible (Ward, 2008).

2.9 Research Question

The research focused on addressing the critical challenge of stabilizing and optimizing the AD process. To enhance AD performance, the study investigated the synergistic effect of Se and biochar. Their potential to stimulate microbial activity and methane production, while biochar was evaluated for its ability to improve digester efficiency by promoting microbial growth and electron transfer.

3 MATERIALS AND METHODS:

This study was carried out on a laboratory scale and held in the Department of Chemistry at Nova University of Lisboa. The study was held between February 2024 and September 2024. All the tests were done in the batch bioreactors. in 50℃ grant bath. The experiments were run at a mesophilic temperature of 50℃, and the pH of all tests was between 7 to 8.

In all the tests, the entrance and effluents of the digesters and the biogas generated by them were characterized. This study was conducted in two different trials. Before starting all the assays, two approaches were conducted to determine the optimal condition for incorporating Se and biochar.

During the studies, the characteristics of the two additives were analyzed. The same inoculum source from the Valorsul and the same substrate were used in both assays.

3.1 Analytical Methods

The study was carried out through two different trials, each assay took approximately 20 days, and all the assays were in a batch reactor. The aim of trial 1 was to assess the effect of adding Se in its free state in three different concentrations which are 2.5 mg/L, 5 mg/L, and 10 mg/L of Se.

Trial 2 aimed to determine the effect of both Se and biochar in addition to the biogas production of AD of FW.

The characterization of the feedstocks and effluents of the digesters and the biogas generated by them was carried out according to the following methods:

- Total solids (TS) and volatile solids (VS) were determined according to the standard methods for the Examination of water and wastewater.
- Chemical oxygen demand (COD) was determined by the dichromate method.
- Biogas production was measured by a gas flow meter.
- Biogas composition was determined by gas chromatography.

3.2 The Study Characterization of the material used in this study

- Solids content: Total solids (TS), fixed solids (FS), and volatile solids (VS).
- Chemical oxygen demand (COD): Total COD was determined using a closed reflux method with potassium dichromate.

3.2.1.1 Substrate and Inoculum Characterization

The substrate and inoculum were characterized by the following parameters: total solids (TS), fixed solids (FS), volatile solids (VS), and total chemical oxygen demand (CODt) and soluble chemical oxygen demand (CODs).

The inoculum origin was from the Valorsul Organic Treatment and Valorisation Station (ETVO) in Belas, Amadora, Portugal, managing Lisbon's metropolitan area's urban solid waste.

A fresh substrate was prepared for each set of experiments using fresh materials bought from local supermarkets in Lisbon. The substrate was prepared in the laboratory in the chemistry department at University Nova on April 23, 2024.

3.2.1.2 Solids Content Determination

TS is an important attribute of digester operation. High-TS in the AD has received a considerable amount of recent attention, on account of its need for smaller digester sizes and lower heating needs (Yi, Dong, Jin, & X, 2014). Furthermore, improved biogas yields were reported in continuous high-TS digesters compared to low-TS digesters operating on the same retention time (Duan, Dong, Wu, & Dai, 2012).

(Xu, 2014) found that the maximum volumetric methane production rate of AD first increased with TS, reaching a peak at 15%-20% TS, and then started to decrease.

(Fernández, 2010) also reported a 17% decrease in organic waste degradation when the TS content increased from 20% to 30%.

To quantify TS, VS, and FS, the tare weight of the crucibles was first determined in a microwave muffle furnace (CEM, model MAS 7000) at a temperature of 105 ± 1℃ for 2 hours , followed by weighing on an analytical scale (Denver Instrument, model TR-204) with an accuracy of ± 0.0001g.

The next step was to take the crucibles to the muffle furnace (Lenton furnace) at a temperature of 550 ± 10℃ for 240 minutes.

Then 5mL of each sample was placed into calcinated crucibles and was taken to a boiling water bath (Precisdig model P Selecta) for evaporation and drying step.

Subsequently, the crucibles were placed in the muffle furnace at a temperature of 105 ± 1℃ for 2 hours and after cooling down were weighed.

Figure 10. Muffle furnace at 550℃

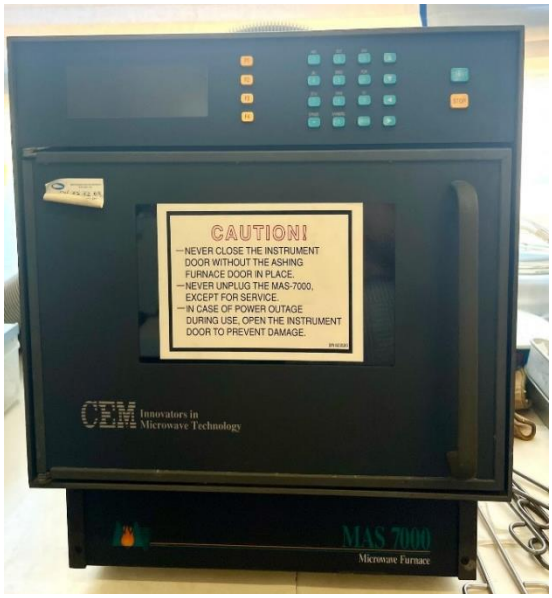


Figure 9. Microwave furnace at 105℃

Figure 11 Drying samples at 100°C

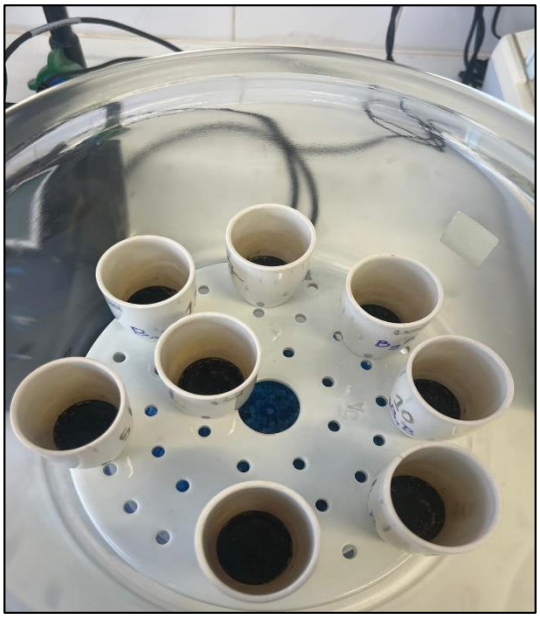


Figure 12. Dried samples for measuring solid content

3.2.1.3 Fixed and Volatile Solids Determination

In AD, the solids content determination includes calculating the total solids, volatile solids, and fixed solids that are presented in organic matter.

$$TS\ (\%) = \left(\frac{weight\ of\ dried\ solids}{weight\ of\ wet\ sample}\right) \times 100 \text{ or } \quad TS = \frac{P1-P0}{Va} \times 1000 \quad (1)$$

$$VS(\%) = \left(\frac{weight\ loss\ on\ igitation}{weight\ of\ wet\ sample}\right) \times 100 \text{ or } VS = \frac{P3-P2}{Va} \times 1000 \quad (2)$$

$$FS(\%) = TS(\%) - VS(\%) \quad (3)$$

which,

TS – Concentration of Total Solids (g/L).

P_0 – Crucible Tare 105 ± 1℃ (g).

P_1 – Crucible Tare + dry sample 105 ± 1℃ (g).

Va – Volume Sample (mL).

FS – Fixed Solids Concentration (g/L).

P_2 – Crucible tare weight at 550℃ ± 10℃ (g).

P_3 – Crucible tare weight + dry sample at 550℃ ± 10℃ (g).

The weight of total solids can be measured by drying the samples in the oven at a specific temperature which in this study was 100℃.

For the estimation of the solids of the I/S mixture, a mathematical method of proportionality about volume:

$$Sx = \frac{Vi * Sx,i + Vs * Sx,s + Mb}{Vt}$$

Which,

Sx – Concentration of TS, VS, or FS in the I/S mixture (g/L).

Vi – Inoculum volume (L);

Vs – Substrate volume (L);

Vt – Total volume of the I/S (L) mixture.

Sx,i – TS, VS, or FS of the inoculum (g/L).

Sx,s – TS, VS, or FS of the substrate (g/L).

Mb – Mass of TS, VS, or FS in the biochar (g).

Figure 13. Process of determination of VS and TS

3.2.1.4 Measuring Total Oxygen Demand (CODt) in Anaerobic Digestion

Total oxygen demand (CODt) is defined as a measurement of the amount of oxygen that exists in any organic matter in the water which includes living or dead plant or animal material, that needs to be fully decomposed in which the bacteria break down the organic matter into the simpler elements by using oxygen. It is an important indicator of water quality and pollution levels. The important note is that the higher level of CODt represents the more organic matter in the water so more oxygen will be needed for the decomposition. The level of CODt is important because a high CODt level can deplete the oxygen level in the water, treat the aquatic life, and be used for pollution monitoring which includes the sewage treatment plant, industrial waste, and agricultural runoff. This study used the volumetric method ISO 15705:2002 to determine the CODt.

1. Volumetric Method (ISO 15705:2002):

This method involves the chemical oxidation of organic matter in the sample by using strong oxidizing elements like potassium dichromate. The excess oxidant is then titrated with reducing elements like ferrous sulfate to determine the amount of oxygen consumed.

Steps:

1. Sample Preparation: 5 mL of sample was diluted in a 100 mL flask with deionized water (Millipore water).
2. Digestion: 5 mL of the diluted sample was removed from the flask and placed in a glass digestion tube. 15 mL of sulfuric acid (95-97%) (Chem-Lab), 5 mL

potassium dichromate (0.5 N) (Panreac), and 0.005 g mercury sulfate then were added to the system.

3. Digestion was carried out in a thermo-reactor (Behr, model Labor-Technik), at a temperature of 100℃, for 60 minutes.
4. Cooling and Dilution: Wait at least 30 minutes for the digested samples to be cooled down and then diluted with deionized water.
5. Titrate the cooled sample with ferrous sulfate solution using a ferroin indicator until the endpoint is reached.
6. Perform a blank test with all reagents except the sample.

$$CODt = \frac{(V1-V2)*N*F}{V}$$

which:

- V_1 = volume of ferrous sulfate used in blank (mL).
- COD= chemical oxygen demand (mg/L).
- V_2 = volume of ferrous sulfate used in sample (mL).
- N = normality of ferrous sulfate solution (N).
- F = molar mass of oxygen (32 g/mol).
- V = volume of sample used (mL).

Figure 14. Preparation digestion at 100℃ for measuring CODt and CODs

Figure 16. Digestion at 100℃ for 75 minutes

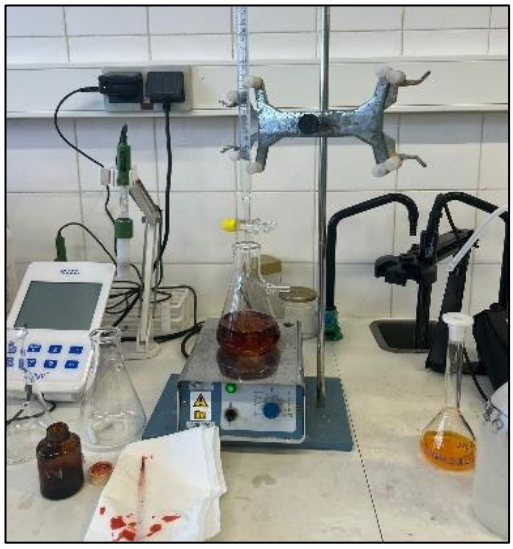


Figure 15. Titration the solution for CODt and CODs

3.2.1.5 Measuring the Soluble COD

1. Volumetric Method (ISO 15705:2002):

This method involves the chemical oxidation of organic matter in the sample by using strong oxidizing elements like potassium dichromate. The excess oxidant is then titrated with reducing elements like ferrous sulfate to determine the amount of oxygen consumed.

Steps:

Measuring 30g of samples and centrifuged at 1000 RFC for 20 minutes then centrifuged again at 5000 RFC for 30 minutes, in this process the solid and liquid were separated

1. Sample preparation: 5 mL of the sample was diluted in a 100 mL flask with deionized water (Millipore water).
2. Digestion: 5 mL of the diluted sample was removed from the flask and placed in a glass digestion tube. 15 mL of sulfuric acid (95-97%) (Chem-Lab), 5 mL potassium dichromate (0.5 N) (Panreac), and 0.005 g mercury sulfate then were added to the system.
3. Digestion was carried out in a thermo-reactor (Behr, model Labor-Technik), at a temperature of 100℃, for 60 minutes.
4. Cooling and Dilution: Cool the digested sample and dilute it with deionized water.

5. Titrate the cooled sample with ferrous sulfate solution using a ferroin indicator until the endpoint is reached.
6. Perform a blank test with all reagents except the sample.

$$CODs = \frac{(V1-V2)*N*F}{V}$$

which:

- V_1 = volume of ferrous sulfate used in blank (mL)
- CODs = chemical oxygen demand (mg/L).
- V_2 = volume of ferrous sulfate used in sample (mL).
- N = normality of ferrous sulfate solution (N).
- F = molar mass of oxygen (32 g/mol).
- V = volume of sample used (mL).

