IMPROVING METHANE PRODUCTIVITY OF FOODWASTE BY ENZYMATIC PRETREATMENT AND ELECTRODE MODIFICATION IN MEC-AD HYBRID SYSTEM

BY

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ABSTRACT

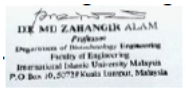
Hydrolysis has been identified as a rate-limiting stage in anaerobic-digestion. While it's been widely used in biomethane production, biomethane only accounts for 50-60%. A Therefore, an integrated AD-MEC system was developed to increase the biomethane content using food waste. However, high electrode's cost in the hybrid system poses an economical challenge to the market. Moreover, the microbial community plays a crucial role in the system, yet, minimal studies address the enhancement of microbial community and diversity. Hence, the characterization of food waste was performed in terms of carbohydrates, lipids, proteins, chemical oxygen demand, moisture content, solids, and volatile solids. The enzymatic hydrolysis of food waste was conducted to obtain the hydrolysate by one-factor-at-a-time (OFAT) through various factors including reaction time, temperature, enzyme loading, substrate concentration, and pH. The results showed that the optimum pH of 7, substrate concentration of 6%TS, the temperature of 50^{o}C, and time of 16h gave the best release of reducing sugars. followed by the statistical optimization using faced centred central composite design (FCCCD) of selected factors, namely enzyme loading and substrate concentration,. The optimum conditions were enzyme loading of 6% (w/v) and a substrate concentration of 10% as the total solids (TS). Another pre-treatment, the acidic-enzymatic treatment using different concentrations of acids were performed. An acid concentration of 0.5% (v/v) showed the best hydrolysis effect achieving a value of 20 g/L reducing sugar,34.2% solids reduction, and 90 g/L soluble chemical oxygen demand (SCOD). However, the biogas production and free amino nitrogen release from acidic-enzymatic treated samples were lesser than only enzymatically treated samples. For MEC system, the effect of electrode modification using multiwall carbon nanotubes (MWCNT) and microbial growth into the electrodes was monitored using scanning electron microscope (SEM) images. The MWCNT growth was in-between the carbon felt fibres and the stainless steel mesh strands. The effectiveness of the electrodes was tested by inserting them into the hybrid system with glucose as the main substrate. Stainless steel mesh-modified cathode showed the highest biogas and methane production with a value of 14.4 ml CH_4/g glucose. In addition, carbon-felt modified electrodes showed a maximum substrate degradation value of 93% and a current density of 4.5 mA/m^2. The SEM imaging of the microbial growth on the electrodes showed that the microbes followed a different growth behaviour in modified and unmodified electrodes. In addition, MWCNT-modified Stainless steel mesh(SSTM) showed a potential hydrogenotrophic growth selectivity, unlike unmodified SSTM, which had a more syntrophic microbial community. Hybrid systems showed a higher hydrolysis efficiency especially modified systems, with a percentage of 39.4% by the 48th hour, followed by unmodified systems. The acidogenesis efficiency results showed that the hybrid systems were dominated by the acetic acid pathway, which is favourable in the hybrid system, unlike the conventional digester, which was dominated by a different pathway. Mixing the original inoculum obtained from a previous AD with cow manure has enhanced and increased the competitiveness of the microbial community. Thus, it was positively reflected on the biomethane production potential and rate, with a value of 38ml/g COD and 1.2 ml/h, respectively. In this study, we successfully enhanced the hydrolysis rate, improved the selectivity of microbes in the system, and introduced a set of commercially available electrodes. Our findings also provided compelling evidence that increasing microbial diversity significantly enhances the overall performance of the system.

ملخص البحث

يعد الهضم اللاهوائي نهج لتحويل النفايات العضوية ، مثل مخلفات الطعام ، والحمأة ، والنفايات الزراعية ، إلى منتجات قيمة مثل الغاز الحيوي. ولكن محتوى الميثان الحيوي من الهضم الحيوي يمثل فقط 50-60٪ ، والباقي (40-50٪) هو ثاني أكسيد الكربون. أدت هذه المشكلة إلى الحد من تطبيق (اللاهوائي لانتاج الطاقة. للتغلب على هذه المشكلة، يمكن انتاج الميثان الحيوي من ثاني أكسيد الكربون داخل النظام باستخدام تطبيق خلية التحليل الكهربائي الميكروبية (MEC) ؛بحيث يتم إدخال مجموعة واحدة أو أكثر من الأقطاب الكهربائية في الهاضمة ، مع توصيل مصدر للطاقة. أولاً، تحليل محتوى نفايات الطعام. ثم تم فحص العوامل التشغيلية لتحلل نفايات الطعام باستخدام الإنزيمات باستخدام طريقة معامل واحد لكل دورة. أظهر الفحص أن الرقم الهيدروجيني 7 وتركيز مخلفات الطعام 6 ودرجة الحرارة 50 والوقت 16 ساعة هم الأفضل لمعالجة بقايا الطعام. وبعد ذلك, تم دراسة تأثير عوامل محددة وهي تركيز الإنزيم وتركيز مخلفات الطعام باستخدام خبير تصميم FCCCD. كانت الظروف المثلى هي تركيز الإنزيم بنسبة 6 ٪ (وزن / حجم) وتركيز مخلفات الطعام بنسبة 10 ٪ من إجمالي المواد الصلبة. تليها المعالجة الحمضية الأنزيمية. أظهر تركيز حمض 0.5٪ (حجم / حجم) أفضل تأثير في معالجة مخلفات الطعام محققًا قيمة 20 جم / لتر سكر مختزل، 34.2٪ اختزال للمواد الصلبة ، 90 جم / لتر SCOD. ومع ذلك، فان إنتاج الغاز الحيوي وانتاج النيتروجين الأميني الحر من العينات المعالجة بالإنزيم الحمضي أقل من العينات المعالجة بالإنزيم فقط. بعد ذلك، تم استخدام المجهر الالكتروني لمراقبة تأثير طلي الأقطاب المستخدمة للخلية الحيوية باستخدام جزيئات الكربون النانوية والنمو الميكروبي في الأقطاب باستخدام. كان نمو الجزيئات النانوية بين الألياف والشبكة بشكل منتظم. بعد ذلك ، تم اختبار فعالية الأقطاب الكهربائية عن طريق إدخالها في النظام. أظهر الكاثود شبكة الفولاذ المقاوم للصدأ المعدل بجزيئات الكربون النانوية أعلى إنتاج للغاز الحيوي والميثان. بالإضافة إلى ذلك، أظهرت الأقطاب الكهربائية المصنوعة من اللباد الكربوني المعدلة بجزيئات الكربون النانوية قيمة قصوى لاختزال السكر بقيمة تبلغ (93 ٪) وكثافة تيار كهربائي بلغ 4.5 مللي أمبير / م 2. أظهر تصوير بالمجهر الالكتروني للنمو الميكروبي على الأقطاب الكهربائية أن الميكروبات اتبعت سلوك نمو مختلف في الأقطاب الكهربائية المعدلة وغير المعدلة. بالإضافة إلى ذلك، أظهر شبكة الفولاذ المقاوم للصدأ المعدل بجزيئات الكربون النانوية انتقائية محتملة للنمو ميكروبات المنتجة للميثان عن طريق اختزال الهيدروجين، على عكس شبكة الفولاذ المقاوم للصدأ غير المعدلة ، والتي كان لديها مجتمع ميكروبي أكثر تنوعا. أظهرت الأنظمة الهجينة كفاءة تحلل مادي أعلى خاصة الأنظمة المعدلة بنسبة 39.4٪ خلال 48 ساعة تليها الأنظمة غير المعدلة. أظهرت نتائج كفاءة التولد الحمضي أن الأنظمة الهجينة قد سادت مع مسار حمض لأسيتيك، وهو مفضل في النظام الهجين. على عكس الهاضم التقليدي، الذي سيطر عليه مسار مختلف. أدى خلط حصيلة الميكروب التي تم الحصول عليه من خلية هضم لاهوائي سابقة مع روث البقر إلى تعزيز وزيادة القدرة التنافسية بين المجتمع الميكروبي. وبالتالي، فقد انعكس إيجابًا على إمكانات ومعدل إنتاج الميثان الحيوي ، بقيمة 38 مل ، و 1.2 مل / ساعة على التوالي.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science in Engineering



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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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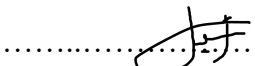

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LIST OF ABBREVIATIONS

AD	Anaerobic digestion/digester
MEC	Microbial electrolysis cell
OFAT	One factor at the time
FCCCD	Face-centred central composite design
TS	Total solids
TVS	Total volatile solids
MWCNT	Multi-wall carbon nanotubes
SEM	Scanning electron microscopy
SSTM	Stainless steel mesh
CF	Carbon felt
FW	Food waste
VFA	Volatile fatty acids
EPS	extracellular polymer substance
pH	Potential of hydrogen
FAN	Free amino nitrogen
COD	Chemical Oxygen demand
SCOD	Soluble chemical Oxygen demand
BSA	Bovine serum albumin
DNS	Dinitrosalicylic acid
HRT	Hydraulic retention time
DIET	Direct interspecies electron transfer
EAB	Electroactive bacteria
HER	Hydrogen evolution reaction
OLR	Organic loading rate
GAC	granular activated carbon
PAC	Powdered activated carbon
PBS	phosphate buffer saline

LIST OF SYMBOLS

CH_4	Methane
CO_2	Carbon dioxide
H_2	Hydrogen
H_2S	Hydrogen Sulphide
CH_3COOH	Acetic acid
H_2SO_4	Sulfuric Acid
NO_2^-	Nitrite
NO_3^-	Nitrate
Fe	Iron
Mn	Manganese
%	Percentage
V	voltage
W	weight
V	volume
mA	milli-Amber
m_2	Meter square
cm_2	Centimetre square
mL	Milli-litre
^oC	Celsius
mg	Milli-gram
Ω	Ohm

CHAPTER ONE
INTRODUCTION

1.1 BACKGROUND OF THE STUDY

The projection of food waste has been increasing the past 25 years, especially in Asian countries, Hodaifa et al., (2019)reported that there would be an increase from 278 to 416 million tonnes from 2005 to 2025. Food waste accounts for 23% of municipal waste, accounting for 30% of the total trash disposed into landfills and incinerators (Abdel-Shafy& Mansour, 2018). This problem has led to uncontrolled fermentation in landfills, emitting greenhouse gases, polluting groundwater, increasing the disposal cost, and damaging incinerators by high-temperature fluctuation due to high water content. On the contrary, food waste has a high content of fermentable substrates such as sugars, fats, starches, lipids, proteins, and cellulose (Moon et al., 2009), which makes it an excellent substrate for producing high-value products (e.g., biofuels and platform chemicals) (Uçkun Kiran et al., 2015).

Anaerobic digestion is an approach to converting organic waste, such as food waste, into valuable products like biogas. The digestion process involves four significant steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Wirth et al., 2012). During hydrolysis, complex organic matters like carbohydrates, protein, and fats are broken down into their monomers, reducing sugars, amino acids, and fatty acids, respectively. Next is acidogenesis, where acidogenesis microorganisms further break down the products of hydrolysis, producing ammonia, H_2, CO_2, H_2S, shorter volatile fatty acids, carbonic acids, alcohols, and trace amounts of other by-products (Kirk & Gould, 2020). Next is acetogenesis, where microbes produce acetate. Finally, acetogens utilize the products of acidogenesis to produce acetic acid, CO_2, and H_2. Methanogenesis is the last step of the pathway. Methanogens produce methane from the final products of acetogenesis as well as from some of the intermediate products from hydrolysis and acidogenesis, following two paths involving the utilization of acetic acid and CO_2 along with hydrogen as shown in the following equations below (Kumar et al., 2012; Salman et al., 2017):

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad (1.1)$$

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad (1.2)$$

Although anaerobic digestion is an attractive solution, biomethane production only accounts for 50-60%; the remaining is CO_2 (Xu et al., 2014; Zeppilli et al., 2019). To separate CO_2 from CH_4, conventional methods for biomethane purification includes the removal of CO_2 without the reduction of CH_4 mass; this includes pressure swing adsorption, membrane separation, or chemical CO_2- absorption (Cerrillo et al., 2017; Hassanein et al., 2017).

Microbial electrolysis cell has been employed in the anaerobic digestion system to upgrade biomethane production. External energy is supplied to the system to drive a thermodynamic non-spontaneous reaction, like the conversion of CO_2 to CH_4 (Aryal et al., 2017; Cheng et al., 2009). In addition to conventional pathways of biomethane production, a unique pathway reaction occurs on the cathode by electro-methanogenesis; the electroactive microbes directly utilize electrons and organic compounds to produce methane(Zakaria et al., 2020). In addition, the enrichment of hydrogenotrophic methanogenesis on the cathode is a key factor in the hybrid system, decreasing the amount of CO_2 produced while increasing the biomethane yield in anaerobic digestion (Anukam et al., 2019; Eerten-Jansen et al., 2011).

The production of biomethane has two different extracellular electron transfer mechanisms, either indirectly by intermediate abiotic electrochemical and microbially catalysed hydrogen production in the cathodic compartment or directly by taking the electrons from the cathode reduction of CO_2 to methane.

1.2 STATEMENT OF THE PROBLEM

Food waste production is increasing with the increase in population. Conventional methods of FW disposal, like incineration and open landfills, are no longer feasible, due to the high operational cost, increased risk projected on the environment, and contribution to global warming (Gao et al., 2017). Although anaerobic digestors have been employed in the treatment of food-waste for biogas production, hydrolysis, which is the first stage, present a significant challenge in the effectiveness of the treatment, hence, limiting the

capacity to handle large scales (Yin et al., 2016). Therefore, The pre-treatment of food-wate is crucial, as it helps speed up the hydrolysis stage, decrease hydraulic retention time, and improves the efficiency of the following stages, especially acidogenesis and methanogenesis (Moon & Song, 2011).

Moreover, Biomethane production through AD only accounts for 60% of the total biogas produced; the remaining 40% is CO_2 (Anukam et al., 2019; Enzmann et al., 2018). Hence, carbon dioxide absorption requires costly downstream processes that also limits the application of anaerobic digestion(Xu et al., 2014).

Microbial electrolysis cell is a new technology representing a new form of green energy. It has attracted considerable attention for the past few years as a promising technology for higher biogas production from organic matter (Huang et al., 2020a). microbe's cathodic reaction is responsible for reducing CO_2 into CH_4 (Kundu et al., 2013).

The high cost of electrodes, especially ones utilizing precious metals such as platinum and palladium have limited the implementation and economical viability of the system (Zakaria et al., 2020). Consequently, finding alternative electrode's material or replacing modifications using precious metal while maintaining the MEC-AD performance is crucial for more economically and sustainable energy production and food-waste treatment.

Lastly, microbial community is the driving force in the system, responsible for the fermentation process and biomethane production rate and volume (Yu et al., 2017). However, a gap exists in the research in terms of manipulating the microbial community, Although multiple studies have reported that inoculums rich in diverse types of microbes performed better than other systems seeded with conventional inoculums(Detman et al., 2021). In addition, there is a lack of study on the effect of mixing inoculum on biomethane production kinetics and enhancement, though mixing two inoculums rich in fermentative and methanogenic microbes could potentially enhance the overall system's performance.