3.3 Collection and Preparation of Material

3.3.1 Inoculum

The inoculum used in all stages of this work was obtained from the Valorsul company, (valorization and treatment of solid waste), which treats municipal solid waste from the Lisbon metropolitan region. This inoculum is the effluent from a thermophilic (52°C) digester at the Valorsul.

Figure 17. Inoculum from the thermophilic digester from the Valorsul

3.3.2 Substrate

3.3.2.1 Food Waste Preparation

In this experiment, FW was used as substrate. FW contains biodegradable solids and a high water content which can be a perfect substrate for AD.

FW composition varies on many factors such as the region, season, culture, economic income, and demographics. To reduce experimental bias, the substrate used for this research was synthetically generated based on an average compositional analysis of FW in the EU, as it was used in previous research (Ariunbaatar J. P., 2015).

It contained 79% fruits and vegetables, 5% pasta and rice, 6% bread and bakery, 8% meat and fish, and 2% dairy products (Ariunbaatar J. P., 2015).

A fresh substrate was prepared for each set of experiments using materials bought from local supermarkets in Lisbon.

At the beginning of making the substrate some raw materials such as meats, fish, potatoes, eggplants, broccoli, carrots, rice, and pasta needed to be boiled at 100℃ water with the addition of salt (maximum of 2%(w/v)). Their weight was verified after boiling. The extra skin and bones of meat and fish have already been cut.

All parts such as stems, peels, and seeds were used for vegetables and fruits. The components were then mixed and blended until a homogenous material was obtained. Then 110g of the homogenized material was placed into individual plastic bags. Each plastic bag of FW contains exactly 110g of mass and was reserved for further use. Table 1 shows the composition of each item used to prepare the desired FW (T, 2022).

Table 1. Composition of the food residue

Components	Weight (g)
White meat	70
Red meat	70
Fish	70
Potato	200
Bread	151
Biscuits	151
Rice	50
Pasta	50
Lettuce, coriander, and cabbage mix	180

Components	Weight (g)
Tomato	170
Eggplant	170
Broccoli	180
Carrot	140
Tangerine	170
Apple	150
Banana	150
Cheese	20
Milk	20

3.3.2.2 Preparation of the Substrate for Digestors:

In this experiment, 110g of FW was prepared in the laboratory of the chemistry of Nova University based on the data from previous research of (Ariunbaatar J. P., 2014), this 110g of substrates then was diluted with with 900 mL of distilled water.

Figure 18. FW mix before grinding and FW after dilution

3.4 Characterization of Biochar

The biochar used in this study was extensively characterized in a previous work (Carvalho, 2019); Therefore, the focus was placed on a leaching test to determine the amount of Se that could potentially leach from the biochar into the digester. The particle size distribution was also determined.

3.4.1 Particle Size Distribution of Biochar

The particle size distribution test was carried out to determine the predominance of particle size in the biochar sample and thus choose which particle sizes would be used in this work. Standardized sieves were used to carry out this test, the stainless-steel sieves were used during the sieving, with mesh sizes of 125, 250, 500, 1000, and 2000 µm.

The sieves were placed in a series in a sieve shaker (Retsch). The sieves were weighed, and 240g of biochar was added to the highest mesh (2000 µm). The Siever worked for one hour which started with 30 minutes in continuous vibration and then half hour in a discontinuous state with a vibration of 70 amp.

These processes took place twice to verify the result. The desired particle size is between 500 and 1000 µm and less than 125 µm. The total mass gained for the higher particle size the amount of 51.7g was obtained and for the smaller particle size 100.2g was obtained.

Figure 19. Process of biochar particle size distribution

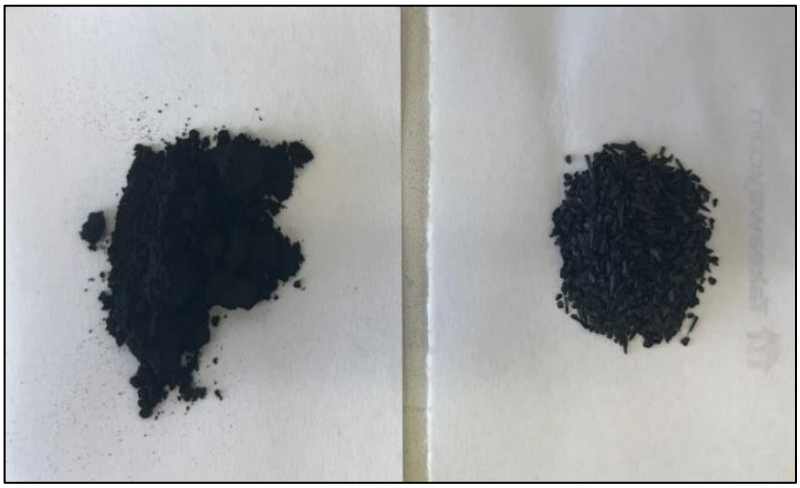


Figure 20. Biochar fractions

3.4.2 Biochar Leaching Test

The biochar leaching test was carried out based on the European standard EN 12457-1. The aim was to determine the chemical elements that the biochar released in the presence of water and whether there were any contaminants present in the leachate.

In this experiment, two 500 mL glass bottles were used, in which 200 mL of demineralized water (Milli-Q), were poured, then the 15g of biochar was added in the two-particle sizes (≤ 0.125mm and 0.5 - 1mm).

The bottles were placed in a rotary shaker (Heidolph, model Reax 20) for 48 hours. The contents of the bottles were then filtered through nitrate filter membranes and a vacuum system shown in Figure 15.

Figure 21. Biochar leaching test

3.5 Selenium

3.5.1 Adsorption Assay

The objective of this part of the experiment was to determine the amount of Se that biochar can absorb. During this assay, 5 different concentrations of Se (20,50,100,150,200 mg/L) were used to determine the maximum amount of Se that the biochar can adsorb with two different particle sizes (larger particle size (0.5-1 mm) and the smaller fraction (<0.125mm).

For each experiment of adsorption, two replicates were prepared. First 10 mg of each biochar particle size was weighed; then added to 25 mL of the Se solution that was made for each Se concentration. After that, they were put in the shaker machine for 24 hours; and then were filtered by the vacuum system filtration.

During the adsorption assay, the amount of Se adsorbed by biochar was not significant due to the negative charge density of Se, the ions were not able to be adsorbed by biochar, so the second approach was taken into consideration.

In the appendix, the isothermal adsorption graphs are presented for the mass of Se adsorbed in the biochar and the percent of the initial mass.

Figure 22. Process of adsorption of Se into biochar

3.5.2 Deposition Assay

The approach to putting Se inside biochar was incorporating selenite (a form of Se) into biochar pores. for this method, 4 different concentrations of Se in the form of sodium selenite powder (Na_2SeO_3) were used.

For making the deposition of Se into biochar, for each sample, the exact desired amount of sodium selenite was measured with the analytical scale, then 3g of each biochar fraction, a larger fraction (0.5-1 mm), and the smaller fraction (≤ 0.125 mm) was added along with 10 mL of distilled water.

For the deposition process, a precise amount of sodium selenite was measured using an analytical scale for each sample. Then, 3g of each biochar fraction (a larger fraction of 0.5-1 mm and a smaller fraction of (≤ 0.125 mm) were added to 10 mL of distilled water. The mixtures were then placed in an agitator and stirred continuously for approximately 24 hours using a magnetic stirrer.

Stirring was performed at room temperature to allow the selenite to be adsorbed and distributed within the porous structure of the biochar. Once the biochar was fully submerged in the solution and the selenite was completely dissolved, the mixed samples were dried at room temperature for 24 to 48 hours until all moisture was removed.

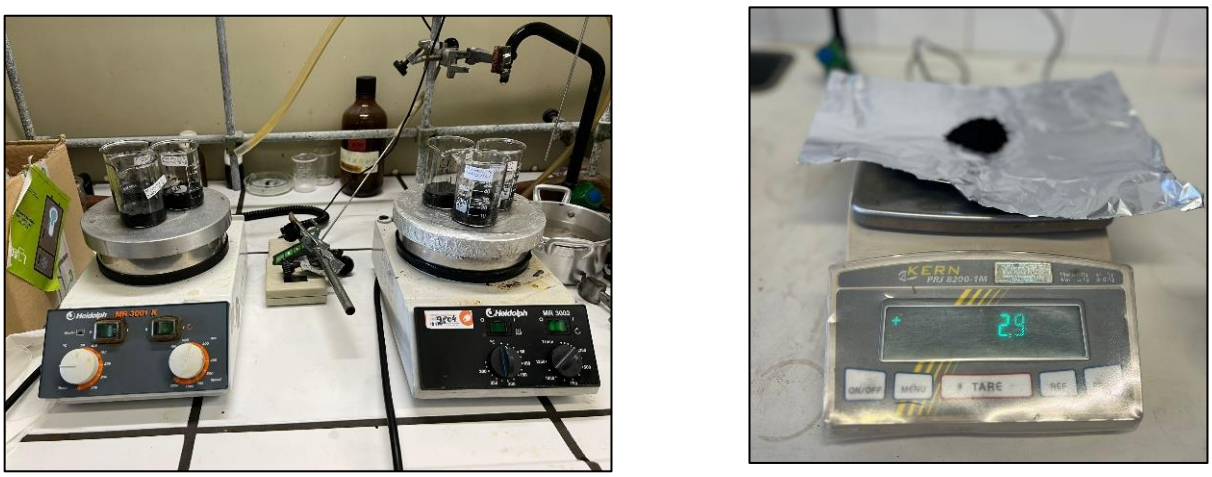


Figure 23. Process of deposition of Se into biochar

3.6 Experimental Trials

The experimental work was divided into two distinct trials. Both trials utilized a combination of substrate and inoculum as the primary influent on the reactor.

Trial 1 focused on assessing the impact of varying Se concentrations (2.5 mg/L, 5 mg/L, and 10 mg/L) on the performance of AD of FW.

Trial 2 investigated the effects of Se deposited in biochar, employing four different Se concentrations (2.5 mg/L, 5 mg/L, 10 mg/L, and 25 mg/L) and two distinct biochar particle sizes, the larger biochar fraction (500-1000µm) and smaller biochar particle size (≤125µm).

3.6.1 Trial 1

Trial 1 aimed to evaluate the behavior of AD with the addition of Se in its free state in 3 different concentrations.

Eight 200 mL bioreactors were used in the first trial, and each bioreactor contained 67 mL substrate and 134 mL inoculum. For each experimental condition, two identical replicates were conducted. The average data from these replicates was used in the subsequent analysis.

Se as the form of sodium selenite was added to the digesters at concentrations of 2.5 mg/L Se, 5 mg/L Se, and 10 mg/L Se. To establish a baseline, a control group of digesters was operated without any added heavy metal. The overall performance of the digesters was evaluated by comparing them to this control group. Bioreactors EXP0-1 and EXP0-2 served as the controls. Bioreactors EXP2,5-1 and EXP2,5-2 received 2.5 mg/L of Se, EXP5-1, and EXP5-2 received 5 mg/L of Se, and EXP10-1 and EXP10-2 received 10 mg/L of Se.

These bioreactors were immersed in a water bath (Grant) at 50℃ (mesophilic temperature). The test was carried out discontinuously, i.e. when biogas production showed a significant reduction (< 20 mL for two consecutive days), the test was terminated.

The contents of the digestors were mixed at least twice a day by shaking them manually for 1 to 2 minutes.

The biogas produced was collected with the Tedlar bags and was measured every two days by reading the level of saline water displaced by gas pressure. The tube was filled with acidified deionized water (Millipore, Elix 5) to measure the volume produced every two days.

Initial l analysis of the gas composition was carried out using a biogas measuring probe (Gasdata¸ model GFM Series) and by gas chromatography (GC), by collecting it in Tedlar bags.

pH for AD should be between 6.8 to 8.5. Lower pH means more acidic conditions inhibit biogas production because the methane bacteria cannot break down so the production of biogas would be stopped.

Most FW feedstocks tend to decompose quickly and can decrease the pH of the digester, to ensure that the pH of the system stayed in the basic range, during the second day of the trial all the digestors were carefully opened and their pH was measured to be sure that the pH of the system remained basic.

In case of the acidic of the system on the first day or the second day of trial, the addition of a buffer such as sodium bicarbonate to the digester may be needed to balance the initial high acidity caused by decomposing FW and would be an extra operating expense. However, in this trial, all the digestors had a pH of around 7 to 8, therefore there was no need to add salt.

Figure 24. Measuring pH in the first and second days of the trials

3.6.2 Trial 2

Trial 2 aimed to evaluate the behavior of AD with the addition of biochar after Se deposition. The biochar was used in two different particle sizes. In this trial, the results and analysis were divided into two separate groups of data and studies for better evaluation of the system.

The first group of experiments is the bioreactors that received the larger biochar fraction (0.5 - 1 mm), and the second group of experiments includes the smaller biochar particle size ($\leq$ 0.125 mm). Table 2 shows the Se concentration and the biochar fraction for each bioreactor trial 2.

Table 2. Biochar particle size and Se concentration in each bioreactor for trial 2

Sample	Biochar particle size	Se concentration (mg/L)
BLANK 3	0 (control)	0 (control)
EXP 0-L1	0.5- 1 mm	0
EXP 0-L2	0.5- 1 mm	0
EXP 10-L1	0.5- 1 mm	10
EXP 10-L2	0.5- 1 mm	10
EXP 25-L1	0.5- 1 mm	25
EXP 25-L2	0.5- 1 mm	25
EXP 0-S1	≤ 125 µm	0
EXP 0-S2	≤ 125 µm	0
EXP 2,5-S1	≤ 125 µm	2.5
EXP 2,5-S2	≤ 125 µm	2.5
EXP 5-S1	≤ 125 µm	5
EXP 5-S2	≤ 125 µm	5
EXP 10-S1	≤ 125 µm	10
EXP 10-S2	≤ 125 µm	10
EXP 25-S1	≤ 125 µm	25
EXP 25-S2	≤ 125 µm	25

The method used in this trial for using these two additives was making the deposition of Se into biochar as explained in the deposition assay.

In the second trial, 14 bottles of 200 mL bioreactors were used, each bioreactor contained 1/3 of the total volume (67 mL) of the substrate and 2/3 of the total volume (134 mL) of inoculum. The overall performance of the digester was evaluated by comparing it with 4 digesters without any heavy metal and consist only 3g of biochar but in two different fractions. One of the digestors didn't receive any Se also any biochar was not added externally and therefore it acted as a control digestor.

Figure 25. Preparation of bioreactors for putting in bath (Grant)

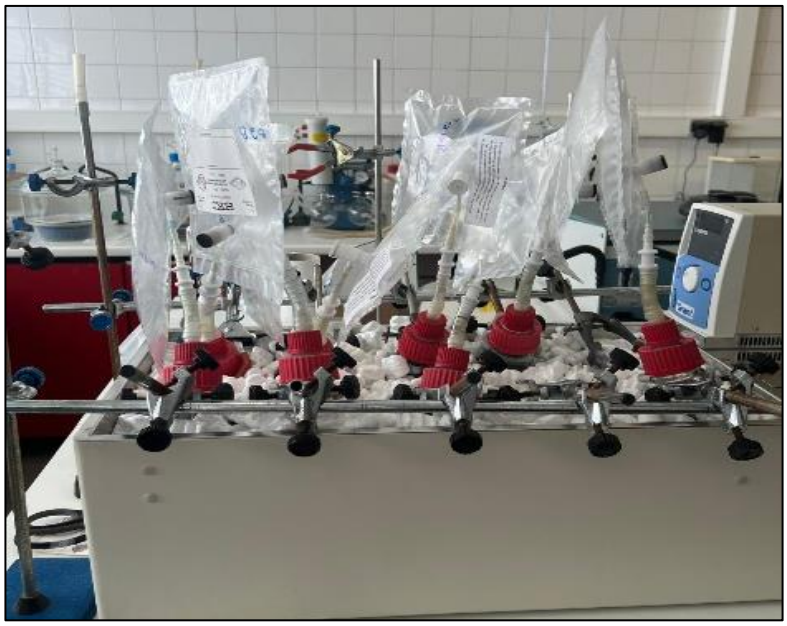


Figure 26. Bioreactors in 50℃ bath (Grant)

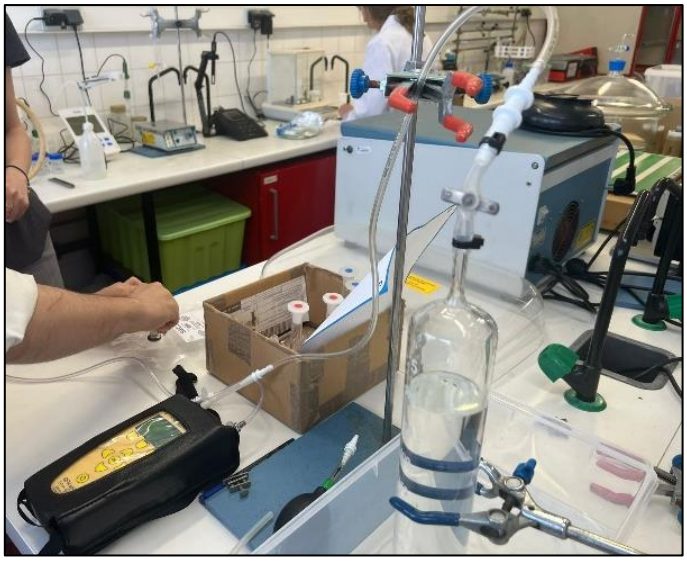


Figure 27. Measuring the composition of biogas by GFM

3.7 Mathematical Modelling of Biogas and Methane Production

Biogas yield Y(t) is the ratio of the volume of gas produced to the mass of volatile solid loaded.

$$Y(t) = \frac{volume\ of\ biogas\ produced}{mass\ of\ volatile\ solid\ loaded} = \frac{V\ biogas}{Mvs}$$

To be able to predict biogas production in AD; the modified Gompertz mathematical model was used to make a fitting curve that allows to generate of a line that predicts the biogas production from AD by using a particular set of data as described by (Luo, 2015).

$$P(t) = P \times \exp\{-\exp\left[\frac{R\ max \times exp(1)}{p}\right] \times (\lambda - t) + 1\}$$

which.

$P(t)$ - Cumulative production of biogas or CH_4, at time t (mL/gVS).

P - Maximum biogas or CH_4 production (mL/gVS).

$R\ max$ - The maximum biogas production rate (mL/gVS*day).

λ - Duration of lag phase (day).

exp (1) - constant= 2.71828

t -Time of assay (day)

The modified Gompertz mathematical model was fitted to the experimental data on the production of using the Solver function in MS Excel. The objective function corresponded to the points obtained in the tests for each experimental condition, by changing the following variables: P, λ and R max. The Nonlinear GRG model was selected to solve the mathematical equation.

4 RESULTS AND DISCUSSION

4.1 Characterization of the Inoculum, Substrate, and Used Mixtures

4.1.1 Characterization of the Inoculum Used in the Trials

Table 3 shows the results for TS, VS, CODt, and CODs, for the inoculum used in the performed trials (assays). The determined values can be compared with the ones obtained by Martins et.al. The inoculum used in (Martins I, 2022) and the ones used in this study were from the same full-scale AD. The origin of each inoculum was from the Valorsul, and both inoculums have the same characteristics.

The inoculum used in this study has a higher CODt, TS, and VS while maintaining relatively similar CODs content. Variations in the quality and quantity of the organic waste introduced into the digestor can significantly influence the microbial community within the inoculum. These variations, in turn, can impact both the COD and solid content of the inoculum.

Table 3. Inoculum characteristics used in this study from the same AD used in other work

	CODt(gO_2/L)	CODs(gO_2/L)	TS(g/L)	VS(g/L)	FS(g/L)
Inoculum in study	23.30 ± 0.10	8.00 ± 0.15	29.02	17.96	11.06
(Martins I, 2022)	23.1	13.8	20.5	13.6	6.9

4.1.2 Characterization of the Substrate

Table 4 shows the characterization of the substrate used and a comparison with the values obtained by other authors. The characterization of the substrate is crucial due to the determination of the stability of the process since inhibitions can occur depending on the substrate used to feed the bioreactor. Se was not detected in the substrate samples analyzed using ICP-AES. The detection limit of the method was 30 (µg/L).

Further investigation into the characteristics of FW revealed that Se was also absent or below detectable levels. This indicates the lack of Se in local food can result in poor or deteriorated AD.

Table 4. Characteristics of substrate used in this study and the other work

	CODt(gO_2/L)	CODs(gO_2/L)	TS(g/L)	VS(g/L)
Substrate in study	38.8 ± 0.0	4 ± 0.06	20.4±2,1	20.3±2.1
Y. meng.et.al (2015)	NA	NA	16.72±0.23	15.63±0.01

4.1.3 Solid Content (TS, VS, and FS) and COD (CODt, and CODs) for Trial 1

Table 5 shows the TS, VS, COD total, and COD soluble values of the initial mixtures, at the start of the process, in each of the experimental conditions of Trial 1.

Regarding TS and VS, it can be seen that the control bioreactor EXP0 and the sample EXP 2,5 presented the lowest concentrations in the digestate, followed by the bioreactor that received the highest concentration of Se (sample EXP 10).

The bioreactor that received the moderate concentration of Se (EXP5) presented the highest levels of TS and VS in the digestate in the initial mixture.

Table 6 shows the characterization of the effluent for trial 1. The control bioreactor EXP0 had the lowest TS and the sample with the highest Se concentration the sample EXP10 had the highest amount of TS.

This difference may indicate a greater development of the microbial community in the reactors that received the Se, thus influencing the number of solids at the end of the process. Analysis of the effluent at the end of the AD process revealed the highest (CODt) in the control bioreactor (EXP0), followed by EXP5 (5 mg/L Se).

The bioreactors with the lowest (EXP2,5) and highest (EXP10) Se concentrations exhibited the lowest CODt compared to the control and the moderate Se concentration bioreactor (EXP5). These results align with expectations, as bioreactors with lower Se concentrations demonstrated greater biogas production and, consequently, more extensive substrate degradation.

Higher total solids (TS) concentrations can indicate a more concentrated substrate, potentially leading to increased biogas production. However, excessively high TS levels can pose operational challenges, such as increased viscosity and mixing difficulties. EXP0, serving as the control bioreactor, exhibited the highest total and soluble chemical oxygen demand (COD) values.

High COD levels in the feedstock suggest a significant amount of biodegradable organic material available for microbial digestion. While this can enhance biogas production, it also indicates a higher pollution load that the system must effectively the sample EXP2,5 with the lowest concentration of Se, demonstrating the lowest total and soluble COD levels, indicating a higher degree of organic matter consumption during digestion. A 3% decrease in effluent COD compared to the influent suggests successful organic matter degradation,

highlighting the efficiency of the AD process. This reflects the effectiveness of microbial activity within the digester.

Table 5. Characterization of the influent in each sample of Trial 1

Sample	TS (g/L)	VS (g/L)	FS (g/L)	CODt (gO_2/L)	CODs (gO_2/L)	pH
EXP 0	25.12 ± 3.98	9.62 ± 2.82	15.50±2.28	21.80±5.28	10.14 ± 0.8	7.85 ± 0.05
EXP 2,5	25.76 ± 0.02	9.06 ± 0.96	16.7 ± 0.9	10.4 ± 5.60	10.4 ± 0.8	7.84 ± 0.02
EXP 5	26.84 ± 0.98	9.36 ± 0.96	17.48 ± 0.02	23.2 ± 4.00	11.2 ± 0.01	7.85 ± 0.02
EXP 10	29.17 ± 0.21	9.88 ± 0.94	19.29 ± 0.73	17.6 ± 4,8	17.6 ± 4.8	7.86 ± 0.02

Table 6. Characterization of the effluent in each sample of Trial 1

Sample	TS (g/L)	VS (g/L)	FS (g/L)	CODt (gO_2/L)	CODs (gO_2/L)
EXP 0	8.58 ± 3.00	4.29±1.70	4.29± 2.37	20.53 ± 5.07	10,93±0.8
EXP 2,5	10.72 ± 0.28	2.14 ± 0.04	8.58 ± 0.32	8.4 ± 0.8	3.2 ± 2.4
EXP 5	10.58 ± 0.76	4.18 ± 1.16	6.47 ± 1.92	16.0 ± 1.6	7.2 ± 3.2
EXP 10	11.1 ± 0.7	3.63 ± 0.05	7.47 ± 0.65	13.6 ± 0.8	4.0 ± 0.8

4.1.4 Solid Content (TS, VS, and FS) and COD (CODt, and CODs) of Trial 2

Table 7 shows the TS, VS, COD total, and COD soluble values at the start of the process in each of the experimental conditions of Trial 2, and Table 8 shows the characteristics of the effluent of all the experiments in Trial 2.

In the entrance, for the majority of the samples, the values of TS and VS are similar to each other both for the higher fraction and lower fraction of biochar with and without the addition of deposited Se; however, A higher value of VS is found for the control bioreactor (EXP0) which is due to the absence of biochar.

For the TS the values of the entrance and the feedstock of the digestors decrease; this indicates that the digestion process has effectively removed a portion of the solid material from the wastewater. This reduction could be due to factors such as settling, filtration, or the conversion of solids into biogas.

For the VS the values of the initial mixture and the feedstock remained relatively unchanged while TS decreased, which suggests that the majority of the solids removed were non-

biodegradable or difficult-to-degrade components. These could be inorganic materials, such as sand or grit, that are not readily metabolized by microorganisms.

The CODt and CODs were decreased significantly for the entrance and feedstock for all the samples which can suggest that a portion of the organic matter has been degraded during the digestion process. This is typically accompanied by biogas production.

Among all samples in Trial 2, the sample EXP10-L exhibited the highest decrease in TS. This suggests that the specific conditions employed in the sample EXP10-L, potentially indicate that the 10mg/L of Se deposited in the larger fraction of biochar was leading the system to have led to the most effective removal of solids.