1.3 SCOPE OF THE STUDY

The research focused on improving biomethane production from FW. AD was integrated with an MEC system to convert CO_2 into CH_4 through the cathodic reaction of hydrogenotrophic and electro methanogenic microbes through the following steps:

- Pre-treatment of the substrate: FW were pre-treated using two treatment methods. Acidic-enzymatic and enzymatic treatment only. Sulfuric acid was used in the acidic treatment, while a cocktail of hydrolytic enzymes produced from rice bran was used in the enzymatic treatment. Performing hydrolysis in a separate process(pre-treatment) from the methanogenesis process can minimize interspecific competition, thus increasing the reaction rate of methanogenesis in the MEC-AD system(Park et al., 2018). Hydrolytic enzymes broke down and solubilized complex organic matter into their monomers. Hence, it eased the substrate uptake by microorganisms and reduced the hydraulic retention time. Enzyme loading, TS concentration, pH, temperature, and reaction time were optimized to obtain maximum sugar and free amino acid recovery. Then the acidic-enzymatic treatment was tested; the acid solubilized the substrate, offering a higher area for the enzymes to attack. The effect of both treatments was monitored on the release of reducing sugar, free amino nitrogen, solubilization of substrate, total solids reduction, and biogas production.

- Electrode modification and biofilm monitoring: Microbes, electrode interaction, and biofilm formation are crucial factors affecting the MEC-AD system. Thus, the set of electrodes was modified with MWCNT. The biofilm formation and interaction with surface-modified 3D electrodes were monitored to improve microbes formation, decrease adaptation time and increase biomethane production.

- The original inoculum obtained from a previous anaerobic digester was enhanced by adding cow manure to the hybrid system with modified electrodes. Cow manure is reported to have a high population of the methanogenic community. Mixing the original inoculum with cow manure gave the essential microbial community for treating high fermentable substrates like food waste. Testing the overall efficiency of the microbial stages was crucial to see the combined effect

of food-waste pre-treatment, electrode modification, and inoculum mixing in enhancing the system.

1.4 RESEARCH OBJECTIVES

The study aimed to achieve the following objectives:

1. To identify and optimize the process parameters of enzymatic hydrolysis of food waste for maximum monomers in the hydrolysate.
2. To determine the effect of electrode surface modification on biomethane production and biofilm formation in the MEC-AD system.
3. To study the fermentation stages and the effect of mixed inoculum on biomethane kinetics production from food waste equipped with modified electrodes using modified gompertz model.

1.5 THESIS ORGANIZATION

This thesis consists of five chapters, including chapter one, which covers background information, problem statements, objectives, the scope of studies, and the overall flow of this study. Chapter two includes the literature on previous research on previous pre-treatment methods of FW, hybrid system of MEC-AD, and factors affecting the system like electrode choice, voltage, microbial community and altering it. Chapter three focuses on the detailed methodology of experiments applied in this study. Chapter four presents the results and discussion of each finding on substrate pre-treatment, electrode modification, and each microbial stage efficiency with mixing inoculum. Chapter five highlights this study's findings, conclusions, and recommendations for future studies..

CHAPTER TWO
LITERATURE REVIEW

2.1 INTRODUCTION

This chapter summarizes previous works in different treatment processes, anaerobic digestors, microbial electrolysis cells and a hybrid system of both. Followed by detailed comparisons of operational factors like the voltage, electrodes, electrode modification, and microbial community, in terms of culture, biofilm and suspension, and methods of enriching methane-producing microbes.

2.2 BIOGAS

Biogas is a renewable, environmentally friendly energy source. It's produced by the microbial breakdown of organic matter, such as food or animal waste, in an anaerobic digestion process (Scarlat et al., 2018). The production of biogas provides a versatile carrier of renewable energy, as methane can be used to replace fossil fuels in both heat and power generation and as a vehicle fuel (Weiland, 2010). Various process types are applied for biogas production, which can be classified into wet and dry fermentation systems. Most often applied are wet digester systems using vertical stirred tank digesters with different stirrer types dependent on the origin of the feedstock. Biogas is mainly utilized in engine-based combined heat and power plants. In contrast, micro-gas turbines and fuel cells are expensive alternatives that need further development work to reduce costs and increase reliability (Tian et al., 2020).

2.3 FOOD-WASTE

Food waste is biodegradable waste discharged from a variety of sources. However, private households are the major source in food waste generation. The projection of food waste has been increasing in the current 25 years, mainly in Asian countries. It was reported by Paritosh et al., (2017) that there would be an increase from 278 to 416 million tonnes from 2005 to 2025. While food waste might not seem like a significant issue to environmental sustainability, almost 30-50% of the total food produced in the world goes to landfills, accounting for 30% of the complete waste disposed into landfills and burnt in incinerators. The uncontrolled fermentation of food waste in landfills causes groundwater contamination and greenhouse gas emissions, with an estimated 3.3 billion tons of CO_2 in the atmosphere annually (Paritosh et al.,2017). In addition, landfills have reached their capacity, incinerators require high capital costs and are insufficient in treating FW with high moisture content. On the contrary, FW is an excellent feedstock to produce high-value biofuels, owing to the high content of the fermentable substrate. Table 2.1 shows the characterization of food waste:

Table 2.1 Characteristics of food waste

Parameter	Content (w/w %)
pH	5.1
Total solid	22.1
Volatile solid	16.8
Moisture	76.6
Total sugar	65.1
Cellulose	3.7
protein	17.3
Lipid	13.9

Source: (Rueda et al., 2020)

2.4 FOOD-WASTE TREATMENT AND HYDROLYSIS

Food waste is readily biodegradable due to the high volatile fraction of total solids. However, the degradation of the substrate into soluble particles is a rate-limiting factor in anaerobic digestion. The pre-treatment of food waste is the process of reducing particle size to increase surface area accessibility by microbes or breaking down complex organic matters like carbohydrates, proteins and lipids to their monomers, reducing sugars, amino acids, and fatty acids, respectively. This process eases the substrate uptake by the microbes, improving biogas production. Hence, improve the hydrolysis kinetics.

2.4.1 Chemical Treatment

Chemical treatment is majorly involved in the solubilization of lignin and hydrolyzing cellulose in the agricultural and food-waste industries (Hodaifa et al., 2019). Chemical pre-treatment involves the usage of strong alkaline or acid to solubilize organic compounds. While alkaline treatment is used for the hydrolysis of proteins, lipids, and lignin, acidic pre-treatment is used for the hydrolysis of carbohydrates (Parthiba et al., 2018). Different acid treatments are performed, including concentrated and diluted acids. The principle of concentrated acid hydrolysis is that crystalline cellulose can be completely dissolved in 72% sulfuric acid or 42% hydrochloric acid, or 77–83% phosphoric acid at a lower temperature, resulting in the homogeneous hydrolysis of cellulose (Chen, 2015) .In the dilute process, acid pre-treatment, acid hydrolysis the hemicellulose portion of the biomass and causes structural changes, thereby improving the enzyme accessibility for hydrolysing cellulose (Achinas et al., 2021).

However, the chemical treatment has multiple drawbacks restricting the application in the food-waste pre-treatment, some of the drawbacks sited in previous literature are as follow:

- The usage of strong alkaline or acids can lead to the formation of toxic by-products such as hydrogen sulfide and ammonia (Carlsson et al., 2012)

- Chemical pre-treatment might disrupt the degradation efficiency of the microbial community in the subsequent treatment of anaerobic digestion, hindering the biomethane production(López et al., 2008).

2.4.2 Thermal Treatment

The process of thermal pre-treatment of food waste involves the disintegration of the cell membrane, which produces organic material solubilization, so it will make it easier for microorganisms to digest the feedstock within a shorter time, in other words, enhancing the solubilization of COD (Chemical Oxygen Demand), which will improve the efficiency of the anaerobic digestion process (Chua et al., 2019).

The process involves heating the food waste in different time intervals under different temperatures. There are two types of thermal pre-treatment:

- Low thermal pre-treatment: temperature range between 50-110 ^{o}C
- High thermal pre-treatment: temperature range between 110-250 ^{o}C

It was reported previously that treating FW pre-treated with low thermal pre-treatment did not show efficient COD solubilization in the temperature range between (50-90 ^{o}C) (Yingcun et al., 2016). Meanwhile, Parre et al., (2020) studied the effect of different temperatures ranging between 25-150^{o}C. The study showed that FW treated under 100 ^{o}C showed the highest solubilization of COD, while FW treated under the temperature range 130-150 ^{o}C showed the lowest solubilization efficiency.

2.4.3 Biological Treatment

Hydrolytic enzymes break down complex substrates into their monomers, allowing a higher surface area to be attacked by the microbes, thus improving the digestion of lignocellulosic biomass in the system, and reducing the hydraulic retention time (HRT) (Wang et al., 2020). Multiple hydrolytic enzymes, such as Protease, Lipase, and Carbohydrase enzymes,

are used in the pre-treatment process. Carbohydrase is a multi-enzyme complex containing mainly arabinase, cellulase, β-glucanase, hemicellulase, Amylase, and xylanase used to hydrolyse carbohydrates(Moon & Song, 2011). Enzymes involved in hydrolysis could be commercial or produced in the laboratory from different substrates. Table 2.2 summarizes previous studies:

Table 2.2 Previous studies using hydrolytic enzymes for the pre-treatment of FW

FW composition	Enzyme	Result	Application	Reference
Veggie, grains, meat, rice, chicken	Carbohydrase, Protease Lipase	9.1 g-SCOD/L/d of organic loading rate	AD feed for methane production	(Moon & Song, 2011)
Starch, sugar, protein, fat cellulose	glucoamylase	74.9g/l	Glucose production	(Ye et al., 2018)
Veggie, grains, meat, fruit	amyl glucosidase	164g/l reduced sugar	Ethanol	(Moon et al., 2009)
Starch	glucoamylase	71g/l Reduced sugar	Ethanol	(Prasoulas et al., 2020)
Food-waste	F.M glucoamylase	89.1 ± 7 g/L glucose	AD-methane production	(Uçkun Kiran et al., 2015)

The primary method used in producing enzyme solids is solid-state fermentation (SSF). (Yin et al., 2016)reported hydrolytic enzymes using Aspergillus awamori from cake waste by SSF to apply FW hydrolysis. FW hydrolysate was used in the production of Biomethane by AD. Interestingly, the biomethane yield was improved by 2.5 folds compared to biomethane produced by untreated FW.

Multiple factors affect the enzymes hydrolysis efficiency, namely:

- enzyme concentration: enzyme concentration increases the products concentration, assuming that the substrate is not limited (Trzcinski et al., 2014)
- substrate concentration: Increasing the substrate concentration increases the product produced until it reaches a point of saturation(Fernández et al., 2001)
- temperature and pH. Increasing temperature and pH can increase the activity, however, excessive heat and extremely acidic or alkaline pH could denature enzymes(Benabda et al., 2019)

Therefore, the optimization of these factors is necessary to ensure maximised hydrolysis (Prasoulas et al., 2020). In addition, different enzymes require different treatment conditions based on their activity.

2.5 ANAEROBIC DIGESTION

Anaerobic digestion is the microbiological breakdown of organic matter in an oxygen-free environment, producing methane. The anaerobic digestion process is divided into four phases; Each pathway has different characteristics and groups of microorganisms (Fuentes et al., 2013). Figure 2.3 shows a simplified schematic representation of the anaerobic degradation process (Demes et al., 2003):

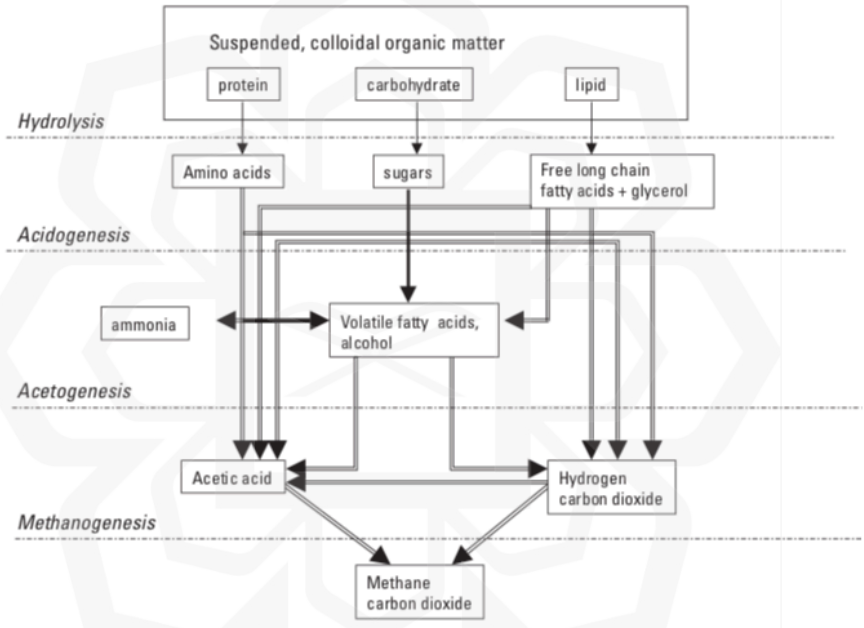

Figure 2.1 Schematic representation of the anaerobic degradation process

Several factors affect the performance of AD, including hydraulic retention time (HRT), depending significantly on the substrate's solubility; the lower solubility, the higher the HRT. In addition, the hydrolysis and biodegradability of specific complex substrates inhibit the AD process. For example, the degradation of starch, cellulose, proteins, and lipids increases the amount of volatile fatty acid, decreasing the pH, which leads to the inhibition of methanogenesis.

2.5.1 Effluent of Anaerobic Digester

Anaerobic digestion is an excellent technology for treating several types of organic waste, like wastewater, municipal, agricultural, Industrial, and animal waste. It can produce bioenergy in the form of biogas and clean water(Khalid et al., 2011). However, the effluent of the digester is rich in nutrients like phosphorus and nitrogen. In the biodegradation process, nitrogenous and phosphorus or polyphosphate into ammonium nitrogen (NH_4-N) and orthophosphate (PO_4-P,) respectively(Kayhanian, 2010; Sánchez et al., 2000). In addition, dissolved oxygen is minimal under anaerobic conditions, adding to that the high concentration of nutrients. The high concentration of nutrients in the anaerobic digester effluent can affect the aquatic organisms receiving natural waters, leading to biodiversity degradation (Pincam et al., 2018). Thus anaerobic digester effluent requires further treatment. Among the treatment process, plants grown in well-designed wetlands are used in treating anaerobic digester effluent (Pincam et al., 2018). These plants are known for increasing sediment deposition, removing excessive nutrients and releasing oxygen into the water and rhizosphere. Another approach used in treating anaerobic digester effluent is fertilizers to be applied to agricultural lands, giving the high content of nutrients in the effluent. Anaerobic digester effluent is subjected to further treatments to meet the standard before being discharged into rivers and discharge streams(Chen et al., 2014).

Sequential nitrification and denitrification are widely accepted as the main processes for nitrogen removal, in which NH_4^+ is oxidized to nitrogen oxide, i.e. nitrite: (NO_2^-) or nitrate (NO_3^-), via nitrification, followed by the reduction of nitrogen oxide to nitrogen gas via denitrification. These processes require a considerable amount of energy for nitrification as well as chemical additives for denitrification. Thus, the treatment process is economically unfeasible(Ruiz et al., 2006). A similar approach was proposed as an attractive alternative to the classical removal of NH_4^+ by co-culture of microalgae and nitrifiers in a single reactor following advantages: (1) microalgal photosynthesis supplies oxygen to the nitrifiers, which results in an effective reduction of the energy consumption for aeration(Karya et al., 2013) (2) nitrogen ions (NH_4^+, NO_2^-, and NO_3^-) uptake by

microalgae lowers the nitrogen loading on the subsequent denitrification (Akizuki et al., 2020).