Several authors have identified a greater development of the microorganism community in reactors that received both biochar and activated carbon (Sinervo, 2017).

Also, higher amounts of solids in bioreactors that received biochar but did not verify solids degradation between the substrate and the end of the AD process, which seems strange given that biogas was produced.

Another important point to be addressed is that in the case of the aforementioned author, the bioreactors that received biochar produced less biogas than the control bioreactor and, therefore, it was to be expected that the concentrations of solids in these bioreactors were expected to be higher at the end of the process (Tan X. L., 2017).

Table 7. Characteristics of the influent in each sample of Trial 2

Sample	TS (g/L)	VS (g/L)	FS (g/L)	CODt (gO_2/L)	CODs (gO_2/L)	pH
EXP0	25.12 ± 3.98	9.62 ± 2.82	15.5 ± 2.28	18.4 ± 2.4	15.2 ± 0.8	7.85 ± 0.05
EXP 0-L	25.98 ± 0.18	5.37 ± 0.35	20.61 ± 0.71	13.6 ± 0.8	18.80 ± 1.13	8.07 ± 0.12
EXP 10-L	25.70(1)	6.74(1)	18.96(1)	22.00(1)	16.4(1)	8.03(1)
EXP 25-L	25.04(1)	6.04(1)	19.04(1)	17.6(1)	18.0(1)	8.14(1)
EXP 0-S	20.13 ± 1.19	4.63 ± 0.41	15.50 ± 0.78	18.4 ± 2.4	17.2 ± 0.8	7.99 ± 0.04
EXP 2,5-S	15.65 ± 0.73	5.54 ± 0.46	10.11 ± 0.27	32.0 ± 1.6	15.0	7.80 ± 0.08
EXP 5-S	19.58 ± 0.22	5.82 ± 0.34	13.76 ± 0.56	24.8 ± 2.4	19.6 ± 1.6	7.97 ± 0.12
EXP 10-S	15.01 ± 1.19	5.02 ± 0.18	9.99 ± 1.01	29.6 ± 0.8	18.0 ± 1.6	7.93 ± 0.80
EXP 25-S	24.76(1)	5.90(1)	19.06(1)	16.0(1)	16.4(1)	8.20(1)

(1) value for one replicate

Table 8. Characteristics of the effluent in each sample of Trial 2

Sample	TS (g/L)	VS (g/L)	FS (g/L)	CODt (gO_2/L)	CODs (gO_2/L)
EXP0	8.58 ± 3.0	4.09 ± 0.45	4.29 ± 2.37	13.6 ± 0.8	9.60 ± 0.81
EXP 0-L	13.22 ± 0.74	4.09 ± 0.45	9.13 ± 1.31	6.40 ± 0.81	12.0 ± 1.6
EXP 10-L	8.20(1)	4.30(1)	3.90(1)	13.60(1)	13.60(1)
EXP 25-L	15.96(1)	2.5(1)	13.46(1)	12.0(1)	10,4(1)
EXP 0-S	12.57 ± 0.09	3.07 ± 1.59	9.5 ± 1.5	10.4	12.8 ± 0.8
EXP 2,5-S	11.85 ± 1.21	4.09 ± 0.19	7.76 ± 1.40	18.4	12.0
EXP 5-S	12.01 ± 0.21	4.29 ± 0.19	7.72 ± 0.48	14.4 ± 0.8	12.0
EXP 10-S	9.93 ± 2.31	2.53 ± 0.79	7.40 ± 1.52	16.0 ± 0.8	12.8 ± 0.8
EXP 25-S	10.72(1)	3.26(1)	7.46(1)	13.06(1)	13.06(1)

(1) value for one replicate

4.1.5 Removal Efficiency of VS, TS, CODt, and CODs of Trial 1 and Trial 2

Graph 1 and graph 2 illustrate the removal efficiencies of each sample of trial 1 and trial 2. Bioreactors supplemented with Se exhibited the highest removal efficiencies for VS and CODs in trial 1 compared to the control bioreactor.

For the sample EXP2,5, this likely explains its superior biogas production in trial 1, the bioreactors with higher Se concentrations (5 and 10 mg/L) did not outperform the control reactor in terms of biogas production, however, in the case of VS and CODs removal efficiencies they had better performance than the control bioreactor, which suggests the microbial community is more effective at decomposing solids but less efficient at converting those solids into methane, you may see high VS removal with lower biomethane yields.

Also, there may be inhibitors present in the digester that affect methanogens specifically.

If the conditions are right for acid-producing bacteria but not for methane-producing bacteria, this could lead to higher VS removal without a corresponding increase in biomethane. The reduced VS removal efficiency observed in the sample EXP10 can be attributed to the toxicity of higher Se concentrations.

Se can adversely affect methanogens, the microorganisms responsible for methane production and organic matter degradation, leading to inhibited activity and decreased VS removal.

Comparing the samples that received both biochar, the TS removal efficiency of the samples was in the same range, however the CODt of the sample that received both additives had an increase in CODt. This can be caused by the synergic effect of Se and biochar which leads to a more active microbial community capable of metabolizing the organic compounds present.

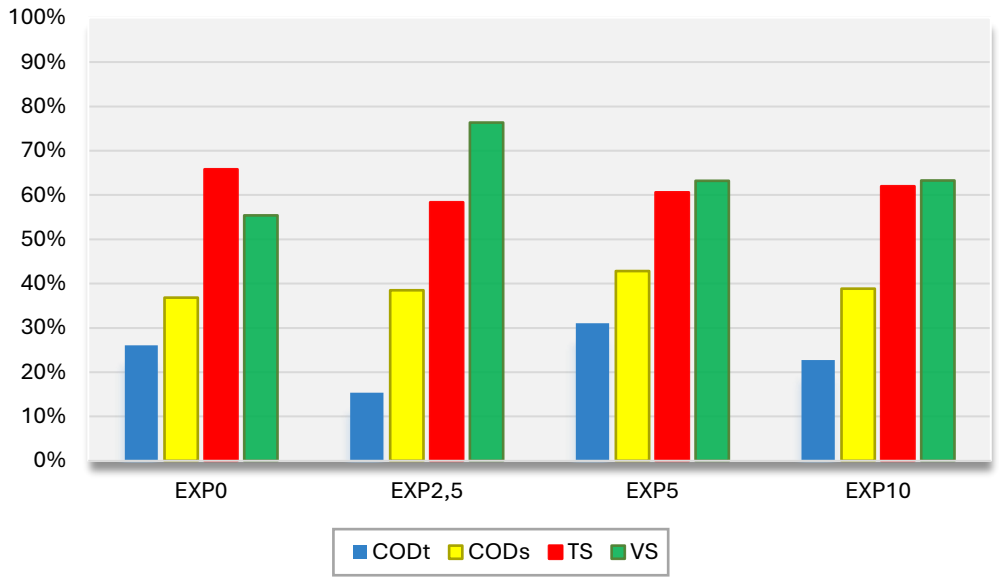
(Yafan Cai, 2018) has reported that the VS removal rate had improved after the addition of trace elements when compared with the control treatment group.

(Martins I, 2022) demonstrated that incorporating conductive materials like biochar into anaerobic digestion (AD) processes can significantly enhance chemical oxygen demand (COD) removal.

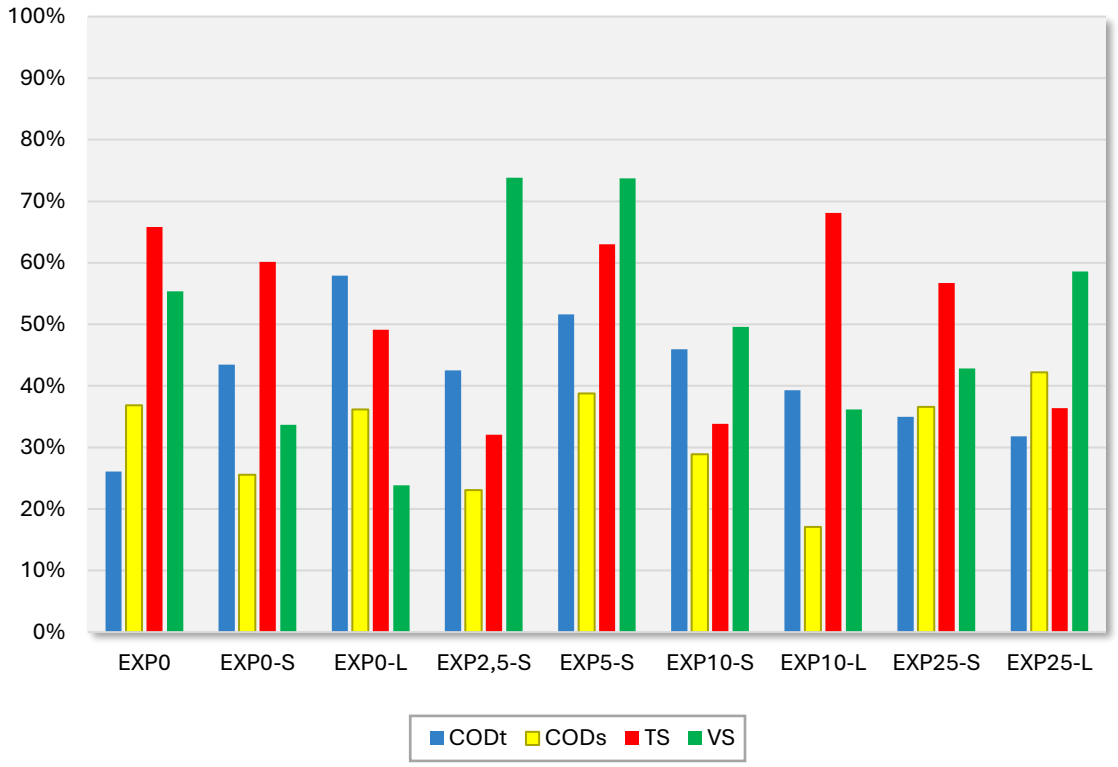
Bioreactors supplemented with Se deposited in biochar demonstrated superior CODt removal compared to the control bioreactor and the samples with the addition of only Se.

However, volatile solids (VS) removal was lower in Se deposited in biochar-treated reactors. This suggests that the biochar may have stimulated bacterial growth in the experimental conditions, leading to increased consumption of organic matter (VS) and ultimately higher CODt removal.

Graph 1. Removal efficiency of VS, TS, CODs, and CODt in each sample of Trial 1.



Graph 2. Removal efficiency of VS, TS, CODs, and CODt in each sample of Trial 2



4.2 Biochar Characterization

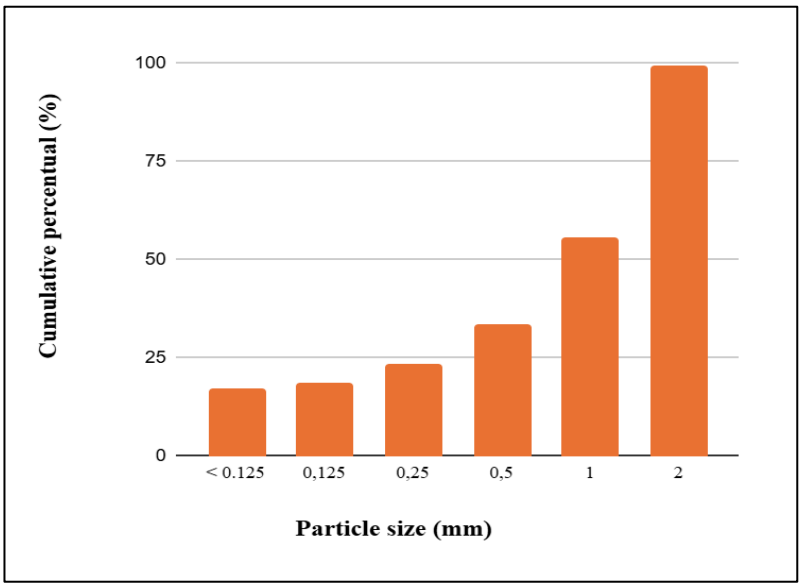
4.2.1 Particle Size Distribution

The decision to study different particle sizes was based on literature showing positive results for biochar particle sizes ranging from less than 125 µm to 1 mm.

Selecting an appropriate particle size is crucial for promoting the initial colonization of microbial cells (Lou,2019).

Graph 3 shows the biochar particle size distribution obtained from the test. The biochar exhibited a relatively uniform particle size distribution across most sizes, allowing for the selection of particles within the ranges studied by other authors: particles 125 µm (17.43 % w/w) and between 500 µm and 1 mm (33.37 % w/w).

Graph 3. Cumulative particle size distribution of biochar



4.2.2 Biochar Leaching Test

The test was carried out to analyze the elements released by the biochar in the aqueous medium, corresponding to the medium in which the AD process is taking place in this study.

Table 9 shows the concentrations of the elements found in the leachate, according to the particle sizes used in both trials of this work.

The concentrations of some elements are presented in mg/L to facilitate the comparison that is made with specific legislation and bibliography. The pH of the water used was 5.5 and the leachate pH ranged from 8.3 to 8.6.

Table 9. Concentrations of elements in the biochar leachate

Particle Size	< 0.125 mm	0.5 - 1 mm
The concentration of biochar in the leaching assays (g/L)	15	15
Element concentrations (mg/kg bs)		
Al	32.66	16
As	1.33	1.33
Cd	0.0	0.0
Cr	1.33	1.33
Cu	3.33	2
Fe	8	32
Hg	0.66	1.33
K	3210	1778
Na	3132	2452
Ni	0.66	0.66
P	98	52
Pb	12.66	10
Se	4.66	4.66
Zn	14	3.33

Na, K, and Zn were identified as having inhibitory potential. However, all had concentrations well below the inhibition limits (Na: 3500 mg/L, K: 2500 mg/L, and Zn: 0.21 mg/L), with the maximum concentrations obtained being as follows: Na - 46.15 mg/L; K - 48.15 mg/L and Zn - 0.21 mg/L, as the leaching test shows, the Biochar must not release toxic substances into the AD process.

The results show that in the biochar the Se concentration is 0.07 mg/L, which is below the toxic range. Se was present in digestion at concentrations ranging from 2.5 to 25 mg/L. While higher concentrations can improve performance, the optimal Se level was determined to be 25 mg/L, balancing benefits with potential toxicity.

Se is a trace element that can enhance AD by increasing biogas production, reducing the lag phase, and improving system stability.

However, it's essential to add Se in appropriate amounts, as excessive levels can inhibit microbial activity and reduce biogas production. The optimal Se concentration may vary depending on specific digestion conditions, and careful monitoring is required to avoid exceeding toxic levels.

Generally, a concentration above 10 mg/L of Se in addition to the AD of FW is considered toxic. However, due to the experimental condition, a higher concentration of 25 mg/L also was examined in the study.

4.3 Trial 1

4.3.1 Analysis of Quantity and Quality of Biogas

Graph 4 shows the cumulative specific biogas production for the experimental conditions tested in Trial 1.

Two identical replicates were used for each sample and the average value between these two replicates was determined.

In this trial, all the bioreactors started producing gas from the first day, a clear lag phase of 0 days can be observed in all experimental conditions.

Lag phase 0 means that the microorganisms started to break down the organic materials without delay, this means that the digestion began immediately, and microorganisms adapted to the system very quickly, leading to a more efficient system and faster biogas production.

During the study, the lower Se concentration experiment had the highest biogas production volume, and the EXP5 and the EXP10 had the lowest production volume.

The sample EXP0 was the control bioreactor that only took substrate and inoculum, and it produced approximately the same volume as the EXP2,5.

During the second week of the trial, the sample EXP0 showed no improvement in biogas production, and its production volume stayed at 100 mL/gVS.

The sample EXP2,5 with the lowest concentration of Se (2.5 mg/L) produced the highest biogas within 14 days of the trial compared to the samples with the highest amount of Se.

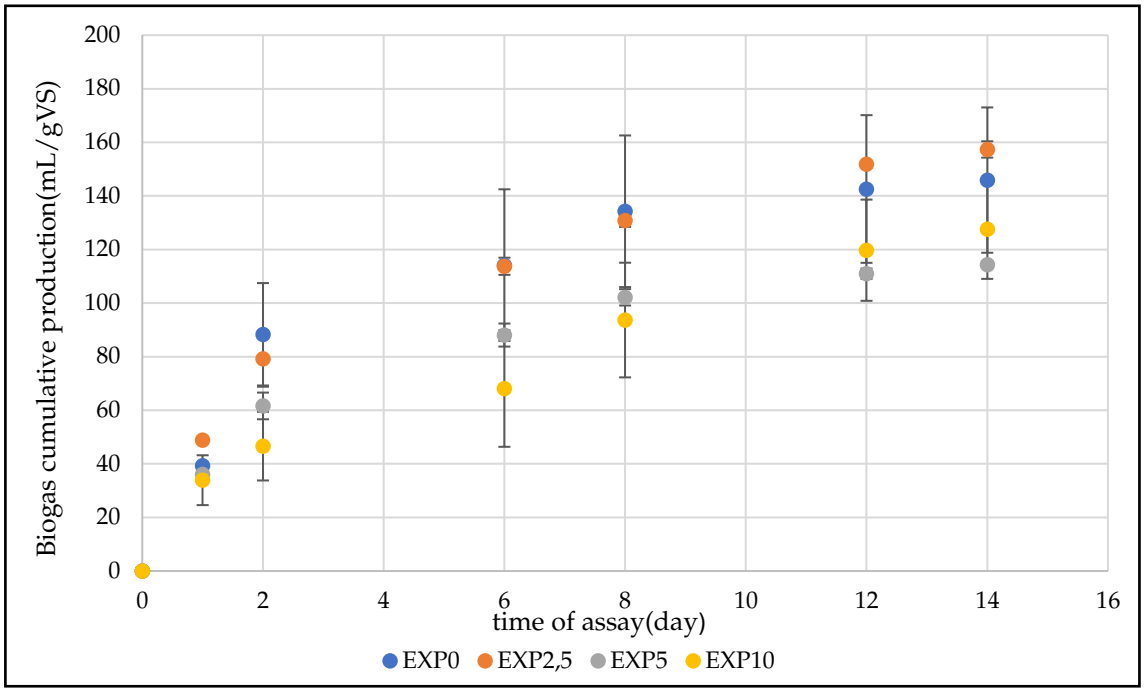
The sample EXP10 was the bioreactor that received the highest concentration of Se and had the lowest biogas production rate until day 8. During the second week of the trial, the production rate gradually increased and went higher than the sample with a moderate concentration of Se the sample EXP5.

In (Javkhlan Ariunbaatar, 2016) it was stated that the supplementation of trace elements increased the biomethane potential of FW with a low trace element concentration. (Javkhlan Ariunbaatar, 2016) experimented with the supplementation of Se and showed an increase of 34.1± 5.6% in biomethane yield.

In (Cai, 2016) it was reported that the addition of Se significantly improved AD performance, particularly at low concentrations of Se, especially during the early stage of AD. However excessive Se concentrations led to toxicity, manifested by the accumulation of VFAs.

The addition of trace elements was associated with the change in the bacterial community structure, which, affected reactor performance. Se significantly changed the bacterial community structure and the change in the community structure directly affected the performance of the AD (Cai, 2016).

Graph 4. Biogas cumulative production for trial 1



4.3.2 Biogas Composition of Trial 1

Graph 5 illustrates the biogas composition when the daily production rate became stable.

The composition of CH_4 for all the samples with Se-treated remained within a similar range during the 14 days of the assay.

It can be seen from the graphs that the methane composition for the best experimental condition of the sample EXP2,5 in terms of biogas production was 17.45% CH_4 on the first day of the trial.

Notably, on day 8 of the assay, all Se-treated samples demonstrated a 28% increase in CH_4 compared to the control bioreactor (EXP0). While the samples EXP2,5 and EXP10 maintained elevated methane levels until day 14, the sample EXP5 experienced a decline after day 10.

The highest Se concentration (EXP10) yielded the most significant biomethane production during the second trial week.

On day 14, the sample EXP10 produced 72.05% CH_4, surpassing the sample EXP5 by 18% and the sample EXP2,5 by 9%.

The results show that Se accelerated methane production. The graphs show that the Se-treated bioreactors reached peak methane levels earlier than the control bioreactor (EXP0).

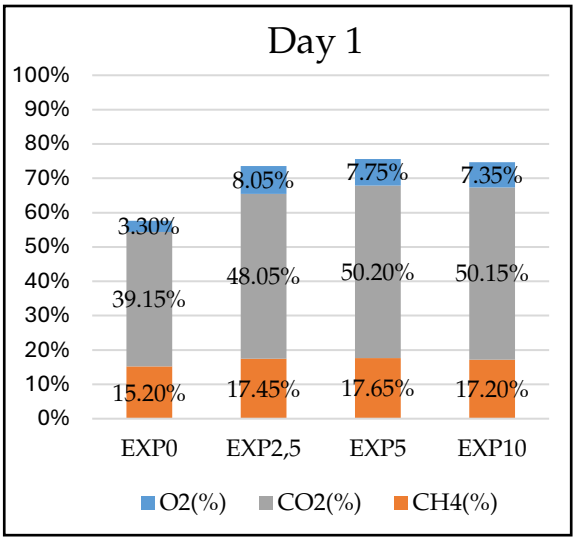
(Yafan Cai, 2018) observed that the methane content of the Se treatment groups rapidly increased from 0% to approximately 60% (by day 10).

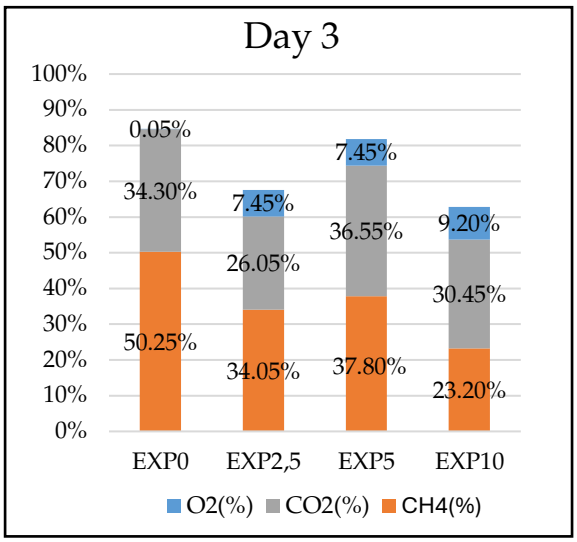
The methane content of the Se treatment group was significantly higher than that of the control treatment group from the third day to the 10th day.

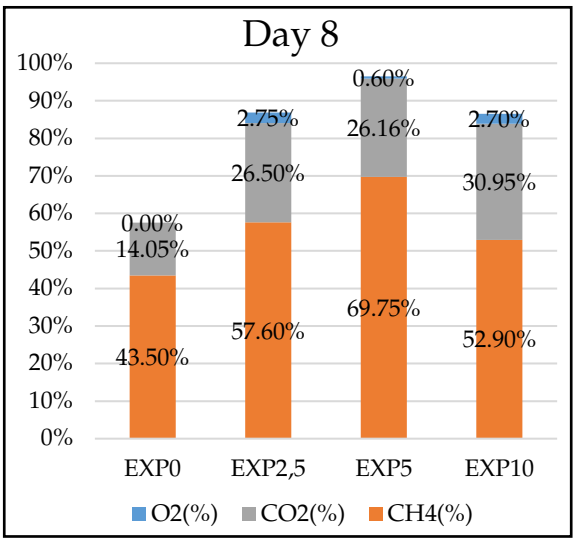
At the end of the digestion period, no significant differences ($P > 0.05$) in the methane content were observed among the Se and control treatment groups.

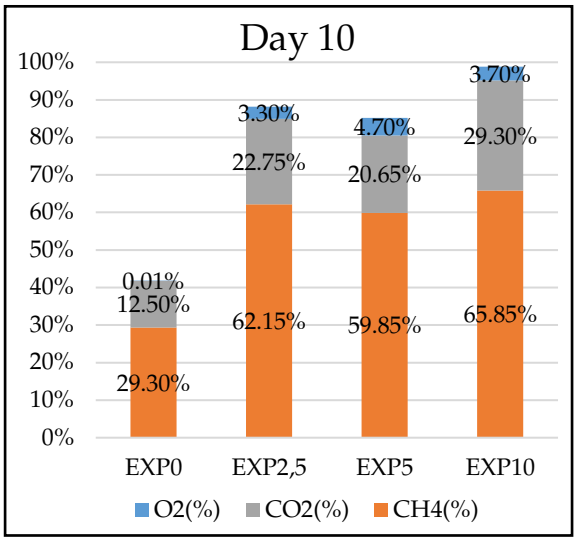
For the Se treatment groups, the methane content increased rapidly in the first three days and then slowly increased.

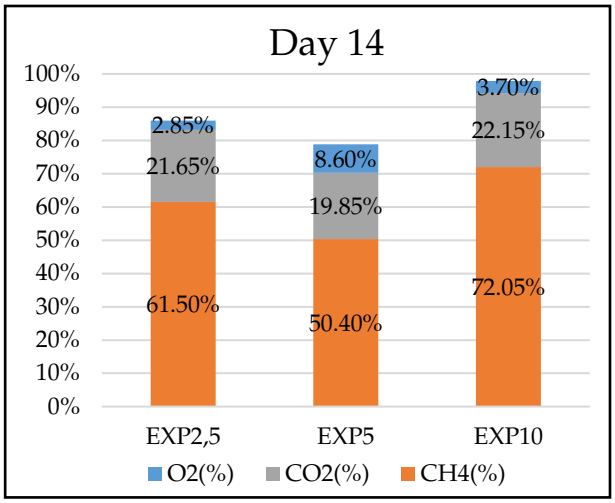
Graph 5. Biogas composition of each sample in trial 1











4.4 Trial 2

Trial 2 aimed to evaluate the behavior of the AD process of FW by the addition of Se (sodium selenite salt) deposited in a biochar matrix.

The AD process conditions were the same as in trial 1 but with a porous material with Se incorporated. In trial 2 the two separated assays were performed as described below.