2.6 MICROBIAL ELECTROLYSIS CELL

Microbial electrolysis cell(MEC) is an electricity-mediated microbial bio-electro-chemical technology initially developed for high-efficiency biological hydrogen production from waste streams(Zhang & Angelidaki, 2014). MEC's technology has several advantages over other conventional ways of biological hydrogen production. Various organic matters such as cellulose, glucose, glycerol, acetic acid, sewage sludge and varied wastewater can be converted to hydrogen in MECs(Pant et al., 2012).MECs have diverse applications. One of the applications is the microbial electrosynthesis of chemicals like methane, ethanol, hydrogen peroxide, formic acid, and acetate(Nevin et al., 2010), or in the recalcitrant pollutants removal of organic and inorganic pollutants and much more like biosensors and resources recovery(Coma et al., 2013).

2.7 HYBRID SYSTEM OF MEC-AD

Microbial electrolysis cell(MEC) is one of the Bio-electrochemical system technologies and an attractive method for biomethane upgrading from CO_2. The system is supplied with an external voltage to overcome the thermodynamic energy barrier (Pawar et al., 2022). First, electrodes are inserted into the reactor seeded with the right microbial community. A potential ranging between (0.2-1.6V) is applied to the system to drive the electrochemical reactions, as shown in Figure below:

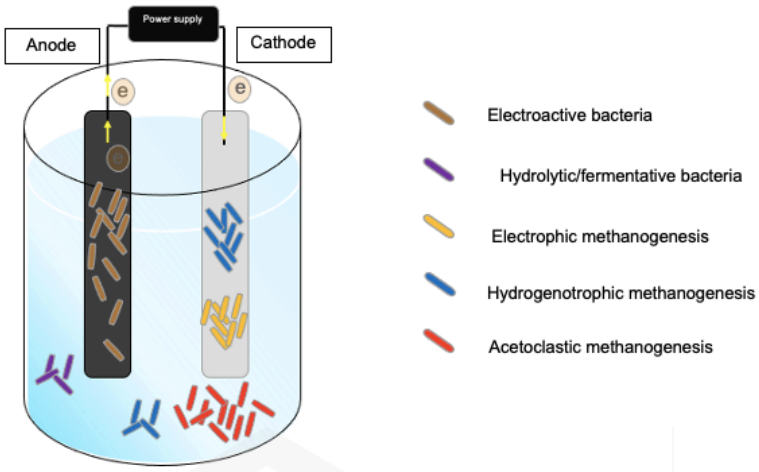

Figure 2.2 Basic MEC-AD system and the microbial community in the system(Lee et al., 2017)

Hydrolytic bacteria break down complex matter into their monomers, followed by fermentation, producing volatile fatty acids by fermentative bacteria. The electroactive bacteria attached to the electrode oxidize the organic matter to CO_2, electrons, and protons. The electrons then travel to the cathode, which is consumed by methanogenesis. In the hybrid system, there are three pathways to produce methane with two mechanisms: directly and indirectly. Directly through the novel pathway by electro-trophic methanogenesis, as mentioned previously, utilize electrons to reduce CO_2 to methane (Appels et al., 2008).

Indirectly, hydrogen reduction occurs abiotically (Choi et al., 2017).Then, hydrogenotrophic methanogenesis utilize H_2 along with CO_2 and produce methane and water, or by acetolactic methanogenesis (Appels et al., 2008) as shown in the figure below:

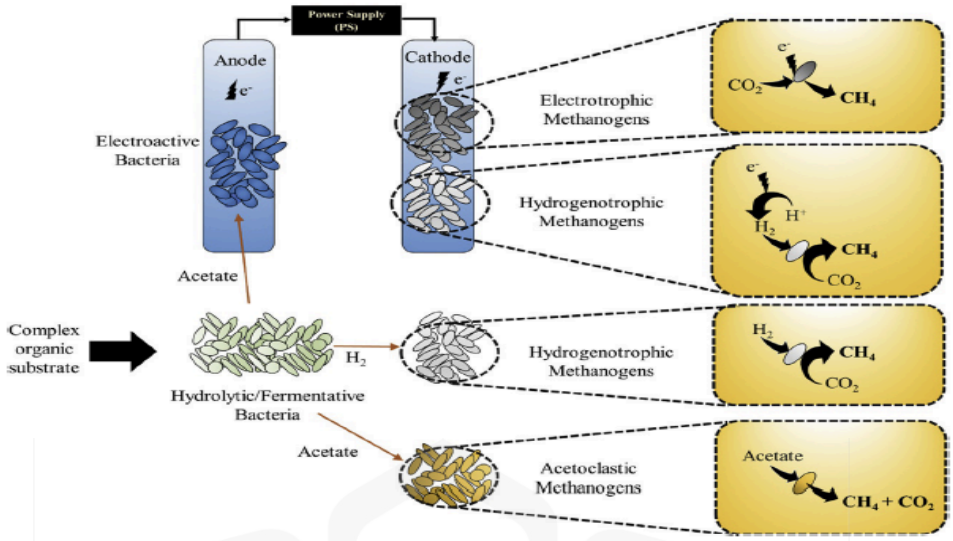

Figure 2.3 Conceptual schematic of various methanogenesis routes in hybrid MEC-AD (Zakaria et al., 2020)

Several studies were conducted using MEC to upgrade methane production from a simple substrate (glucose), high-strength wastewater, and food waste. For instance, (wang et al., 2019) studied and compared the performance of conventional AD with an integrated system of MEC-AD using synthetic wastewater; the results showed an increase in biomethane production by 30% when incorporating the MEC system. Another study using food waste as substrate showed a rise in biomethane production by 1.7 folds and four times faster than conventional AD (Park et al., 2018).

2.8 FACTORS AFFECTING THE PERFORMANCE OF THE HYBRID SYSTEM

2.8.1 Voltage

The integrated system of microbial electrolysis cell and anaerobic digester is affected by the same factors affecting conventional digesters: pH, temperature, organic loading rate, and hydraulic retention time. In addition, the applied voltage, electrode type and microbial community.

Combining anaerobic digestors with a bio-electrochemical system has proven to enhance the biomethane production rate and the biomethane percentage (Cai et al., 2018). The applied voltage plays a crucial role in initiating the electrochemical reaction by *Geobacter* and electro-methanogenesis in the system. Applying low voltage as low as 0.5 can substantially improve methane production by affecting bioelectrode and bulk solution microbial communities. However, the effect of voltage on the AD microbial suspension consortia enhancement was not thoroughly tested (James et al., 2018; Que et al., 2018). A recent study reported that applying voltage potential enriched and enhanced DIET-associated organisms and methanogen's performance. However, the applied potential negatively affected higher organic loading rates (Xen et al., 2020). Thus, it is crucial to study the effect of voltage on the system fed with different substrates. Two studies showed that increasing the voltage up to 2V substantially enhanced the system's performance, despite the increased potential for failure due to electrolysis(Song et al., 2016). Harb et al., (2020) studied the effect of increasing the voltage over 2V. The results showed a deterioration in the system's performance with a voltage over 2.25V. However, adding granular activated carbon (GAC) to the reactor at a high applied voltage of 2.75 has substantially increased methane production compared to controlling the reactor by up to 25 folds. Oppositely, (Mer et al., 2020) reported that the COD, VFA removal and biomethane produced were the highest when the voltage applied was as low as 0.1V. Not only improving methane production but also the secretion of extracellular polymeric substances (EPS) which improves the electron transfer and interaction between microorganisms. On the other hand, (Jay G. Park et al., 2020) reported no significant effect of voltage on the microbial community, namely hydrogenotrophic methanogens, while there was an improvement in methane production.

2.8.2 Electrode's

Electrode material plays a significant role in the bio-electrochemical system; Biofilm formation and density depend significantly on the biocompatibility and morphology of the electrodes. The anodic reaction of electroactive bacteria plays a significant role in boosting

methanogenesis performance. Thus, choosing the electrode material for microbial interactions and growth is crucial. The selected electrode should have high conductivity, excellent chemical stability, high mechanical strength, biocompatibility, high surface area, low cost and low overpotential (Aryal et al., 2017).On the other hand, multiple studies reported using non-precious materials like stainless steel. Stainless steel is reported to be a good alternative for metal electrodes and modification with the precious metal catalyst. Stainless steel is an abundant, low-cost, conductive material with a low overpotential and high stability in alkaline solutions (Aryal et al., 2017; Selembo et al., 2009). The table below summarizes different MEC-AD with different substrates and electrodes modification:

Table 2.3 Previous MEC-AD's electrodes modification and substrates

Anode	Cathode	modification	Biogas enhancement	Substrate	Reference
Carbon felt	Carbon felt	Chitosan poly-neutral red	75% 62.5%	Syntheticwastewaterr	(Seelajaroen et al., 2020)
Platinum foil	Stainless steel mesh	heating	75%	Wastewater	(Liu et al., 2017)
Carbon cloth	Stainless steel mesh	Plasma-treatment	80%	nm	(Rozenfeld et al., 2019)
carbon-modified copper foam	carbon-modified copper foam	Multi-wall carbon nanotube	86%	Food waste	(Anna et al., 2020)
graphite carbon mesh coated with Ni	graphite carbon mesh coated with Ni	-	3%	Food waste	(Park et al., 2018)
Carbon fibre brush	Carbon cloth	Magnetite/zeolite nanocomposite	15%	n.m	(Noori et al., 2020)
graphite fibre fabric	graphite fibre fabric	Ni, MWNT	53%	distillery wastewater	(Feng et al., 2018)
Carbon felt	Carbon felt	Neutral red	500%	Sodium bicarbonate	(Yang et al., 2020)
sludge-modified titanium	a platinum-coated titanium mesh tube	Electrodes were not modified	150%	food waste	(Huang et al., 2020b)
Nickel plate	SS316	Cathode: Ni-Co-P deposit	215.7%	Sodium acetate	(Chaurasia et al., 2020)

2.8.3 Carbon Electrodes

Multiple modifications to carbon electrodes are made to improve bacterial adhesion, conductivity, and overall performance. Carbon-based electrodes, namely, carbon brush, fibre, and felt, have been widely employed in the system owing to their high surface area, biocompatibility and low cost (Baek et al., 2021;Seelajaroen et al., 2020). In addition, newly developed 3D-porous carbon electrodes are reported to be a great host for biofilm development and bacterial growth (Baek et al., 2018; Jourdin et al., 2020a). However, carbon-based electrodes provide slow catalysis for cathodic HER, which seems critical for enriching hydrogenotrophic methanogens (Liu et al., 2016). Previous studies reported improving carbon-based electrodes' cathodic HER with precious metal catalysts (ex., Nickel, platinum, titanium). However, these catalysts are expensive (Kim et al., 2017; Rozenfeld et al., 2019; Singh et al., 2021). Like modification of carbon fibre with self-supported N-doped C/Fe3O4-nanotube composite arrays (Wang et al., 2021), carbon black with humic-acid (Huang et al., 2019), carbon felt with carbon derived from mango wood biomass (Li et al., 2020), preparing porous carbon cloth using Nickel (N-doped) (Yuan et al., 2019). Other studies reported electrode surface modification in terms of surface charge, functional group, and roughness (Cheng, 2019). Among the tightly studied 3D-carbon material is reticulated vitreous carbon. The 3D electrode has a highly porous structure but with lower biocompatibility (LaBelle & May 2017). Modifying the electrode surface with carbon nanotubes(CNT) has substantially improved the biofilm formation on the porous electrode, along with increased biogas production by multiple folds (Jourdin et al., 2014).

2.9 MICROBIAL CULTURE

Bacteria are the driving force in the MEC system, with their ability to degrade organic matter, generating electricity and biogas (Salar-Garcia et al., 2020). Methane can be produced via three ways: direct electron transfer from the cathode to electro-trophic methanogens coupled with CO_2 reduction to methane, through hydrogenotrophic methanogenesis of H_2 produced via cathodic hydrogen evolution reaction (HER), and

through the direct interspecies electron transfer (DIET) between electroactive bacteria (EAB) and electro-trophic methanogens in cathode and anode electrodes (Huang et al., 2020).

Generally, methane production in AD is composed of 70% acetolactic methanogenesis and 30% hydrogenotrophic methanogenesis(Siegert et al., 2015). However, the microbial community could be altered to increase the methane-producing culture. Hence, increasing the biomethane production selectivity and substrate degradation.

2.9.1 Effect of Microbial Culture Source

Inoculum is crucial in providing the initial microbial community, which highly affects the biomethane production rate. MECs inoculated with a wide diversity of inoculants from natural freshwater environments, and engineered reactors (e.g., wastewater treatment plants) typically converge to communities containing predominantly *Geobacter sulfurreducens* (Yates et al., 2012). *Geobacter* is exo-electrogenic anaerobic microbes mainly utilize VFA's and hydrocarbons; they coexist and cooperate with other fermentative or syntrophic VFA (Volatile Fatty Acids) degrading bacteria such as *Smithella, Bifidobacterium, and Clostridium* (Lovley et al., 2011).

To test the effect of inoculum community structure on the biomethane production from acetate, two inoculum sources were used: (AD) sludge dominated by *acetolactic Methanosaeta* and an anaerobic bog sediment where hydrogenotrophic methanogens were detected. The study tested the effect of inoculum mass on the system's performance. Interestingly, chambers inoculated with anaerobic bog sediment showed better performance in COD removal (>80%) and biomethane production compared to anaerobic sludge(Rajput et al., 2019). In addition, increasing the inoculum mass has increased the biomethane production up to 0.27 mL mL^{-1} cm^{-2} for systems inoculated with AD bog but with no effect for systems fed with AD sludge. Similarly, Elsayed et al., (2020) tested the effect of using three different inoculums, namely fresh cow manure, activated sludge, and excess sludge, on the biomethane production by AD of primary sludge with fruit and vegetable waste. The results showed that reactors

inoculated with activated sludge had the highest CH_4 content, with a value of 200 ml/g VS. The inoculum of activated sludge clearly showed higher methanogenic activity.

Agricultural residues are used as substrates in the production of biomethane using AD. However, due to the high content of lignocellulose, it is hard for microorganisms to uptake. On the contrary, recent studies reported that the type of microorganisms involved, highly affects the degradation and biogas production rate. To test the hypothesis, Liu, Sun, Müller, & Schnürer, (2017) studied the effect of using three different inoculum sources(wastewater treatment plant, stillage from ethanol production process, agricultural biogas plant) on the biogas production from agricultural substrates. Generally, inoculums that had high concentration of ammonia showed lower performance and microbial diversity; ammonia has strong inhibitory effects on methanogenesis, with minimal effects on hydrolytic and acidogenic microbes (Chen et al., 2016). Reactors inoculated with waste-water treatment plant sludge had the highest methane production and microbial diversity. The high diversity of microbial community simulated the diversity of multiple degradation pathways, hence reducing the retention time and increasing the biomethane yield. Similar to the previous study, Rajput & Sheikh, (2019) reported that reactors inoculated with digested manure has outperformed reactors inoculated with acclimatized sludge and septic tank sludge in the production of biogas from sunflower straw.

While the inoculum source is very important to the system, the inoculum to organic loading ratio is as important. Providing the optimum amount of inoculum to substrate is crucial in providing a balanced population of microbial community for the treatment and biomethane production process. In addition, The biodegradation rate and lag time relies greatly on the concentration of microorganisms and consortia provided by the inoculum (Hidalgo et al., 2016). Higher organic loading rates (OLRs) can induce a process instability due to the accumulation of volatile fatty acids (VFAs) followed by irreversible acidification of digesters.