The first assay was performed using the smaller biochar's fraction (≤ 0.125mm); in-which Se was to achieve concentrations of 2.5 mg/L Se, 5 mg/L Se, 10 mg/L Se, and 25 mg/L Se in the digester.

In the second assay, it was used the larger size fraction but with a concentration of 10 mg/L Se with better performance than the previous experiment and, a superior one with 25 mg/L of Se.

4.4.1 Analysis of Quantity and Quality of Biogas

4.4.1.1 Group 1: smaller biochar fraction (≤ $0.125mm$)

Graph 6 shows the cumulative biogas production per volatile solids mass within 14 days of the trial. In all the experiments two duplicate bioreactors were used for each condition and the average values between these two replicates are represented in this study.

The sample EXP0 acted as a control reactor that only received the inoculum and the substrate.

The sample EXP 0-S contains only the biochar (3g) with (≤ 0.125mm) particle size.

The sample EXP2,5-S contains biochar with deposited Se to achieve a concentration of 2.5 mg/L Se in the digestor. The sample EXP 5-S contains biochar (3g) of a smaller fraction (≤ 0.125mm) with deposited Se to achieve a concentration of 5 mg/L Se in the digestor.

The sample EXP10-S contains biochar (3g) of a smaller fraction (≤ 0.125mm) with deposited Se to achieve a concentration of 10 mg/L Se in the digestor.

The sample EXP 25-S contains biochar (3g) of a smaller fraction (≤ 0.125mm) with deposited Se to achieve a concentration of 25 mg/L Se in the digestor.

All digesters started to produce biogas on the first day of the experiment. During the trial, experimental samples with additives consistently exhibited higher cumulative biogas volumes than the control group, suggesting that the incorporation of Se deposited by biochar altered the microbial environment of the AD systems.

In the end, the experimental treatment groups achieved their maximum cumulative methane yields, with the sample EXP10-S reaching 280 mL/gVS.

This enhanced performance can be attributed to the facilitated electron transfer between microorganisms, promoting more efficient organic matter degradation and biogas production.

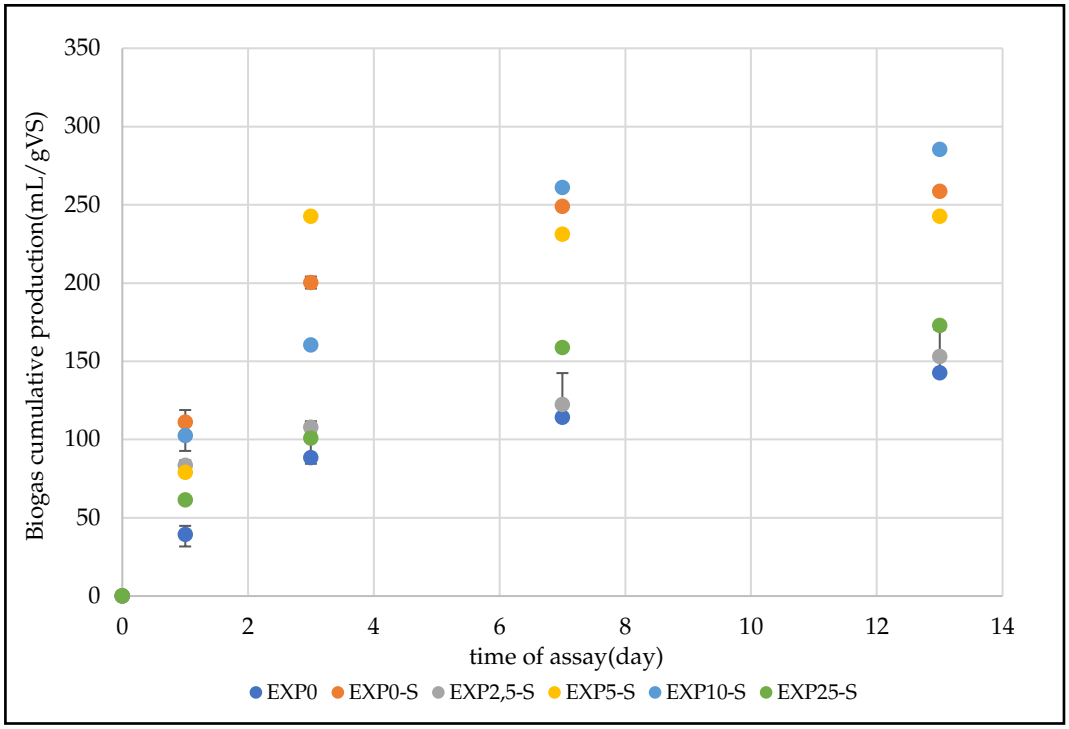
Additionally, the combination of high Se concentration (10 mg/L) and smaller biochar particles in the sample EXP10-S likely contributed to a more diverse and active microbial community, further enhancing organic matter degradation and biogas production.

The control bioreactor, the sample EXP0, exhibited the lowest biogas production volume per unit of VS within the initial 14-day trial period; followed by the samples EXP 25-S (highest concentration of Se) and the sample EXP2,5-S (lowest concentration of Se).

In contrast, the sample EXP0-S and the sample EXP5-S demonstrated nearly identical biogas production during the latter stages of the trial.

This stable cumulative biogas production suggests that the AD process has reached a steady-state condition, indicating efficient system operation and microbial adaptation to the feedstock.

Graph 6. Biogas cumulative production for Trial 2 Group 1



4.4.1.2 Group 2: larger biochar fraction (0.5-1mm)

The sample EXP0-L contains only the biochar 3g with 500-1000 μm particle size. Samples EXP10-L and EXP25-L contain biochar with deposited Se, achieving a concentration of 10 mg/L and 25 mg/L, respectively, inside the digestors.

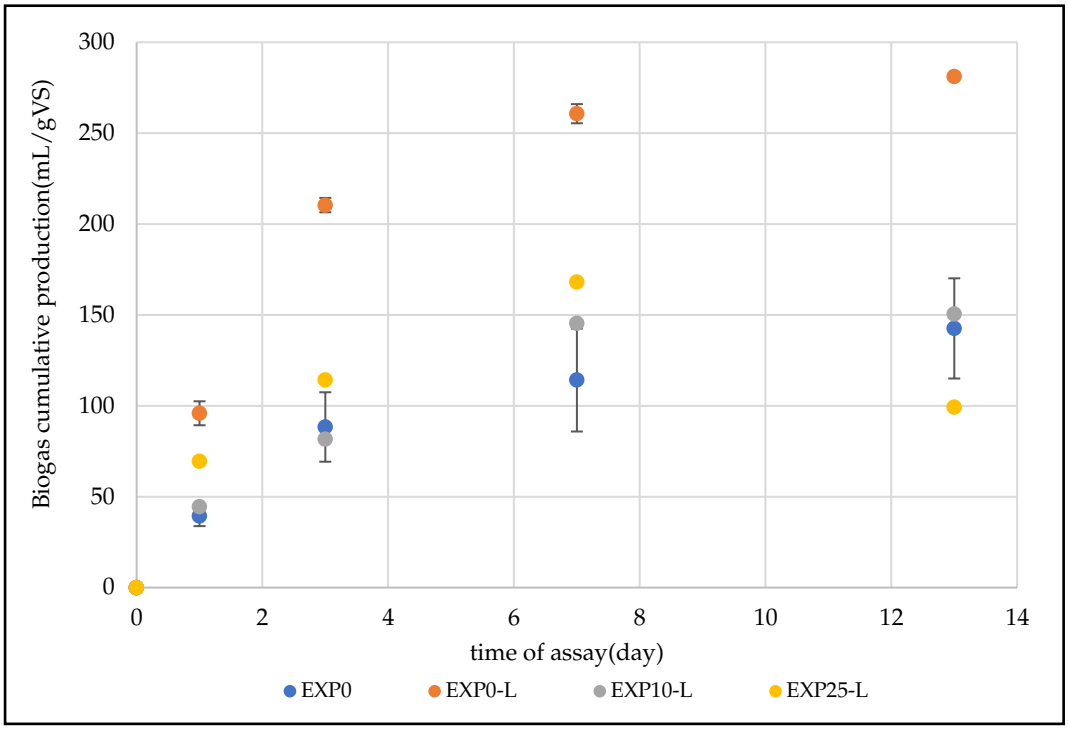
The sample EXP0-L that received only the larger fraction of biochar had the highest accumulative biogas production within the 14 days of the experiment; followed by the bioreactor the sample EXP25-L.

This higher production rate is due to the biochar's porous structure which can provide a suitable habitat for microorganisms involved in AD.

This increased surface area can promote microbial growth and diversity, leading to more efficient organic matter degradation.

The sample EXP0 had the least biogas production, and the production rate stayed stable during the second week of the trial.

Graph 7. Biogas cumulative production for Trial 2 Group 2



4.4.2 Biogas Composition of Trial 2

Graph 8 shows the biogas composition for trial 2 group 1 (the smaller biochar fraction) when the daily production rate became stable.

It can be seen from the graph that the methane composition for the best experimental condition of the sample EXP10-S in terms of biogas production was 25% CH_4 on the first day of the trial.

The sample EXP10-S had an increase of 2% in methane composition compared to the control bioreactor (EXP0), which had a 12% increase in CH_4 on day 7.

The bioreactors with the supplementation of Se deposited into smaller biochar fraction addition had approximately the same biomethane production trend during the 14 days of the trial; however, for the bioreactor with the lowest concentration of Se the sample EXP2,5-S, the composition of methane stayed in the range of 20% CH_4.

The sample EXP2,5-S exhibited a 40% reduction in methane composition compared to the control (EXP0).

Graph 9 illustrates the composition of biogas for all the experiments of trial 2 group 2 (the larger biochar fraction). The sample EXP0-L had the highest biomethane production compared to the control bioreactor. It had an increase of 12% CH_4.

Compared to the control bioreactor (EXP0), the sample EXP10-L exhibited a 27% increase in methane (CH_4) production. While the samples EXP10-L and EXP10-S received the same Se concentration, their biochar particle sizes differed.

Despite this variation, both samples produced biomethane with similar compositions. However, the sample EXP10-S showed a 20% increase in carbon dioxide (CO_2) production compared to the sample EXP10-L.

(Carvalho, 2019) found that adding biochar to a bioreactor improved biogas production. The best results came from using the highest concentration (15 g/L) of the smallest biochar particles (under 250 microns). This setup reduced the lag phase by 22% and increased total biogas production by 31% compared to the control without biochar. The study suggests biochar might benefit the process by supporting bacteria growth and accelerating substrate breakdown. Interestingly, biochar didn't affect the methane content of the biogas, which remained between 80% and 85% for all conditions.

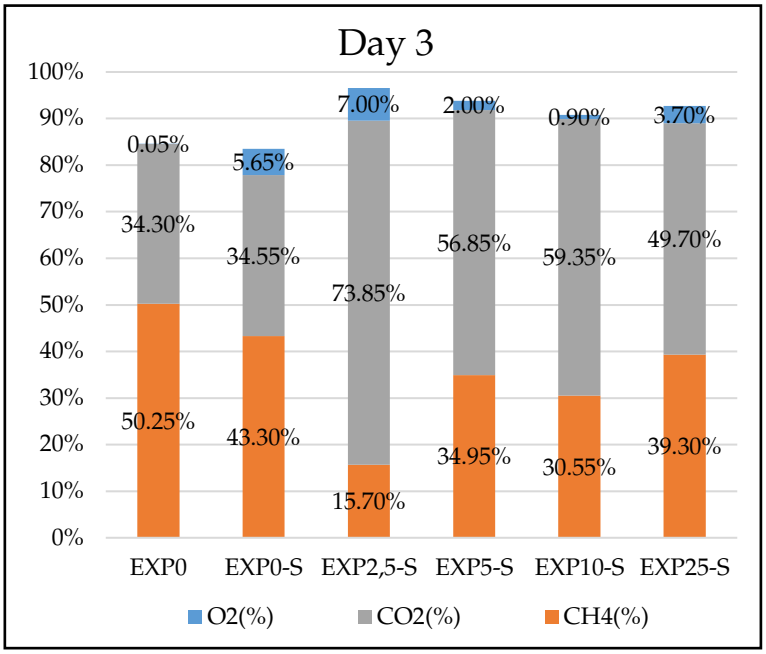
(Yafan Cai, 2018) observed that the methane content of the Se treatment groups rapidly increased from 0% to approximately 60% (by the 10th day). The methane content of the Se treatment group was significantly higher than that of the control treatment group from the third day to the 10th day. At the end of the digestion period, no significant differences ($P > 0.05$) in the methane content were observed among the Se and control treatment groups. The methane content for the Se treatment groups increased rapidly in the first three days and then slowly increased.