2.9.2 Biofilm and Suspension

The microbial community in the suspension is very similar to the ones on the anode, mainly, electroactive *geobacter*. The specie is specialized in making electrical contacts with extracellular electron acceptors and other organisms like electro-trophic methanogenesis. This gives *Geobacter* an important role in the diversity of anaerobic environments. *Geobacter* species appear to be the primary agents for coupling the oxidation of organic compounds to the reduction of insoluble Fe(III) and Mn(IV) oxides in many soils and sediments (Lovley, 2011).

Among the archaeal community, hydrogenotrophic methanogenesis are reported to dominantly present in the suspension, similar to cathodic biofilm. Hydrogenotrophic methanogenesis utilizes hydrogen and oxidise carbon dioxide to biomethane (Park et al., 2020). Gao et al., (2017) inoculated an MEC-AD system with an anaerobic leachate sludge. The anode was mainly dominated by *Desulfuromondales*, which are a type of species capable of growing by transferring electrons from the oxidation of H_2 or organic compounds (i.e. long chain fatty acid) to insoluble Fe(III) oxides (Malvankar et al., 2012). *Pseudomonas* was another genus of bacteria that was enriched on the anode surface, which are known for degrading aromatic compounds. The cathodic chamber was mainly dominated by *Methanobactin* species, an acetolactic methanogenesis which are capable of direct interspecies electron transfer (DIET) with exo-electrogenic bacteria such as *Geobacter* species using hydrogen, formats, insoluble electron shuttles, or conductive materials (Jas et al., 2016).Similarly, system's inoculated with waste activated sludge were primary dominated by *Geobacter*, and with *Methanocorpusculum* from the archaeal genera, both microorganisms were responsible for the enhancement and production of methane in the system (Sun et al., 2015).

2.9.3 Altering the Microbial Community using additives

The addition of activated carbon increases the surface area for microbial attachment and growth (Iel et al., 2020), Holmes et al., (2019) performed a study using powdered activated carbon (PAC) and granulated activated carbon(GAC).PAC is used to enrich the growth of methanogens and syntrophic VFAs-oxidizing bacteria(Homes et al., 2015). Studies have shown that iron-based materials like zero-valent iron, iron-biochar, or magnetite can adsorb some of the salts, which makes the reactor a more hospitable environment for microorganisms involved in wastewater treatment. (Hwang et al., 2014; Sebastian et al., 2019). Magnetite is an ideal adsorbent of harmful salt, in addition to its high insoluble surface area, which acts as a host for microbial enrichment. (Chen et al., 2020) reported that the proportion of bacterial genera of *Pseudomonas* has doubled in digesters amended with magnetite. Pseudomonas is known for its ability to transfer electrons to insoluble electron acceptors and electrodes and accept electrons from various extracellular electron donors. This ability to transfer electrons extracellularly would be needed for electron transfer to an electron-accepting methanogen or a magnetite particle via DIET (Arnold et al., 1986; Bosire & Rosenbaum, 2017). In addition to Pseudomonas, two other genera were substantially enriched by magnetite's addition: Soehngenia and Thermanaerothrix.

2.9.4 Enriching Methane Producing Microbes using carbon based materials

Methanogenesis is an extraordinary microbe responsible for the anaerobic digestion of organic compounds like the reduction or dismutation of carbon dioxide, methyl compounds, or acetate to methane, or methane and carbon dioxide in several ecosystems and consortia (Jal et al., 2014). In addition, Methanogenesis has an insignificant reducing potential compared to other aerobic and anaerobic microbes. Diverse environments are home to Methanogenesis, like the deep ocean, rice paddies, wetlands, landfills, and the gastrointestinal tracts of termites, ruminants, and humans. There are three types of Methanogenesis: acetolactic, methyl-trophic, and hydrogenotrophic Methanogenesis. Of the three classes of Methanogenesis, class one and most of class

two belong to hydrogenotrophic Methanogenesis, namely *Methanobacterium, Methanobrevibacter, Methanosprillum, Methanococcus, Methanogenium, and Methanoculleus* (Berghuis et al., 2019)

Carbon material improves the direct interspecies electron transfer between bacteria and methanogens, thus improving biomethane production. Several studies were performed using different types and forms of carbon to enhance biomethane production. Salvador et al., (2017) Studied the effect of carbon nanotube(CNT) on a pure culture of methanogens and typical fatty acid degrading in syntrophic methanogenic coculture. Interestingly, the activity of hydrogenotrophic methanogens was higher compared to acetolactic methanogens. In general, biomethane production in pure cultures was improved substantially compared to the syntrophic cultures system. Likewise, granular activated carbon(GAC) is reported to enhance biomethane production by multiple folds. Capson-Tojo et al., (2018) Exploited GAC in an AD, the biomethane production has noticeably improved and doubled for supported reactors. Similarly,Chowdhury et al., (2019) enhanced the performance of a food-waste AD by adding GAC to the system; the Lag phase was shortened from 7 to 3 days, and the biomethane production increased to 80% compared to the control reactor. Correspondingly, the addition of GAC to AD-treating food dogs has substantially increased the COD removal from 30% to 80% and VFA's removal from 54-64% to 78-81%, thus increasing the biomethane production to 772–1428mmol vs 80mml for control reactor (Dang et al., 2017).

Lin et al., (2018) studied the effect of graphene on enhancing biomethane production; the study showed that using an optimal amount of 0.5g/L can significantly improve production. However, increasing the amount of graphene added can substantially decrease the biomethane yield. The bacterial population in AD has increased by 40% when integrated with MEC, compared to control; hence, increasing the removal of organic matters and the conversion of volatile fatty acids (VFAs)(Lee et al., 2017). Packed activated carbon(PAC) is also reported to enhance biogas production by multiple folds in anaerobic digesters (AD) and integrated systems of AD-MEC Matsumoto et al., (2012) suggested that carbon fibres have a high capacity for adsorbing microbial cells due to less negative zeta potential and the large Hamaker constant for interaction between carbon. Hence, Barua et al., (2018) tested the effect of incorporating

carbon fibre into AD to enhance methanogenic co-digestion and biomethane production. Interestingly, biomethane production has increased by 2.4 folds compared to control reactors. Increasing the surface area available for microorganisms and adding conductive systems to improve (DIET) between electroactive microbes and Methanogenesis can improve the performance of the system (Baek et al., 2021; H et al., 2015; Viggi et al., 2014). Baek et al., (2021) added a conductive high-surface carbon brush to multiple anaerobic digester systems. Interestingly, the methane generation rate was 57-82% higher for all modified digesters than for the control. Moreover, the VFA's consumption rate has substantially increased owing to the high microbial density growing on the added brush, improving the system's performance (Baek et al., 2018).

Li et al., (2015) reported that magnetite accelerates biomethane production; the lag phase was reduced from 12 to 8 days. Similarly, Chen et al.,(2020) used magnetite to enhance AD's performance in high-salinity organic wastewater; biomethane production has increased by 1.54 folds.

Examining the effect of combining magnetite and external voltage on an anaerobic dairy digester by Gen et al., (2020) showed that both strategies were effective in enhancing the process performance and stability. However, adding magnetite improved the stimulatory effect. At the same time, external voltage contributed little to the methane yield, and the digester incorporated with magnetite alone had a stable performance comparable to that of the digester where both strategies were combined.

Conductive granular graphite (GG) as fillers was developed to enhance direct interspecies electron transfer (DIET) between syntrophic electroactive bacteria and methanogens to stimulate the methanogenesis process (Liu et al., 2012; Lovley, 2011). A few studies reported that adding phosphate buffer solution (PBS) to the system could potentially enhance biomethane production by facilitating the release of organics into a mixed liquorice-water anaerobic digester (Sasaki et al., 2010). (Xi. Xu et al., 2020) Adding (PBS) has improved methane production by 1.8 folds. In addition, reactors enhanced with PBS had a more diverse microbial community than the control.

Moreover, the PBS addition could enhance the growth of acetolactic methanogens (Methanosaeta) and inhibit a portion of hydrogenotrophic methanogens (Methanobacterium) Hagos et al., (2018) reported employing electrodeposited cobalt

phosphate to MEC-AD coupled reactors to improve the performance of the stainless steel and carbon cloth electrodes. Interestingly the system's performance has enhanced by 48%, and CH_4 production has improved by 80% compared to the control reactor utilization of endogenous hydrogen. Electrodeposited cobalt phosphate is deemed a valid alternative to noble metals as an electrocatalyst (Palma et al., 2019).

2.10 KINETIC MODELLING FOR BIOMETHANE PRODUCTION

Kinetic modelling is a mathematical representation of the dynamic process occurring during anaerobic digestion, it describes the conversion of substrate to biomethane gas and carbon dioxide with the aid of microbial consortia . Multiple kinetic modelling have been developed to simulate and analyse the behaviour of anaerobic digesters for the biomethane production as follow:

- First-order kinetic model : estimating the specific biomethane production rate based on the degradation of the substrate(Mata-Alvarez et al., 2014)
- Two phase kinetic modelling: The separation of hydrolysis from methanogenesis in two phase anaerobic digestion process (Mata-Alvarez et al., 2014)
- Monod kinetic modelling : Studies the microbial growth and utilization of the substrate in the anaerobic digester(Durruty et al., 2011)
- Modified gompertz model: Describes the cumulative biomethane production overtime in the digester(Pind et al., 2003)

The modified gompertz model has been. Widely utilized in recent research on biomethane kinetics. The equation below is the expression of the model:

$$M(t) = fd \cdot \exp\{-\exp\left[\frac{Rm.e}{fd}(\lambda - t) + 1\right] t > 0\} \qquad (2.1)$$

where M(t) - the accumulative CH_4 yield at the time of t (mL/g COD); fd - the maximum CH_4 potential (mL); λ - the lag-phase (d); Rm - the maximum CH_4 production rate t - the digestion time (d); and e - the exponential e (2.71828).

2.11 SUMMARY AND RESEARCH GAP

Food waste has high fermentable substrates; with the proper pre-treatment, this substrate can be utilized in bioenergy production. Although fungal mash was reported to treat food waste for biogas production effectively, it has not been implied to the pre-treatment of substrate for the hybrid system. In addition, multiple novel electrodes have been proposed for the hybrid system. In contrast, they have significantly improved the biomethane upgrade and substrate degradation. The fabrication price of these electrodes acts as the bottleneck to commercializing these products. This study focuses on using commercially available, cheap electrode material, with the proper modification, to enhance the system's performance and increase the selectivity of methane-producing microbes. Lastly, the importance of the microbial source and culture has not been thoroughly studied in the literature review. Mixing two inoculums increases the diversity and competitiveness between the microbes. Hence, active microbes will dominate. Mixing an inoculum rich in fermentative microbes with an inoculum rich in methane-producing microbes will give the right and efficient community to the hybrid system. Acknowledging these issues, a well-studied and designed MEC and AD hybrid system could potentially tackle the FW projection into the environment while producing high biomethane content as green energy for heat and electricity generation.

CHAPTER THREE
MATERIAL AND METHOD

3.1 OVERVIEW

This chapter summarizes the sample and inoculum collection, the flowchart of the overall work, the sample collection and characterization method, and pre-treatment optimization using OFAT, followed by FCCCD optimization using a design expert. Monomers quantification, electrode modification, and observation. In addition to system monitoring through substrate degradation, microbial attachment, current density and biogas production. Lastly, the hydrolysis and acidogenesis efficiency were explained, along with the kinetic modelling of biomethane production, using Gompertz-modified modelling.

3.2 FLOWCHART OF STUDY

The following section explains the flow diagram of the experimental process in the project as shown in Figure 3.1, followed by detailed description of the study's flowchart. The study started with the collection of food-waste, followed by multiple pre-treatment to maximise reducing sugar release. Then, electrodes modification and surface monitoring, followed by electrode's performance monitoring. Lastly, the efficiency of each stage was monitored, and inoculum mixing's kinetic in biomethane production was observed.

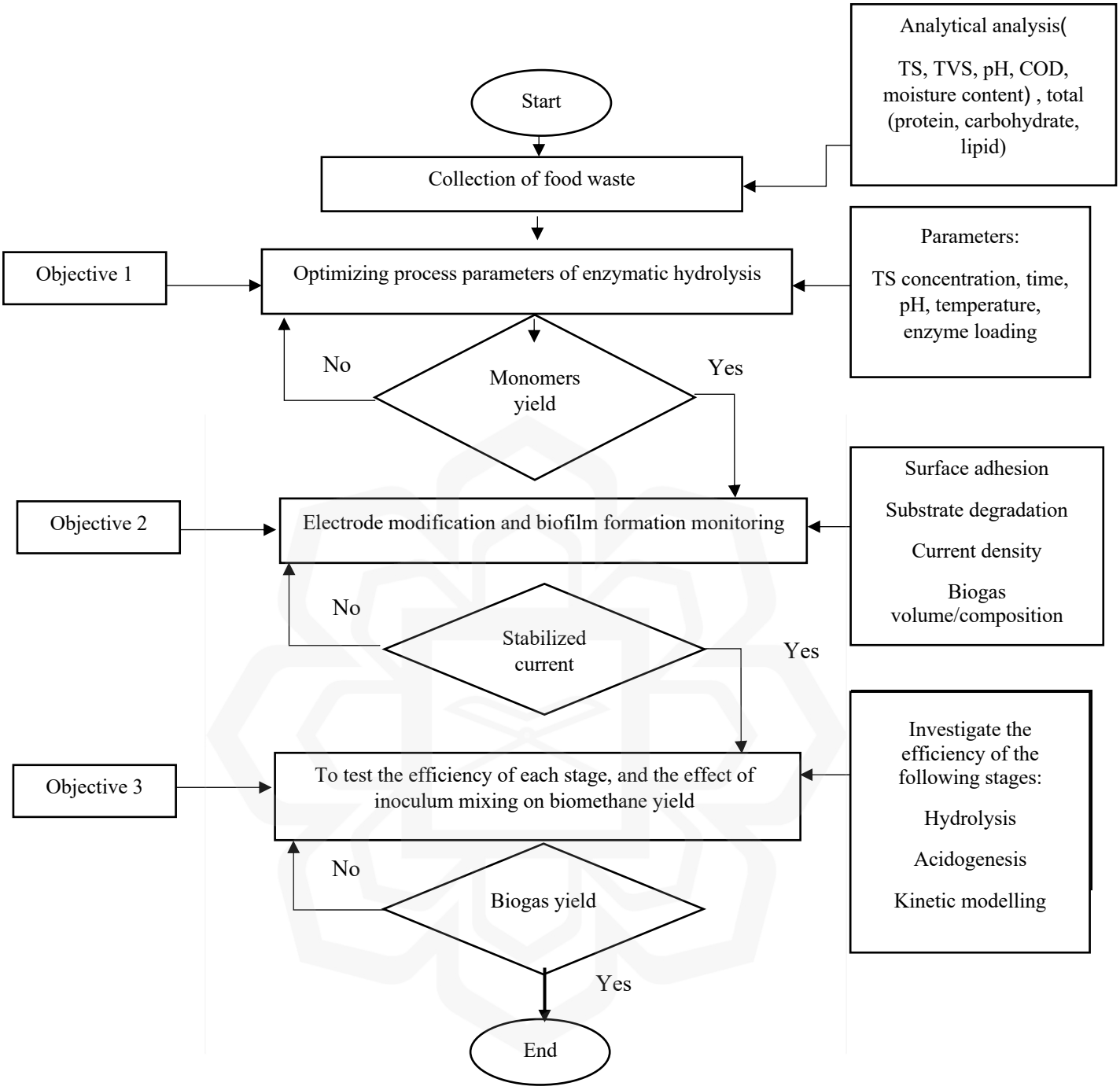

Figure 3.1 Flowchart of the study Methodology

3.3 SAMPLE COLLECTION

Different eating habits affect the food composition. To maintain a consistent food-waste composition, FW was prepared in the lab by mixing 80% rice, 10% vegetables, and 10% chicken, bones, shells were removed and, Fw was mixed and left out overnight to mimic food-waste conditions. Next, equal amount of water was added and FW was blended and sieved to achieve a size of 1-2mm (Hassanein et al., 2017). Food composition was analysed and other parameters, such as moisture content, TS, VS, COD, fats, protein, and carbohydrate, using the standard methods.

3.4 INOCULUM COLLECTION AND PREPARATION

The initial microbial source was collected from previous anaerobic digester of POME from Sime-Darby plantation at Carey island, Selangor .Cow manure was collected from farm fresh at UPM Industry Centre of Excellence. The samples were kept in the chiller until further usage. Effluent of previous anaerobic digester was centrifuged at 8000rpm for 5min. Then the supernatant was discharged, and precipitate was used as the seeding sludge for the systems. Equal amounts of cow manure and water were mixed, then the microbes in the cow manure were allowed to grow for two weeks.

3.5 FOOD-WASTE CHARACTERIZATION

3.5.1 Total Carbohydrate

Total carbohydrates were measured using Anthrone method. Standard glucose curve was prepared using different concentrations of glucose(0.1-1g/L) reacted with anthrone reagent. 1ml of food-waste sample was boiled in 6ml concentrated hydrochloric acid. Distilled water is then added to make up 1000ml of total volume. Then one ml of the mixture was mixed with 4ml of ice-cold anthrone reagent, heated in boiled water for 15min. and then measured

at wavelength of 490, the concentration of sugar was measured against glucose standard curve (Hedge, and Hofreiter, 1962).

3.5.2 Total Proteins

Total proteins were measured using Bradford reagent.
Protein standard curve was prepared by preparing Five to eight dilutions of BSA standard with a range of 5 to 100 µg protein. 0.2ml of sample was added to 5ml of Bradford reagent, the samples were left under room temperature for 30min, for the protein to react with the reagent. Then 10ml of dilution solvent was added to the samples. The samples were analysed under a wavelength of 595 nm and measured against protein standard curve (Ernst & Zor, 2010).

3.5.3 Total Lipids

One gram of sample was homogenized in 10ml of ethanol for 1min, then 20ml of chloroform was added to the sample and homogenized for 2min. the cake was re-extracted with 1ml methanol and 20ml chloroform, and the extractants were combined. then 25% of the volume was calculated and 0.8% KCL was added to the sample. the sample was placed in a separatory funnel. The chloroform layer was removed and dried in the oven, the weight of extracted lipids was measured (Ellefson et al., 2010) .

3.5.4 Total Solids

Weighing dish was measured. Then 1 ml of sample is added to the dish and placed in the oven at 120 ^{o}C for 3h (Reeb et al., 1999). Then, the dish is weighed and total solids is measured as follow:

$$\frac{\text{Final weight} - \text{Initial weight}}{\text{total sample volume}} \text{x}100\%. \qquad (3.1)$$

3.5.5 Total Volatile Solids

The same sample for measuring total solids is used (Reeb et al., 1999). The sample was placed in a pre-heated furnace at 550^{o}C for 20min.

$$\frac{\text{Final weight} - \text{Initial weight}}{\text{total sample volume}} \text{x}100\% \qquad (3.2)$$

3.5.6 Moisture Content

Same steps taken for calculating total solids were repeated. Moisture content was measured as followed:

$$\text{Moisture content} = \frac{\text{initial weight} - \text{dry weight}}{\text{dry weight}} \text{x}100\% \quad (3.3)$$

3.6 ENZYME HYDROLYSIS AND ACIDIC-ENZYMATIC TREATMENT

Enzymes were chosen based on the FW composition used(carbohydrates, proteins, fats, and oils). A purchased fungal mash rich in cocktail of enzymes produced by solid state fermentation, namely cellulase, Amylase, protease, and Lipase produced from rice bran, were used in this research. The activity of amylase enzyme was measured using the standard method of DNSA (Jain et al., 2020) since starch was the main constituent in food-waste. The enzyme loading (1-5% w/v), total solid concentration(2-8%w/v), pH(4-9), and temperature(40-60), and time(8-24h) were screened using OFAT as shown in appendix A , then optimized to achieve maximum sugar and FAN (Free Amino Nitrogen) recovery from FW.

Then the substrate concentration in terms of TS and enzyme loading (w/v) were optimised using Face cantered central composite design (FCCCD) in the response surface method (RSM). Condition of the design is as follow:

- Study type: Response surface method (RSM)
- Design: FCCCD
- Level: 2
- 2 factors: Substrate concentration (TS%) and enzyme loading (w/v)
- Type of design: full
- Response: reducing sugar concentration

Following the optimization, acidic-enzymatic pre-treatment was conducted using different acid concentration of (0.5-1.5%) (w/w) using 18M sulfuric acid. The samples were treated by acid first, sample's pH were adjusted to 7, followed by enzymatic treatment. , soluble chemical oxygen demand(SCOD), and reduction of total solids and volatile fatty acids were measured using standard methods. To test the effect of the pre-treatment, hydrolysed samples were fed to 500ml anaerobic digester, biogas produced was measured by water displacement method as previously illustrated in figure 3.2. Two cylinders placed upside down were placed into a water-bath, with a tube inserted in each cylinder, one connected to the digester to be filled with the biogas produced, the 2nd one

was to collect the biogas, The water's pH was adjusted to 3, to avoid solubilization of CO_2 gas. The biogas composition was analysed using PG810 multi gas analyser of CH_4, CO_2, H_2

3.6.1 Total Reducing Sugar

DNSA involves the oxidation of aldehyde functional group in glucose, and ketone in fructose. Darker sample colours indicates higher level of reduced sugar (Jain et al., 2020).

DNSA reagent is prepared by adding the following chemicals to 1L of distilled water:

- Dinitro salicylic acid: 10 g
- Phenol: 2 g (optional)
- Sodium sulphite: 0.5 g
- Sodium hydroxide: 10 g

Then, 1ml of DNS is added to 1ml of sample, boiled for 15min, and measured at 540nm wavelength.

3.6.2 Free Amino Nitrogen

Free amino nitrogen was measured using Ninhydrin reagent. Ninhydrin reagent is prepared by Dissolving 10 g Na_2HPO_4, 6 g KH_2PO_4, 0.5 g ninhydrin and 0.3 g fructose in a total of 100 mL. A dilution solution is prepared by dissolving 2 g potassium iodide in 600 mL distilled water and then add 400 mL of 96 % ethanol (Hill & Stewart, 2019).

2ml of sample was mixed with 1ml of Ninhydrin solution, then the samples were boiled for 16min, cooled down to 20^{o}C and 5ml of dilution solution was added.

$$Free\ amino\ nitrogen\ \left(\frac{mg}{L}\right) = 2Fx\frac{(\mathrm{As}-\mathrm{Ab})}{(Ag-Ab)} \qquad (3.4)$$

A_S = average absorbance of the sample

A_G = average absorbance of the glycine standard solution

A_B = average absorbance of the blank value (H_2O)

F = dilution factor of the sample

2 = concentration of the glycine standard solution in mg/L

3.6.3 Soluble Chemical Oxygen Demand (SCOD)

Samples were centrifuged at 8000rpm for 5min, supernatant was collected, diluted as needed, and then 2ml of sample was added into COD digestion tubes, heated at 150ºC for 2h, cooled then measured using spectrophotometer .

3.6.4 Biogas Production

A 500ml anaerobic digester were set-up. The digesters was inoculated with 10% previous anaerobic digester effluent. The digester operated under pH 7 , and temperature 37 ºC. On The first cycle, the reactor was fed with equal amount of untreated food-waste and water . The 2nd cycle it was fed with equal amount of enzymatically treated FW and water. On the third cycle the reactor was fed with equal amount of acidic-enzymatically treated FW and water. The biogas volume was monitored.

3.7 Electrode's Modification

Both electrodes, anode which is carbon felt, and cathode which is stainless steel mesh, were modified with multi-wall carbon nanotube. Electrodes were first washed with a solution consisting of acetone and ethanol 1:1. Then washed with water and dried in the oven.

3.7.1 Stainless Steel Mesh Modification

The methodology for SSTM modification was followed by (Wang et al., 2016) with some adjustments to the original method. The cathode was modified through submerging the mesh while mixing in a solution prepared using 95% ethanol with MWCNT prepared based on a ration of 1:2 (ml/mg) respectively. The solution was homogenized for 30min.Then the mesh was dried under 120 ^{o}C for 20min. The process was repeated until the mesh was coated completely.

3.7.2 Carbon Felt Modification

The method for the treatment was used based on two previous studies modification of electrodes using MWCNT with binding materials and organic solvents (Peng et al.,2010)(Dong et al., 2013), however, in this study only the binding reagent was used.
anode was modified through submerging the carbon felt while mixing in a solution prepared using distilled water, MWCNT, and PNP binding reagent of a ratios (1:2:0.4)((ml/mg.mg)) respectively. The electrode submerged in the mixture was homogenised to ensure the accessibility of MWCNT into the fibre's strands. The felt was then dried in the oven under 170 ^{o}C for 20min. the electrodes were then observed under SEM (Scanning Electron Microscope).

3.7.3 Scanning Electron Microscopy Imaging (SEM)

A square sample with the dimension of (0.5x0.5)cm were cut from each electrode. The samples were conductive, hence, no coating was needed. Samples were observed under SEM using different magnifications.

3.8 Proposed Design For An Integrated System of MEC-AD

The design of a bio-electrochemical system is crucial for the production of biomethane. A single-chamber system was proposed with carbon felt for anode and stainless steel mesh(304) for the cathode. Since the electrodes distance is crucial in the MEC-AD system, the distance between the electrodes was fixed at 1.5cm as previously optimized by (Choi & Lee, 2019). The electrodes were modified using MWCNT to improve surface properties for biofilm formation.

The system consisted of a single chamber with one set of electrodes connected to a DC power supply. The influent of the system was the outlet of enzymatic pre-treatment. The gas effluent volume was measured using water displacement method as shown in figure 3.2:

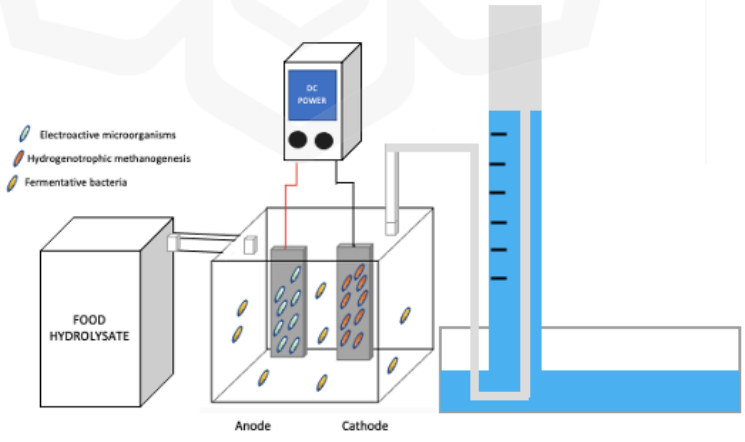

Figure 3.2 Proposed design of an integrated system of MEC-AD

A digester and two MEC-AD hybrid systems were set up. The first system was set up using unmodified carbon felt anode and MWCNT modified stainless steel mesh cathode. The 2rd system was set up using MWCNT modified carbon felt anode, and unmodified stainless steel mesh. A (10 Ω) resistor was placed between the electrodes to monitor the current. The digester was inoculated with 10% of anaerobic sludge obtained from simedarby's digesters. The reactor was fed with modified growth medium contained glucose 5 g/l; peptone 10 g/l; yeast extract 5 g/l; starch 1 g/l; sodium chloride 0.5 g/l; sodium acetate 0.5 g/l; cysteine hydrochloride 0.5 g/l. The pH of the substrate was adjusted to 7 with every feeding. The current drop at the resistor was used as a sign for substrate replenish. A voltage of (0.9V) was applied to all hybrid systems. The biogas volume was monitored using water displacement method (Selvankumar et al., 2017). The water's pH was adjusted to 3 to avoid CO_2 solubilization. The biogas composition of all four systems was monitored using CH_4,H_2 and CO_2 gas analyser daily. 1ml of reactors media was taken to monitor substrate degradation and pH.. Current at the resistor was measured daily using an ammeter.

3.8.1 Substrate Degradation

The substrate degradation was monitored based on the glucose reduction. 1ml of sample was collected after feeding to measure the initial glucose concentration, then 1ml of sample was collected after 24h, before the following feeding. The sample was diluted 10 times, then glucose concentration was measured. The glucose reduction percentage was measured as follow:

$$\frac{\text{Final glucose concentration} - \text{Initial glucose concentration}}{\text{Initial glucose concentration}} x\ 100\% \qquad (3.5)$$

3.8.2 Current Density

Current was measured on the resistant connected to between the anode and cathode using an ammeter. The current density refers to the current generated per unit area of the electrode as follow:

$$\frac{\text{Current generated}}{\text{Electrodes area}} \qquad (3.6)$$

3.8.3 Sample Preparation for Biofilm Formation on Electrodes(SEM)

Biofilm formation on the electrodes was observed. Sample pre-treatment was needed prior to observation as follow:
0.5x0.5cm of sample was cut , washed with phosphate buffer, then fixed using 2.5% of glutaraldehyde for 4h. Then the samples were washed and dehydrated with ethanol 50% 75% and 100% for 15min respectively.
Then the samples were dried and coated with gold for further analysis.

3.9 INVESTIGATING THE FERMENTATION STAGES EFFICIENCY

The main purpose of the study was to offer system's stability and improve the performance of the system. Hence, the efficiency of hydrolysis of untreated food-waste, acidogenesis of treated food-waste were monitored.

3.9.1 Hydrolysis Efficiency

To test the efficiency of hydrolysis, three reactors, namely conventional digester, MEC-AD with unmodified electrodes, MEC-AD with modified electrodes were fed with

untreated food-waste. The COD in different timepoints 2h, 4h, 8h, 16h, 24h, 48h was measured(Gao et al., 2019). The biogas volume and composition was measured. The Hydrolysis efficiency (%) was calculated using Equation:

$$\text{Hydrolysis efficiency (\%)} = \frac{COD_{solt} - COD_{sol0} + COD_{CH_4}}{COD_{t0} - COD_{sol0}} \quad (3.7)$$

Where:

COD_{solt}: Amount of hydrolysis products in liquid phase at time t (in mg COD)

COD_{sol0}: Amount of soluble COD at time 0 (in mg COD)

COD_{CH4}: Amount of hydrolysis products in gas phase (CH_4) at time t (in mg COD)

3.9.2 Acidogenesis Efficiency

To test the acidogenesis performance, the VFA production was monitored in three reactors, namely conventional digester, MEC-AD with unmodified electrodes, MEC-AD with modified electrodes. The reactors were fed with treated food-waste with a concentration of 6g COD/L. Biogas was measured under different timepoints as follow: 0h, 2h, 4h, 8h, 16h, 24h, 48h. Volatile fatty acids were measured at time 0 and 48, to monitor the accumulation of VFA's (Liu et al., 2012).

$$\text{Acidogenesis effciency (\%)} = \frac{COD_{VFAt} - COD_{VFA0} + COD_{CH_4}}{COD_{t0} - COD_{VFA0}} \quad (3.8)$$

Where:

COD_{VFAt}: amount of VFA'S produced in terms of COD at time.