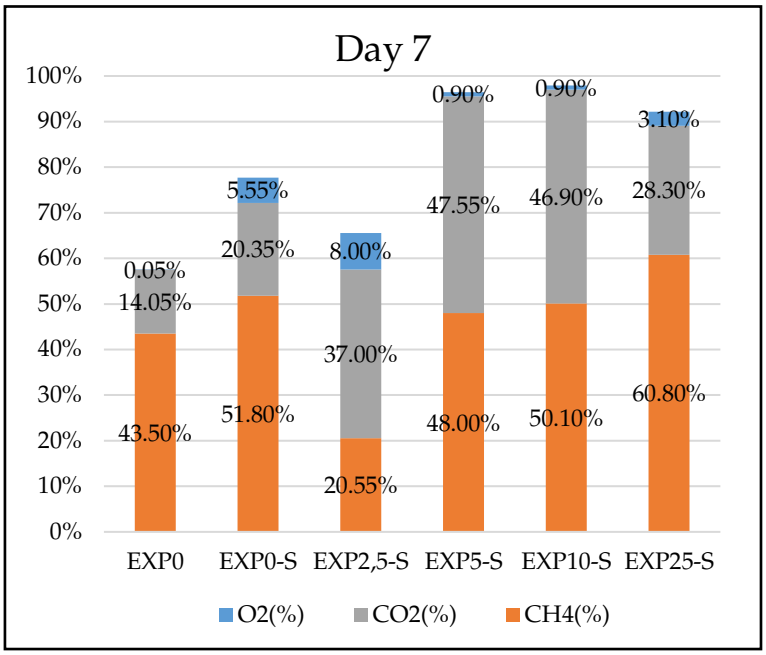
(Yi, Dong, Jin, & X, 2014) observed at low Se concentrations (0.1 mg/L) significantly enhanced methane yield and production rate, increasing them by 121% and 113%, respectively. Conversely, high Se concentrations (30-120 mg/L) inhibited methane production by

reducing hydrolytic enzyme and F420 activities, resulting in a 23.1-100% decrease in methane yield and production rate.

4.4.2.1 Group 1: Smaller biochar fraction (≤ 0.125 mm)

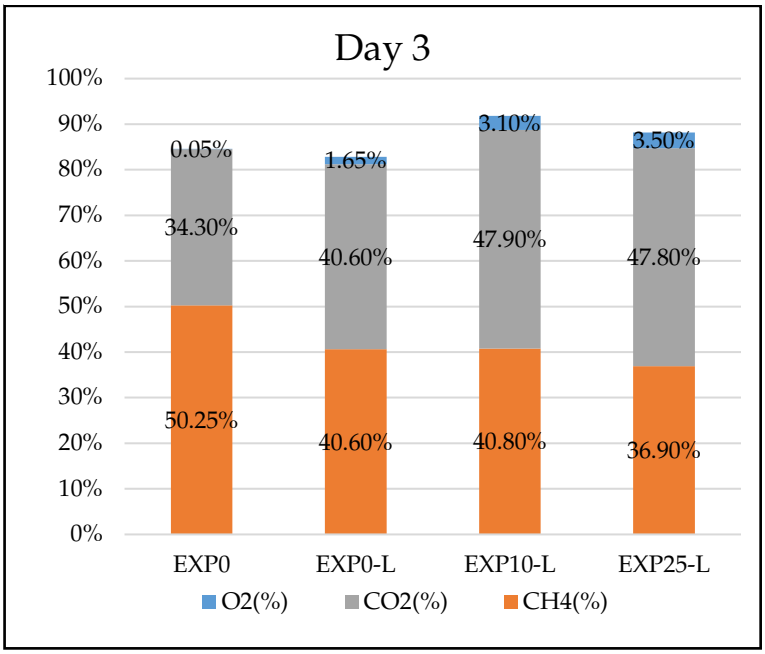
Graph 8. Biogas composition of each sample of trial 2 group 1

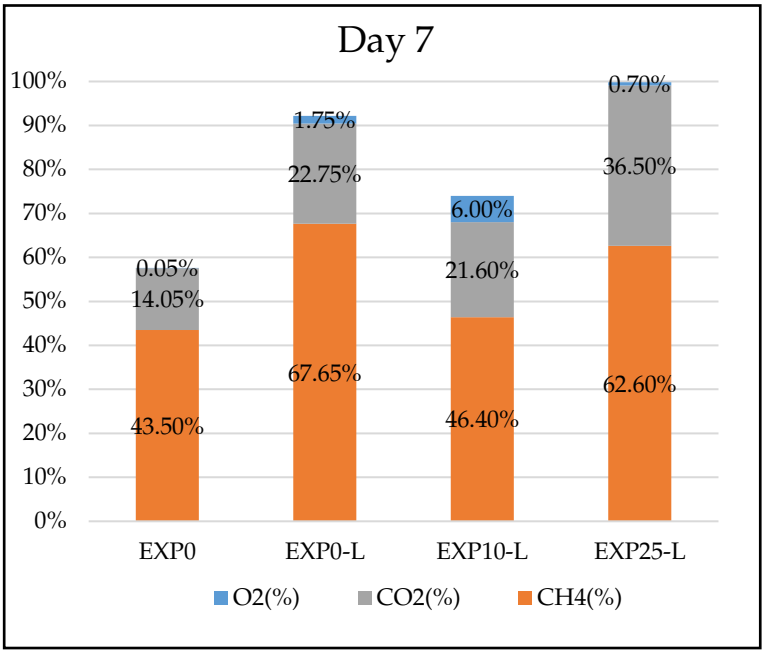




4.4.2.2 Group 2: Larger biochar fraction (0.5 -1 mm)

Graph 9. Biogas composition of each sample of trial 2 group 2





4.5 Gompertz Mathematical Module

Table 10 shows a summary of the values obtained for the kinetic variables of the Gompertz model, for the experiments of Trial 1 and Trial 2, as well as the coefficient of determination (R2). The model was applied to biogas production in Trial 1 and Trial 2.

The experimental curve and the curve obtained by adjusting the Gompertz model are shown in the graphs.

In all the tests except for the experimental sample EXP10 for trial 1, there was good convergence between the kinetic and experimental values.

The absence of a lag phase in all experimental conditions indicates a well-acclimated inoculum, capable of rapidly initiating digestion.

Factors contributing to this include a diverse microbial community with broad enzymatic capabilities, optimal environmental conditions (temperature, pH, nutrients), and the substrate's ease of biodegradability.

Additionally, the substrate's physical properties (particle size, distribution) and any residual microbial activity from previous digestion cycles may have further accelerated the process. While a zero-day lag phase suggests a highly efficient system, it's essential to monitor the digester's performance to ensure it's not operating near its maximum capacity. A longer lag phase might be necessary for the microbial community to adapt to changes in the substrate or operating conditions.

Regarding methane production, the bioreactor supplemented only with biochar (samples EXP0-L, EXP0-S) demonstrated a 58% increase compared to the control (268 mL/gVS for EXP0-L and 230 mL/gVS for EXP0-S, versus 142 mL/gVS, respectively).

Several studies have reported similar findings regarding the positive impact of biochar on methane production (Igalavithanaa, 2018), (Waqas, 2018), (CBF, 2020). One of the main reasons for this enhancement in biogas production of the samples with the addition of biochar deposited by Se is that the biochar's porous structure provides a suitable environment for microorganisms involved in AD where Se can improve the uptake of nutrients by microorganisms, promoting their growth and activity. This means that porosity associated with the samples facilitates the electron transfer between microorganisms (which stay in clusters inside the pores) promoting in this way the metabolism responsible for the reactions intervenient in AD processes.

(Sunyoto, 2016); observed a 10% increase in accumulated methane volume, while others reported increases of up to ranging from 122% to 32% (Shen, 2016), (Fagbohungbe, 2017) and (Le Zhang a, 2019). This variability might be attributed to the differences in substrates used across these studies.

Regarding the factor (Rmax) a higher value of Rmax in the experiments that received only biochar suggests a well-acclimated microbial community capable of rapidly degrading organic matter and producing biogas.

When comparing the sample EXP2,5 from Trial 1 (addition of only Se) to the sample EXP2,5-S from Trial 2 (2.5mg/L Se deposited in smaller biochar fraction), the sample EXP2,5 exhibited a 6.38% increase in cumulative biogas volume production.

This suggests that the addition of biochar, even at a small particle size, may have had an antagonistic effect on biogas production when combined with a low Se concentration.

Comparing the addition of only 5 mg/L Se (the sample EXP5) and the sample with 5 mg/L Se deposited in 3g biochar (EXP5-S), the sample EXP5 that received only Se had a lower biogas production rate, a 46% decrease in biogas production rate, it appears that the combination of Se and biochar (the sample EXP5-S) created a synergistic effect, leading to a higher biogas production rate than the sample that received only Se the sample EXP5.

The biochar likely provided a favorable environment for microbial growth and activity, while the Se enhanced the efficiency of organic matter degradation and biogas production.

A comparison of the sample EXP10 (addition of only 10 mg/L Se) and the sample EXP10-S (10mg/L Se deposited by 3g biochar with smaller particles (≤0.125mm) revealed a significant 63.80% increase in biogas production for the sample EXP10-S. This enhancement is attributed to the synergistic effects of Se and biochar.

The combination of these additives, coupled with the increased surface area provided by biochar particles, may have fostered a more diverse and active microbial community, the biochar provides a larger surface area for nutrient adsorption, potentially improving nutrient availability for microbial growth and activity. This could be particularly beneficial at lower Se concentrations, where nutrient limitations might be more pronounced leading to improved organic matter degradation and biogas production.

In contrast, the sample EXP10-L (10 mg/L Se deposited in 3g larger biochar particles (0.5-1mm) exhibited only a 9.74% increase in biogas production rate, suggesting that the particle size of biochar can significantly influence the effectiveness of the system since smaller particles have a larger surface area, providing more habitat for microorganisms and facilitating nutrient adsorption.

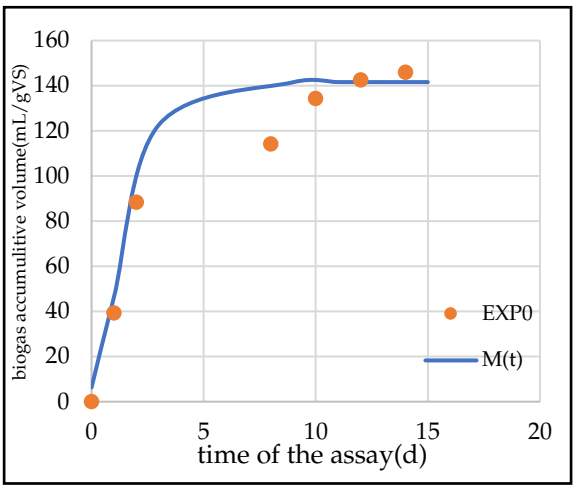
Comparative analysis of samples with larger biochar particles (0.5-1mm) and varying Se concentrations (10 mg/L and 25 mg/L) demonstrated a 30.30% increase in biogas production rate for the sample with the higher Se concentration.

Despite a negligible difference in Rmax (maximum specific growth rate), the enhanced biogas yield suggests a synergistic interaction between Se and larger biochar particles (0.5-1mm). This combination likely created a more conducive environment for microbial growth and activity.

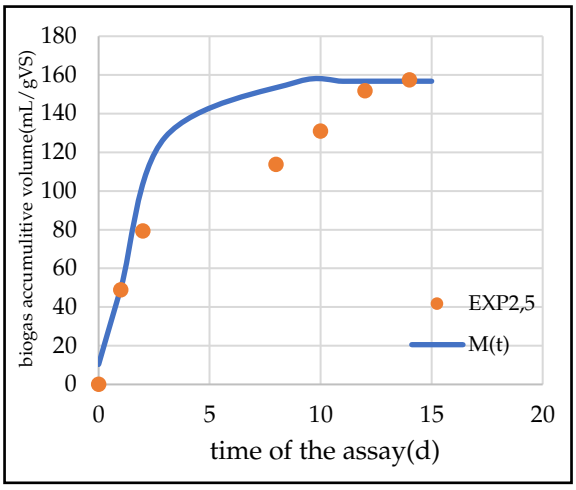
Table 10. Kinetic constants of the Gompertz model applied to biogas volume in trial 1 and trial 2 and coefficient of determination (R^2).

Sample	Cumulative volume (mL/gVS)	P (mL/gVS)	R_{max} (mL/gVS*day)	λ (days)	R^2
EXP0	145	142	53.40	0.13	0.9488
EXP2,5	157	156	49.79	0.0	0.9359
EXP5	114	111	37	0.0	0.9598
EXP10	127	130	25	0.04	0.8360
EXP0-L	268	262	87.94	0.0	0.9890
EXP10-L	140	142	47.57	0.0	0.9492
EXP25-L	190	196	45.48	0.0	0.9582
EXP0-S	230	210	106.88	0.0	0.9566
EXP2,5-S	147	140	52.48	0.0	0.9938
EXP5-S	182	186	41.31	0.0	0.9941
EXP10-S	246	248	60.04	0.0	0.9853
EXP25-S	173	178	38.75	0.0	0.9639

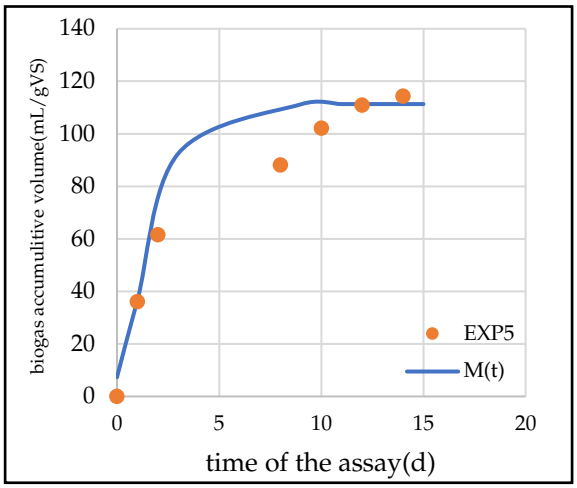
(a) Curve for the control bioreactor EXP0 for Trials 1 and 2.