COD_{VFA0}: Amount of VFAs at time 0 (in mg COD)

The conversion factor of acetic acid to COD was (1.067) as reported previously by (Khatami et al., 2021)

3.9.3 High-Performance Liquid Chromatography (HPLC) Analysis of Volatile Fatty Acids

Volatile fatty acids, namely acetic acid, propionic acid, and butyric acid were analysed. The analysis shows the amount of each volatile acid, to understand the favorable pathway used. HPLC with RI detector was used. Zorbax C18 column was used to analyse the samples. While (0.25mM) sulfuric acid was used as the mobile phase, The operation temperature was 40 °C. Two standard curves of the main three acids were also prepared, namely acetic acid and propionic acid.

3.10 The Effect Of Mixing Inoculum On The Biomethane Yield

Three rounds of experiments were performed with modified systems using three different inoculations. On the first round, system was inoculated with the original inoculum, obtained from POME previous anaerobic digester at Sime's Darby. On the 2nd round, the system was inoculated with cow-manure that was previously fed with food-waste for one-month, due microbial adaptation purposes. On the third round, a mixture of the original inoculum and cow-manure were inoculated to the system

Then Modified Gompertz model was employed to study the methane yield by the system, using the Equation below:

$$M(t) = fd \cdot \exp\{-\exp\left[\frac{Rm.e}{fd}(\lambda - t) + 1\right] t > 0\} \qquad (3.9)$$

where M(t) - the accumulative CH_4 yield at the time of t (mL/g COD); fd - the maximum CH_4 potential (mL); λ - the lag-phase (d); Rm - the maximum CH_4 production rate t - the digestion time (d); and e - the exponential e (2.71828).

The biomethane volume was measured as follow: 0h, 2h, 4h, 8h, 16h, 24h, 48h. The data vs timing were keyed into Mat-lab software along with equation 3.9 to generate the response on the biomethane production kinetics.

3.11 Chapter Summary

The chapter summarised the methods of food-waste characterization, multiple food-waste treatment, and optimization. Next, the electrodes preparation and modification using MWCNT was explained, followed by electrode-microbes interaction, and performance. Lastly, the methodology of calculating the efficiency of the first two fermentation processes were explained, and the kinetic study using different inoculums were presented.

CHAPTER FOUR
RESULTS AND DISCUSSION

4.1 OVERVIEW

This chapter includes the results and discussion of the enzymatic pre-treatment and optimization, acidic-enzymatic treatment of food-waste, and their effect on the release of monomers, and biogas production was monitored. In addition, the electrodes modification and microbial interaction on the electrode's surface was observed. The substrate degradation, current density, and biogas production of three different systems mentioned previously were discussed. Lastly, the efficiency of the first stages of fermentation was monitored and calculated, and the biomethane kinetic study produced from different inoculums was reported.

4.2 FOOD-WASTE CHARACTERIZATION

Food-waste was analysed as previously described. The substrate had high content of rice, followed by equal amounts of meat and vegetables. Hence high value of carbohydrates, followed by lipids and proteins. Different studies reported different results, as food-waste composition varies based on different eating habits. However, the food-waste composition aligns with a study by Kiran et al., 2015, where almost 70% of food-waste consisted of grains, followed by vegetables and meats. The Table 4.1 shows the characteristics of food-waste obtained for this study:

Table 4.1 Food-waste characterization

Food-waste characterization	Value
Total carbohydrates (g/L)	66.5
COD (g/L)	112.5
TS%	30
VS%	96.5
Moisture%	70
Total proteins g/L	16
Total lipids g/L	9.55
rice %	70
vegetables %	15
proteins %	15

4.3 ONE FACTOR AT THE TIME OPTIMIZATION STUDY OF ENZYMATIC PRE-TREATMENT

The effect of substrate concentration, enzyme loading, pH, time, and temperature on reducing sugar release in the enzymatic treatment were monitored. Based on the results plotted. In terms of enzyme loading as shown in figure (4.1), there was a noticeable increase in the reducing sugar at a loading of 3% in comparison loadings of 2% and 1%. On the other hand, increasing the loading up to 5% had no substantial increase. This might be attributed to the amount of available substrate. In the study of OFAT. Substrate concentration for different enzyme loading is consistent. Hence, the enzyme activity will increase with the increase of enzyme, until a certain point, in which there is no more available substrate for the available enzyme-reaction sites as explained by Shaarani et al., 2021.

a measure increases in reducing sugar was noticed between substrate concentration of 4 to 6%. However, samples with higher concentrations namely 6% and 8% showed no major increase in releasing reducing sugar, the values were 12 and 13.5 g/L respectively as

shown in figure (4.2). Increasing the substrate concentration is expected to increase the reducing sugar release concentration. However, at a certain substrate concentration increase, a substrate inhibition occurs (Reis et al., 2011). Kokkonen et al., 2021 reported that the inhibition is mostly attributed to the formation of unproductive enzyme-substrate complex after the simultaneous binding of two or more substrate molecules to the active site.

In terms of pH, neutral pH showed the best performance in releasing sugars, reducing the acidity of the mixture improved the sugar release, while alkaline pH showed the least reducing sugars. Multiple previous studies have also reported that neutral pH is more favorable to the enzyme amylase as previously reported by (Kant Yadav & Prakash, 2011)

Previous studies reported that the optimum temperature for amylase is 37 ^{o}C (Fernández et al., 2001) and multiple other studies. However, in this study, the enzymes activity was the highest at 50 ^{o}C, however, no major effects were observed with temperature increase as shown in figure 4.4. The results aligns with a previous study by Mahdavi et al., 2010 reported that his findings showed that amylase had the best activity at temperature 50 ^{o}C. The same enzyme can have different optimum conditions, depending on the initial enzyme production, microbial strain, and substrate.

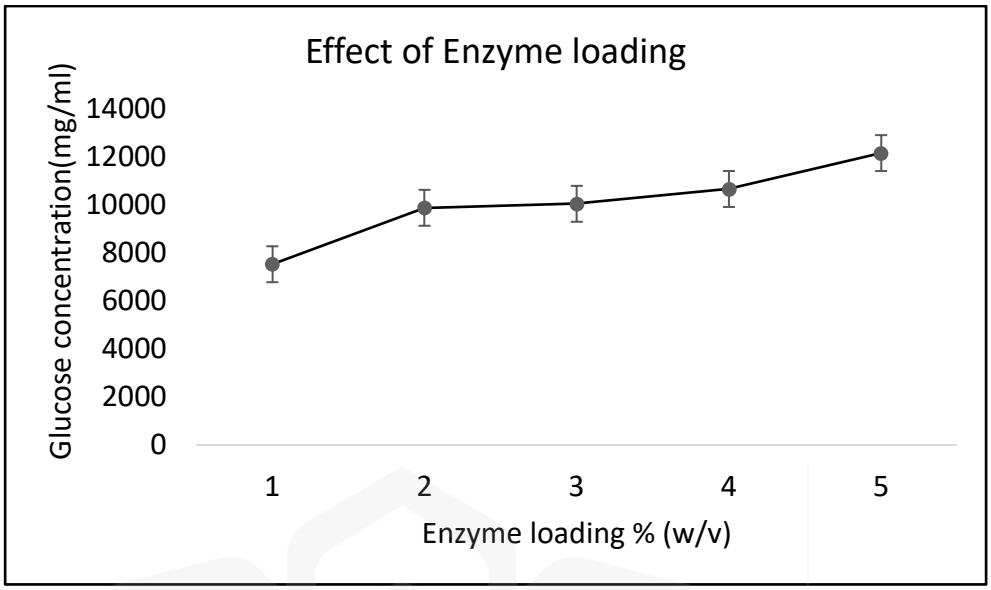

Figure 4.1 The effect of enzyme loading on the release of reducing sugars

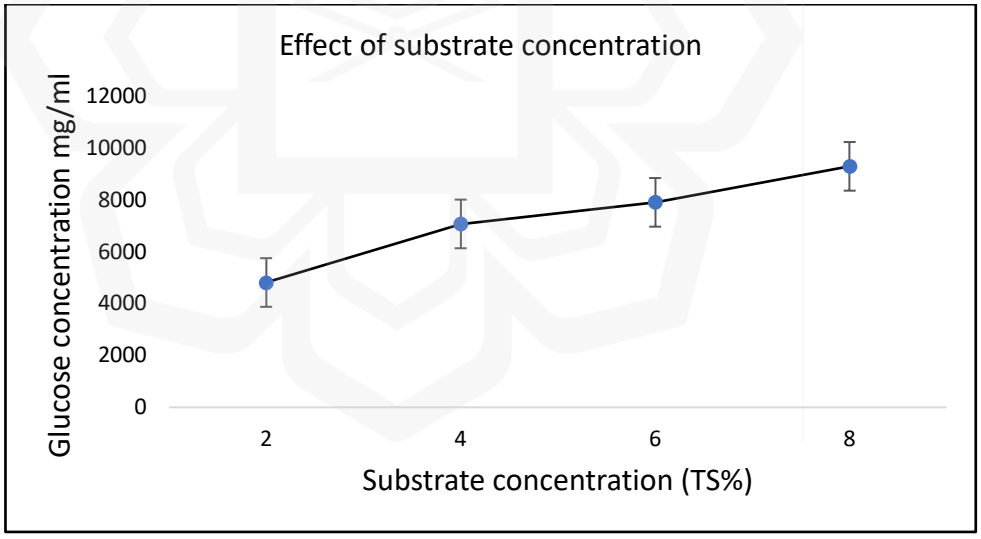

Figure 4.2 The effect of substrate concentration on the release of reducing sugars.

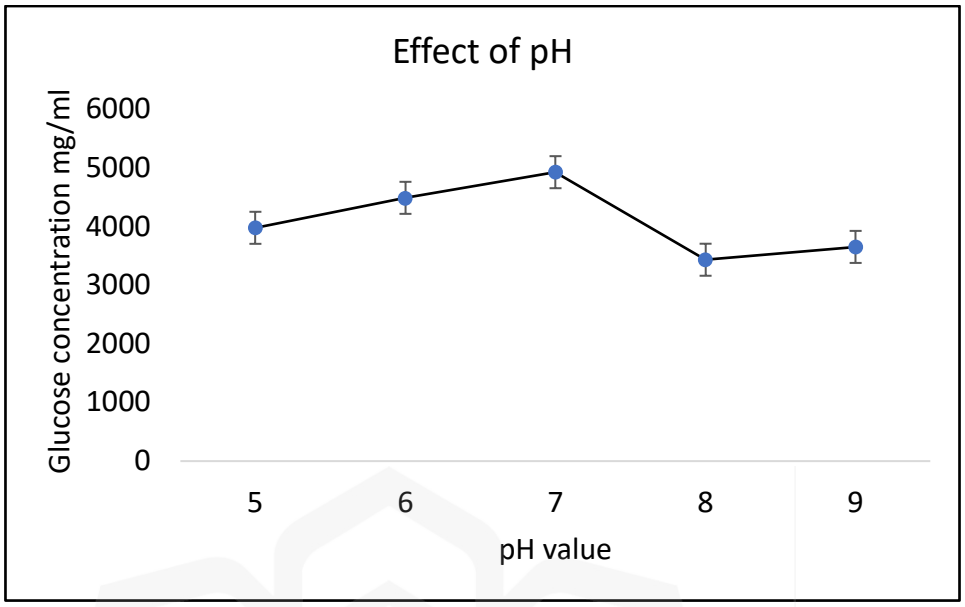

Figure 4.3 The effect of pH on the release of reducing sugars

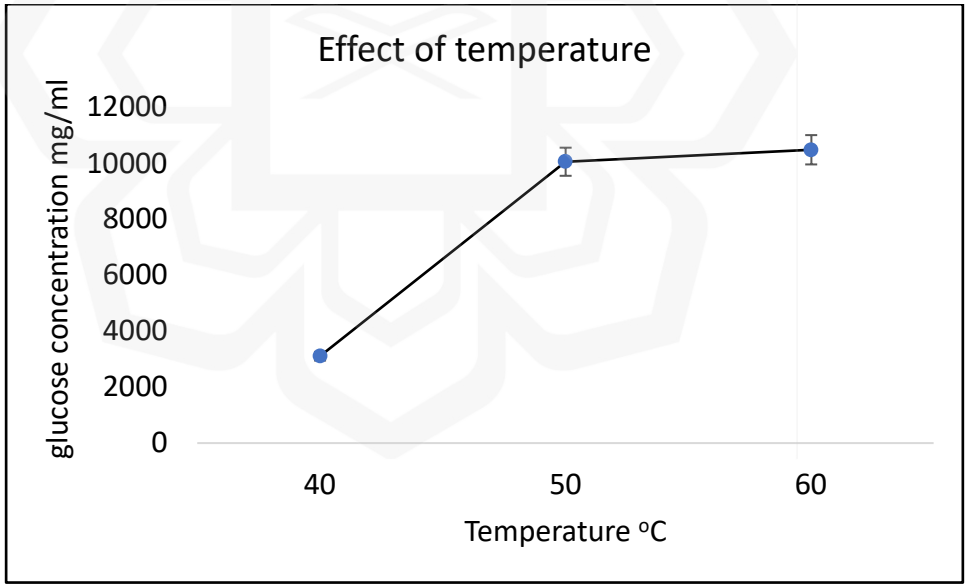

Figure 4.4 Effect of temperature on the release of reducing sugars

Based on the OFAT results previously explained, the levels for optimization were determined. The interaction between two factors, namely enzyme loading and substrate concentration will be studied. pH, temperature, and time will be fixed, since the enzyme amylase's optimum pH is 7 and since temperature had no major effect on the enzymes activity over 50^{o}C. The low and high level and Centre point of each factor is as tabulated in Table 4.2:

Table 4.2 Design of experiment operational factors and levels.

Factor	Low level	Centre point	High level
Substrate concentration (%)	6	8	10
Enzyme loading (%)	4	6	8
pH	-	Fixed at 7	-
Temperature, ^{0}C	-	Fixed at 50	-
Incubation time, hour	-	Fixed at 16h	-

4.4 OPTIMIZATION OF ENZYMATIC TREATMENT

The optimum condition and interaction of the factor's enzyme loading, and substrate concentration were determined using FCCCD under the response surface methodology (RSM). At central point conditions (6% enzyme loading and 8% substrate concentration), the maximum reducing sugar release of 14.88 g/L was achieved at an enzyme loading of 8% and substrate concentration of 10%. Analysis of variance (ANOVA) was performed, the 3D curve was provided as shown in the Figure 4.5, and the results are described in Table 4.3. The P-value was 0.0019 ($P<0.05$), and F-value 12.37 which indicates that the terms were significant. Lack of fit value is 0.2945 (>0.005) which is insignificant. The model, and models A, B, A^2 were also significant. However, models B^2 and AB were not significant. these models' terms could not be excluded to assist the structure of the model.