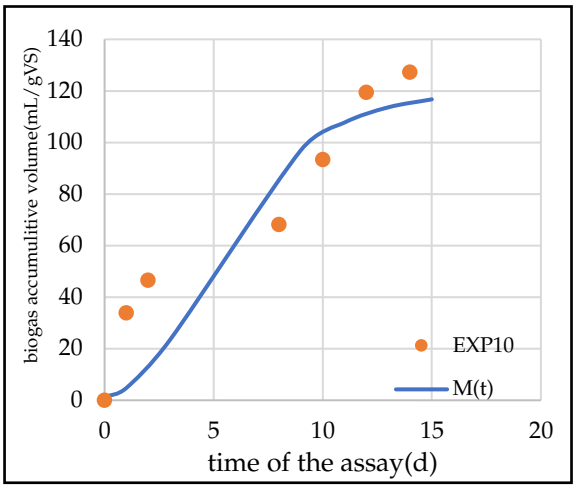
(b) Curve for the sample EXP2,5 for Trial 1.



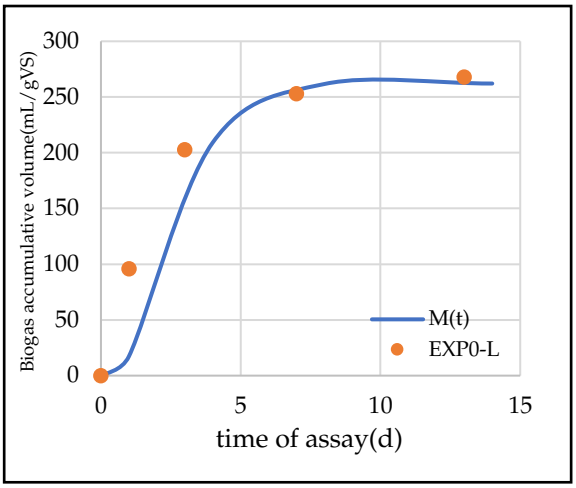
(c) Curve for the sample EXP5 of Trial 1.



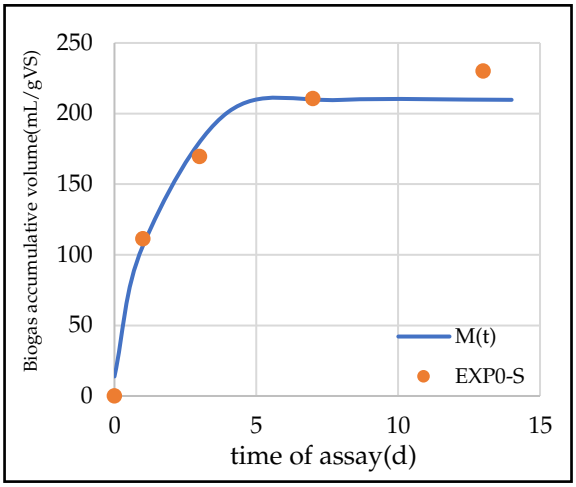
(d) Curve for the sample EXP10 of Trial 1.



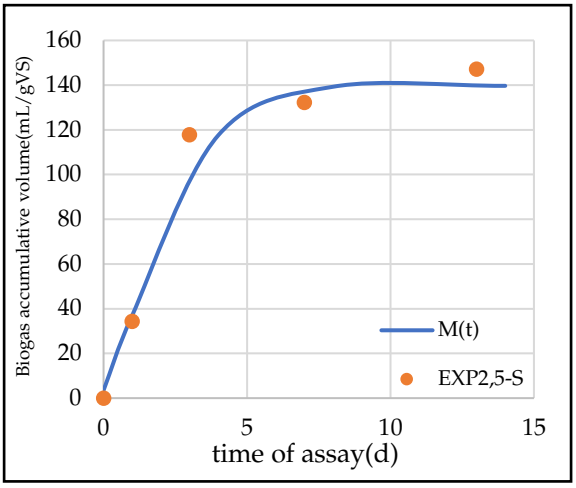
(e) Curve for the sample EXP0-L of Trial 2.



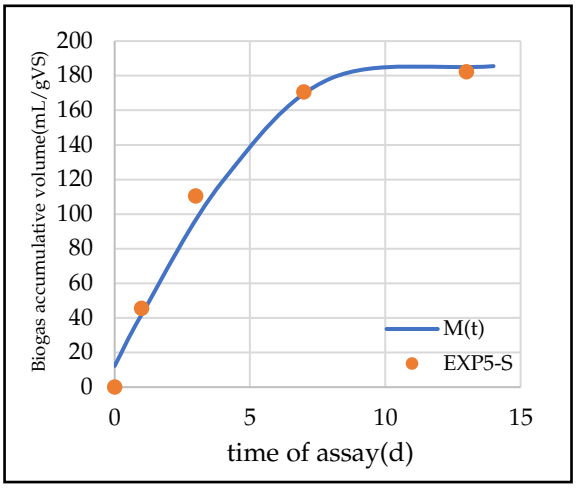
(f) Curve for the sample EXP0-S of Trial 2.



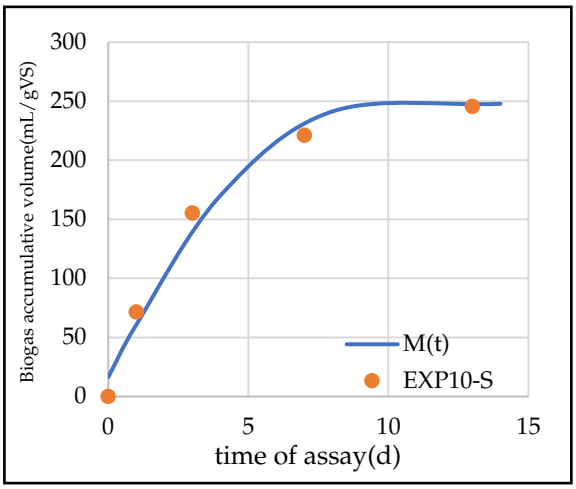
(g) Curve for the sample EXP2,5-S of Trial 2.



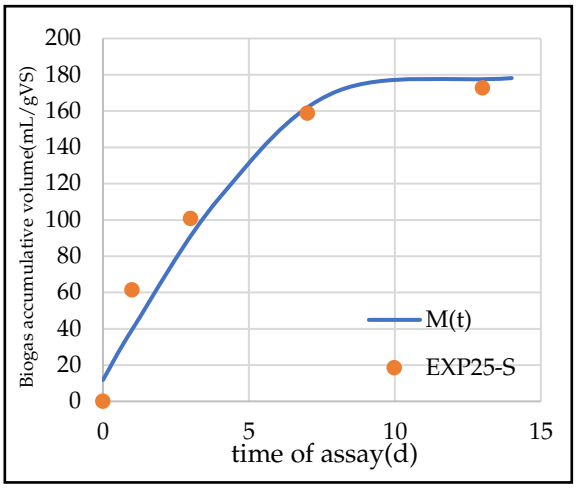
(h) Curve for the sample EXP5-S of Trial 2.



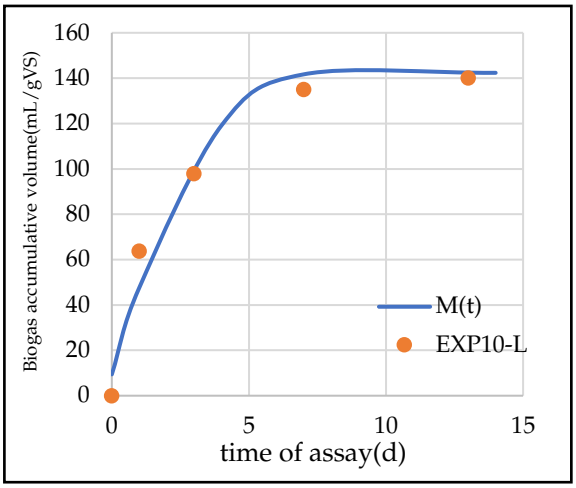
(i) Curve for the sample EXP10-S of Trial 2.



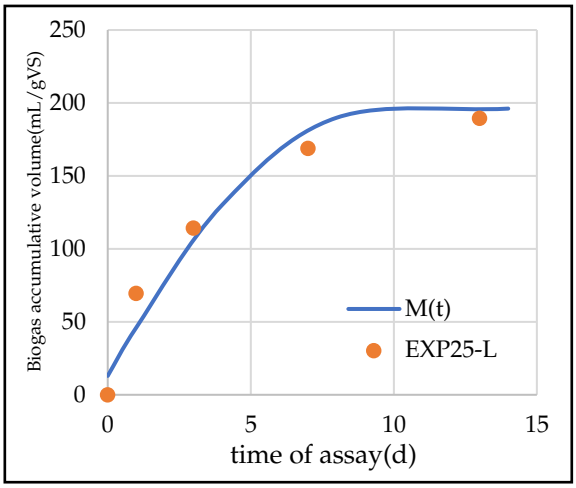
(j) Curve for the sample EXP25-S of Trial 2.



(k) Curve for the sample EXP10-L of Trial 2.



(l) Curve for the sample EXP25-L of Trial 2.



Graph 10. Adjustment of the Gompertz model to the experimental results of trials 1 and 2 of this work

4. CONCLUSION

This study highlights the challenges affecting the AD process—such as slow biodegradation of complex substrates, low methane yield, and system instability which can be addressed through the addition of Se and biochar. The findings demonstrate that Se, particularly at low concentrations, enhances methane production in biogas, while biochar further improves AD performance.

Key conclusions include:

- Low concentrations of Se (2.5 mg/L and 5 mg/L) added as sodium selenite increase biogas production, with the 2.5 mg/L concentration yielding an 8 % improvement compared to the control.
- Higher concentrations of Se (10 mg/L) led to inhibitory effects when used alone, indicating that Se concentrations above 5 mg/L may negatively affect the AD process.
- The combination of Se and biochar enhanced AD performance. In particular, Se deposited in biochar with smaller particle size showed superior results at 10 mg/L, outperforming the control with biochar in 18 % and the one without biochar in 75 %.
- Biochar not only improved biogas yield but also reduced the toxic effects of higher Se concentrations. This suggests that biochar enables the system to tolerate higher Se levels by enriching microbial communities and promoting interspecies electron transfer. In conclusion, a synergistic relationship exists between Se and biochar in enhancing the AD of FW. Biochar shows great promise as an additive, improving the system. stability and allowing for the use of higher concentrations of Se without negative effects. This approach could be key to optimizing AD processes for biogas production.

4.6 Future Challenges

The cost of the additives is a primary obstacle to the widespread adoption of different elements in full-scale anaerobic digesters. Most research on additives in this context has been conducted in laboratory settings or, at best, on the pilot scale, limiting our understanding of their performance under real-world conditions. Batch studies often fail to accurately simulate the dynamic environment of continuous or semicontinuous full-scale digesters.

The addition of trace elements to aid the AD process and their effect on the system still needs more study. Further research involving the use of Se in different oxidation states such as Se nanoparticles where Se is in the elemental state (less toxic) will be a priority.

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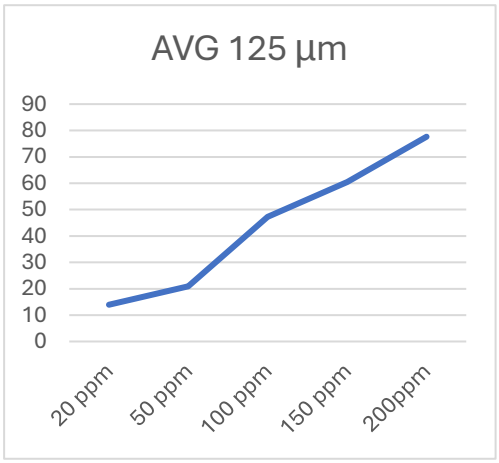
APPEN-DIX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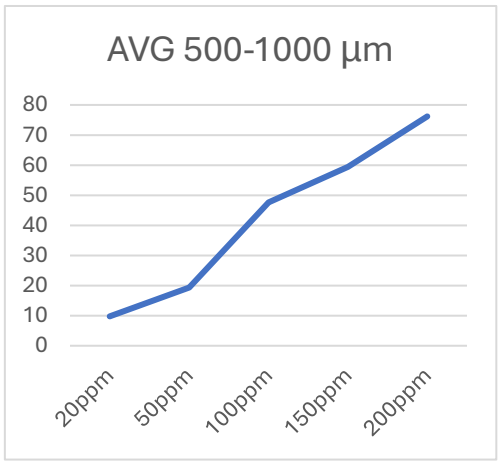
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