Table 4.3 ANOVA Results for Reducing Sugar Release

Sum of Square	Value	Mean Prob > F	F		Source	Squares
Model	61.87	5	12.37	13.09	0.0019	significant
A	*31.37*	*1*	*31.37*	*33.19*	*0.0007*	
B	*21.21*	*1*	*21.21*	*22.44*	*0.0021*	
A^2	*8.67*	*1*	*8.67*	*9.17*	*0.0192*	
B^2	*1.63*	*1*	*1.63*	*1.72*	*0.2305*	
AB	*0.59*	*1*	*0.59*	*0.63*	*0.4543*	
Residual	6.62	7	0.95			
Lack of Fit	*3.76*	*3*	*1.25*	*1.75*	*0.2945*	*not significant*
Pure Error	*2.86*	*4*	*0.71*			
Cor Total	68.49	12				

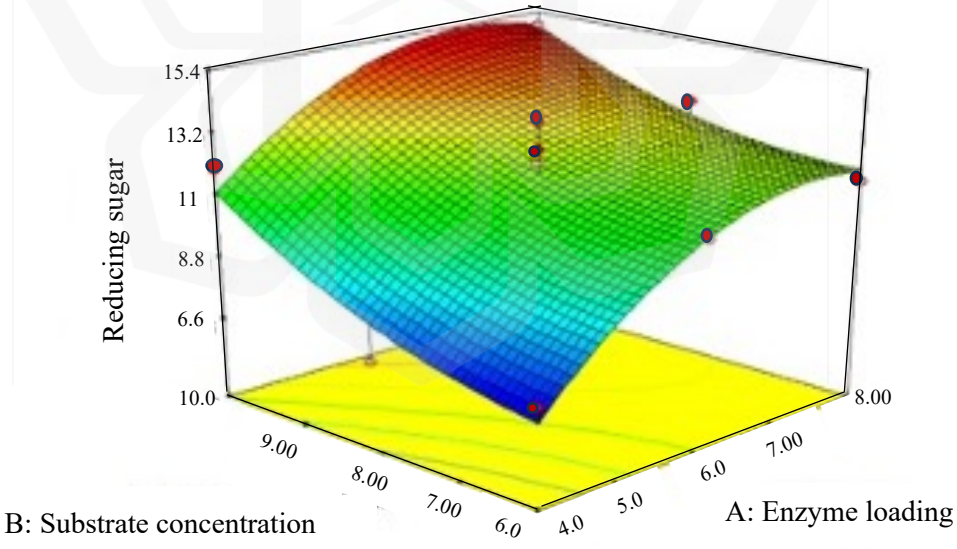

Figure 4.5 The interaction between substrate concentration and enzyme loading

Based on the regression analysis, the best model for the relation of reducing sugar (Y) with enzyme loading(A) and substrate concentration(B) is fitted in the
Equation 4.1 below:

$$Y= 11.82+2.29A+1.88B- 0.38AB-1.56A^2 +0.98B^2 \quad (4.2)$$

Theoretically increasing the enzyme loading would increase the release of monomers, namely reducing sugar, the predicted to theoretical values were tabulated in Table 4.4 and were plotted in figure 4.6. The optimization results showed that enzyme concentration of 8%, which is the highest concentration did not show major effect in increasing the reducing sugar yield percentage substantially. though the maximum sugar release was observed in samples with an enzyme loading of 8% with substrate concentration of 10% with value of 14.88 g/L. samples with the same substrate concentration at lower enzyme loading of of 6% showed relative results, with a sugar release of 14.1 g/L. Likewise, samples with substrate concentration of 6% treated with enzyme loading 8% had no substantial effect the concentration of sugars compared to samples treated with enzyme loading of 6%. The main aim of the study is to maximise the substrate loading while minimizing the enzyme loading. Therefore, enzyme loading of 6% and substrate concentration of 10% will be used for further studies since there were no major difference in the reducing sugar released with samples of higher enzyme concentration with the same substrate concentration.

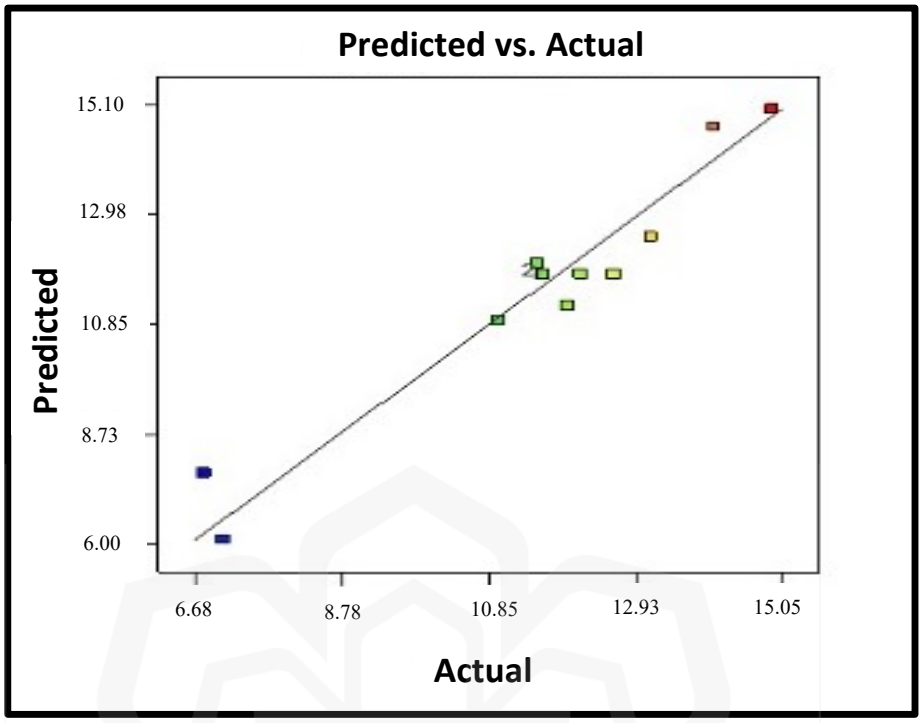

Figure 4.6 Predicted vs actual value of reducing sugar release (g/L)

Table 4.4 Predicted value vs actual value of enzymatic optimization

Run	Enzyme loading %	Substrate concentration(TS%)	Actual Value reducing sugar (g/L)	Predicted Value Reducing sugar (g/L)
1	4	6	7.09	6.68
2	8	6	11.54	12.02
3	4	10	11.97	11.21
4	8	10	14.88	15.01
5	4	8	6.8	7.97
6	8	8	13.16	12.54
7	6	6	10.99	10.92
8	6	10	14.05	14.68
9	6	8	11.63	11.82
10	6	8	12.16	11.82
11	6	8	11.6	11.82
12	6	8	11.63	11.82
13	6	8	12.63	11.82

4.5 Acidic-Enzymatic Pre-Treatment

Although the enzymatic treatment of food-waste was successful, the substrate conversion only accounted for 35% of the substrate's total volume. As it can be observed from the previous results, increasing the enzyme loading had no substantial effect on the treatment, hence, the pre-treatment should be improved to increase the conversion percentage. Diluted acid treatment is to break down polymer's structure to make it more susceptible to an enzymatic attack. Based on previous experiments, substrate concentration of 10% with enzymatic loading of 6% is used for the co-treatment.

4.5.1 Total Reducing Sugar

The co-treatment of food-waste has greatly improved the hydrolysis of carbohydrates and the release of reducing sugars (RS) compared to control and samples treated with enzymes only as shown in figure 4.7. Samples pre-treated with low concentration of aicd 0.5%, followed by enzymatic hydrolysis has showed an increase in the sugar monomers by 49.2% and 256% compared to samples treated with enzyme only and control respectively. However, the release of sugar reduced with higher acid concentration. Samples treated with 1.5% of acid followed by enzyme showed a drop by 16% compared to samples treated with enzyme only. The decrease of reducing sugar with the increase of acid concentration can be owned to two reasons:

- Chavan et al., (2015) reported that heated sulfuric acid destroys glucose slowly. Glucose is gradually dehydrated to carbon and water; thus, it is advised to reduce the total treatment time with higher acid concentration.
- The neutralization of the sample's pH prior to the enzymatic treatment. Braham et al., (2021) reported that during neutralization, Na_2SO_4 salts are produced along with water, salts increase the ionic strength of the sample, changes and increase in the ion strength affects the stability of the enzyme, leading to lower enzyme activity.

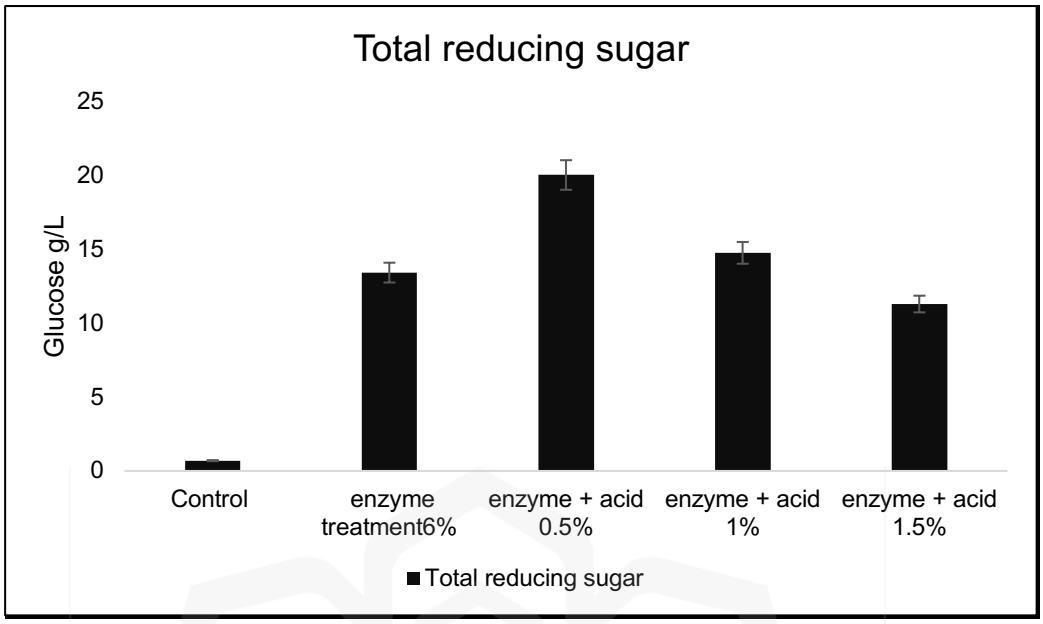

Figure 4.7 The effect of different treatments on the release of RS release from food-waste

4.5.2 Total Free Amino Nitrogen

The hydrolysis of proteins has decreased with acid pre-treatment by 26% in comparison to samples treated with enzymes only as shown in the figure 4.8. acidic pH has a negative impact on the proteins three-dimensional structure. Talley & Alexov, (2010) reported that acidic pH changes the attractions between the groups in the side chains of the protein, due to the high concentration of hydrogen ions in the acidic medium. denatured proteins lose their original folded shape; therefore, prtoeins have new structure that does not bind to the active site of the enzyme for hydrolysis, which explains the reduction of free amino nitrogen recovered. Increasing the acid concentration had no measure effect on hydrolysis of proteins, the FAN values were relatively close to all three samples treated with different concentrations of acid.

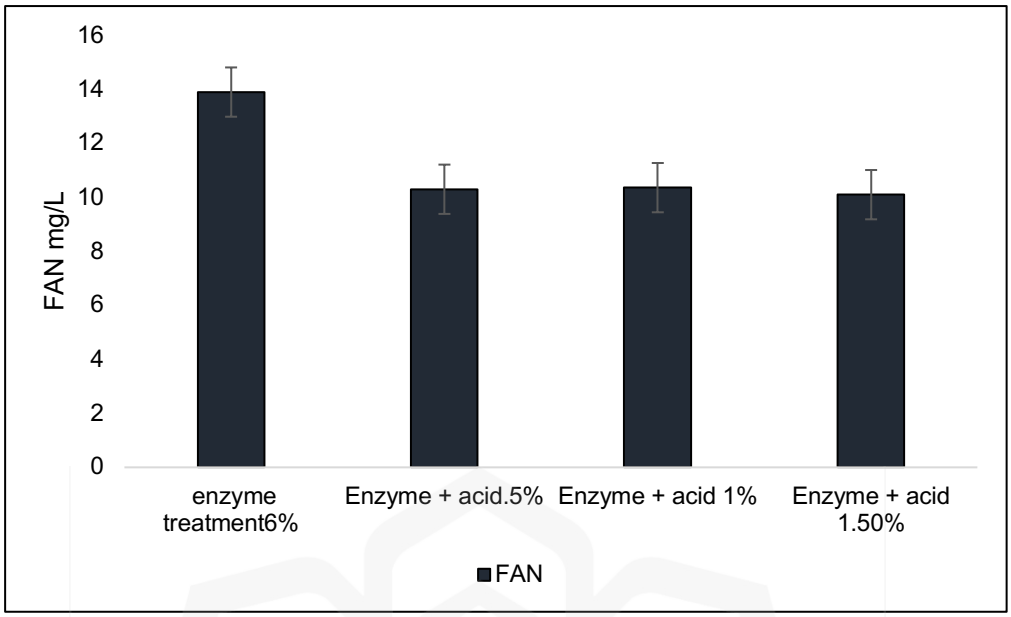

Figure 4.8 The effect of different treatments on the release of FAN from food-waste

4.5.3 Total Solids

Total solids (TS) value refers to insoluble compounds in the sample, solids reduction means substrate hydrolysis and solubilization. The total solids solubilization achieved by enzymatic treatment and the co-treatment was by 24% and 34% respectively as shown in the figure below. However, acid concentrations over 0.5% reduced the solubility of the substrate. As explained previously, high concentration of salts affects the enzymes activity. In addition (Prasoulas et al., 2020) reported that lower pH causes the denaturation of proteins, hence reducing the release of FAN, since denatured proteins cannot be hydrolyzed neither are they soluble due to their change of structure.

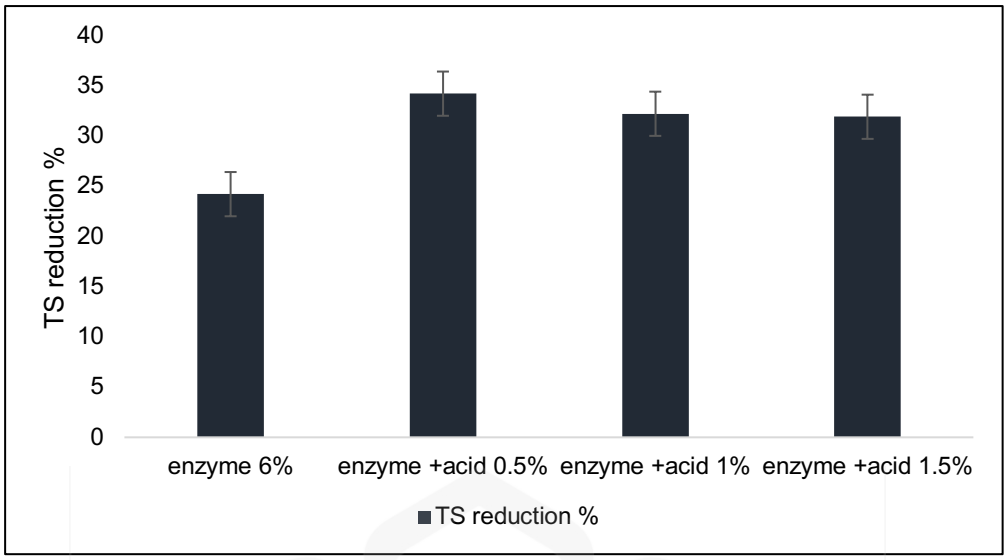

Figure 4.9 The effect of different treatments on the TS reduction of food-waste

4.5.4 Soluble Chemical Oxygen Demand

The increase in the SCOD values shows the successfulness of the acid solubilization of the substrate. The SCOD values from different treatment conditions were plotted in the figure below. The SCOD has increased by 104.5% in samples pre-treated with 0.5% of acid concentration. However, increasing the acid concentration has no major effect in solubilizing food-waste compared to samples treated with enzymes only. As mentioned in the previous sections, higher acid concentration has a negative effect on the enzyme's activity, complex substrate, and their monomers.

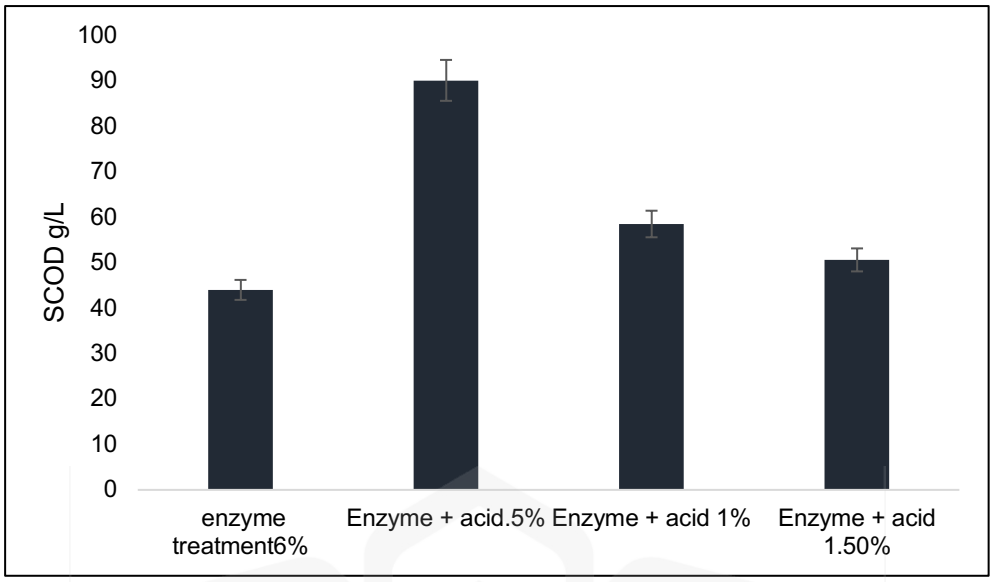

Figure 4.10 The effect of different treatments on the soluble chemical oxygen demand

4.5.5 Biogas Production

The biogas values of three systems fed with untreated food-waste, enzymatically treated FW and acidic-enzymatically treated FW respectively were monitored, to examine the effect of the treatment on the anaerobic digestion process and monitored for five dats. The figure below shows the daily biogas production. Digesters fed with enzymatically treated FW showed the best performance in the production of biogas, by 91% and 600% in comparison to acidic-enzymatic treated FW, and untreated FW respectively. The enzymatic treatment substantially increased the release of reducing sugar in the suspension, resulting an increase in the biogas production. Similar studies reported the effectiveness of enzymatic pre-treatment. Speda et al., (2017) reported that enzymatic treatment has enhanced the degradation of lignocellulose and improved the daily biogas production by multiple folds.

Samples treated with co-treatment had the highest soluble chemical oxygen demand,however, the cumulative biogas production of the digester was substantially lower compared to enzymatically treated FW digesters. During acidic treatment of FW, the pH

drops drastically during the acidic treatment, the pH is then adjusted 7 for the enzymatic treatment, producing Na_2SO_4 salt and water. High salinity mainly included cations of Na, K, Ca, Mg, and Fe, which could restrain the AD seriously and dehydrate cell walls through the action of osmosis, hence, disrupts the biogas producing microbes (Anwar et al., 2016).

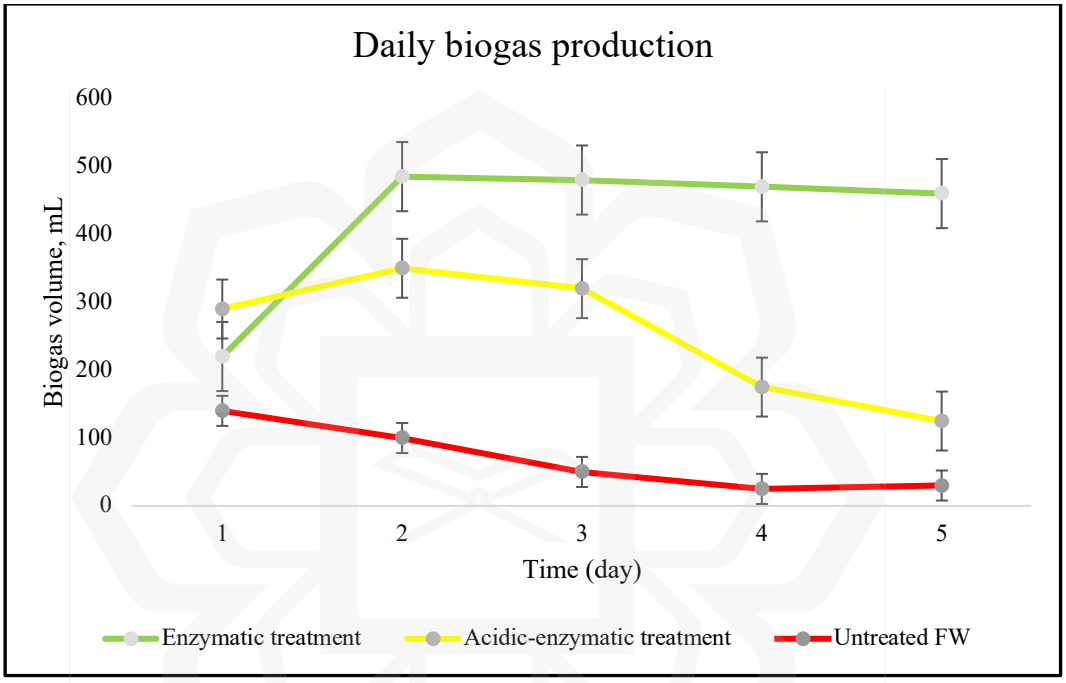

Figure 4.11 Daily biogas production of digesters fed with substrate under different treatment conditions

4.6 Electrodes Modification and Biofilm Formation

4.6.1 Stainless Steel Mesh Modification with Multiwall Carbon Nanotubes

Electrodes were washed using ethanol and acetone (1:1) and dried in the oven for 20 min to remove any impurities on the surface. Multiple modification methods were performed to ensure a homogenous dispersion of MWCNT on the surface of the mesh. The figure 4.12 includes multiple SEM images under different modification methods. The SSTM had a smooth, clean surface, as shown in figure 4.12a. Then SSTM was modified with MWCNT using the method suggested by Tsai et al., (2017). However, the dispersion of CNT in ethanol was poor, leading to poor adhesion and dispersion of MWCNT on the SSTM. To improve the MWCNT dispersion in ethanol, sonication time was increased from 15min to 1h. However, no improvements were observed regarding the interaction area between the SSTM and MWCNT, but the particle aggregates growing on the mesh were reduced.

To maintain a homogenous dispersion of MWCNT and to increase the area of interaction between MWCNT and SSTM, the sonication time of MWCNT-ethanol was increased, and a moderate mixing speed fixed at 100 rpm was incorporated into the procedure. Multiple modifications were performed to ensure complete coverage of MWCNT on SSTM. As a result, MWCNT aggregates were reduced, and their growth on MWCNT has improved.

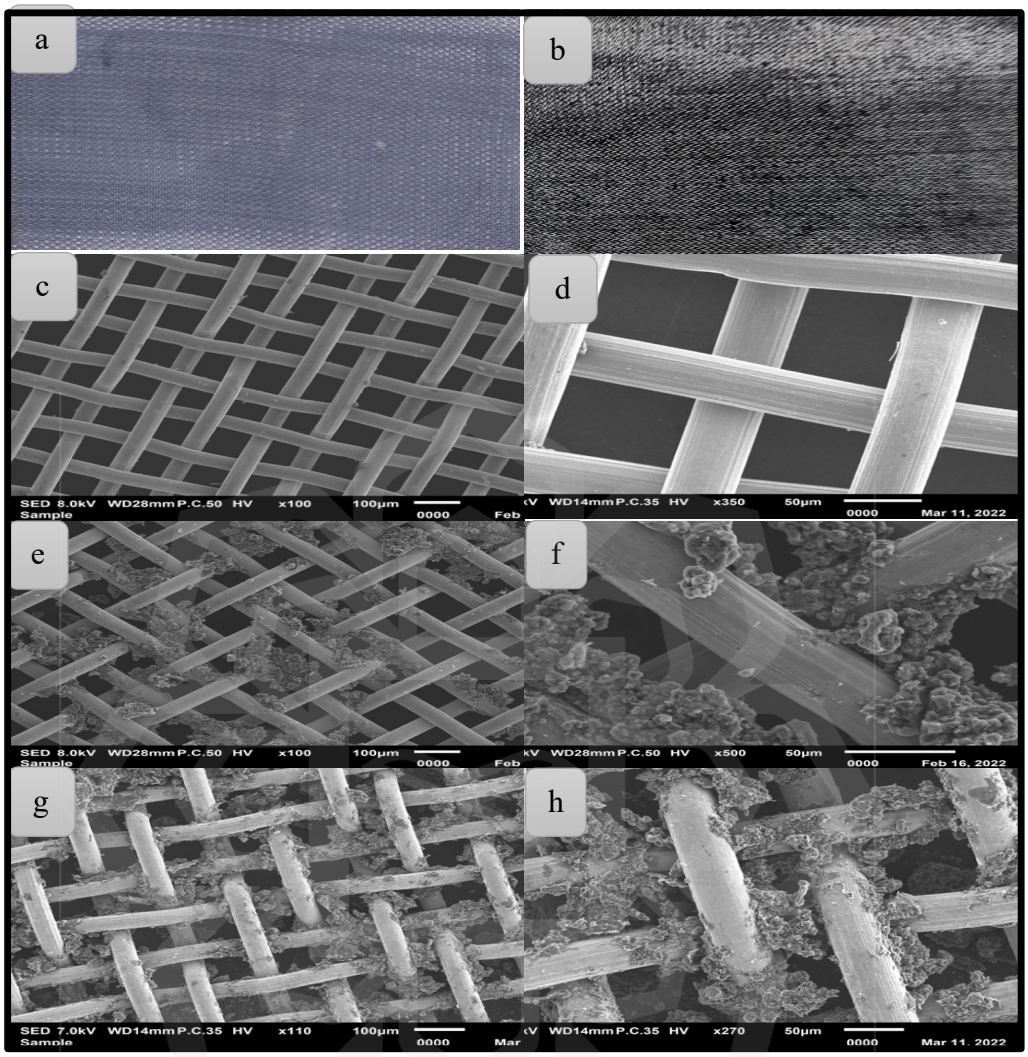

Figure 4.12 SEM imaging of different treatment conditions of SSTM and MWCNT: (a)Unmodified SSTM, (b)Modified SSTM, (c)Control-1, (d)Control -2, (e)Submerged with mixing, (f)Submerged with mixing, (g)Multi-layer-1, (h)Multi-Layers-2

4.6.2 Carbon Felt Modification Using Multi-Wall Carbon Nanotubes

Carbon felt was used as an anode. Carbon felt, and MWCNT are known to be hydrophobic. Hence, a dispersion reagent was required to improve the dispersion of MWCNT in distilled water. PNP powder was used to improve the dispersion of MWCNT in water as a binding reagent between MWCNT and carbon felt. Since carbon felt is hydrophobic, sonication was required throughout the modification process to ensure the fibre's contact with MWCNT. The modification process was repeated multiple times until the electrodes achieved a smoother and covered surface. The samples were observed under SEM; images (a)&(b) are of the original carbon felt surface, and images(c)&(d) are of modified carbon felt electrodes:

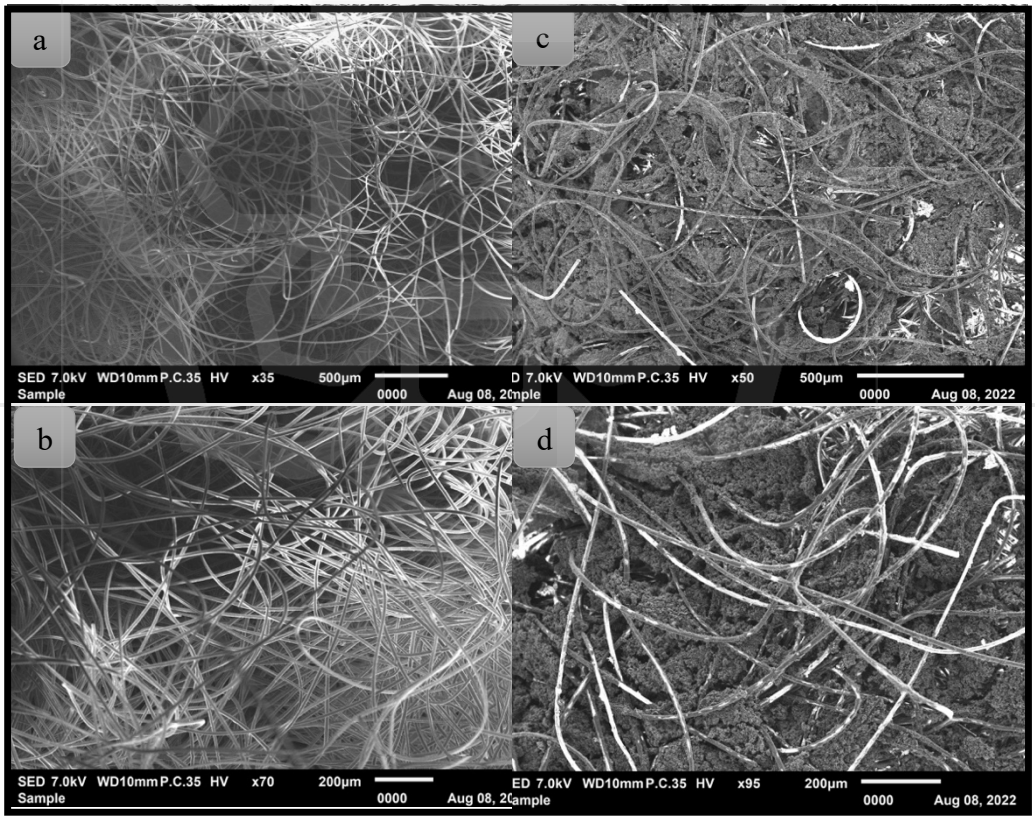

Figure 4.13 Different magnifications of SEM imaging electrodes: (a&b) Unmodified (c&d) of modified electrodes

MWCNT are reported to increase the methanogenic population on the electrodes, which will be monitored further in the hybrid system. Based on the SEM imaging, images (a)&(b) of the original carbon felt show smooth surface and fibre dispersion, with a high volume of the void between the fibres. In images (c)&(d), it can be observed that a large density of MWCNT growth was in between the fibres, with a light coating of MWCNT on the fibre strands. Occupying the space between the fibre strands increases the surface area provided for the microbe's growth. Hence increasing the microbial population while providing a bridge between the fibres to improve the microbe's interaction with less EPS substance. It also has been reported previously that MWCNT increases the biocompatibility and electrical conductivity of the material (Feng et al., 2020), hence increasing the current density.

4.7 Microbial attachment on electrodes

4.7.1 Carbon Felt

Microbes had different behavioural growth on modified and unmodified electrodes based on SEM's electron images. Images (a&b) show the general distribution, bacterial growth, and colonization of microbes on modified and unmodified electrodes. In contrast, images (c & d) are closer imaging of microbial growth behaviour of modified and unmodified, respectively. Based on the image (a) of modified electrodes, microbes thoroughly covered the fibre's surface and the MWCNT in between the fibres with the distribution of irregular individual colonies on different areas on the fibres. On the other hand, microbes had a completely different behaviour in the unmodified carbon felt, as shown in image (B). The microbial behaviour was big lumpy biofilm formation and growth in some fibre regions, rather than a full coverage like modified fibres. As can be seen from image (b), the microbial density on the unmodified CF was much less than the modified CF.

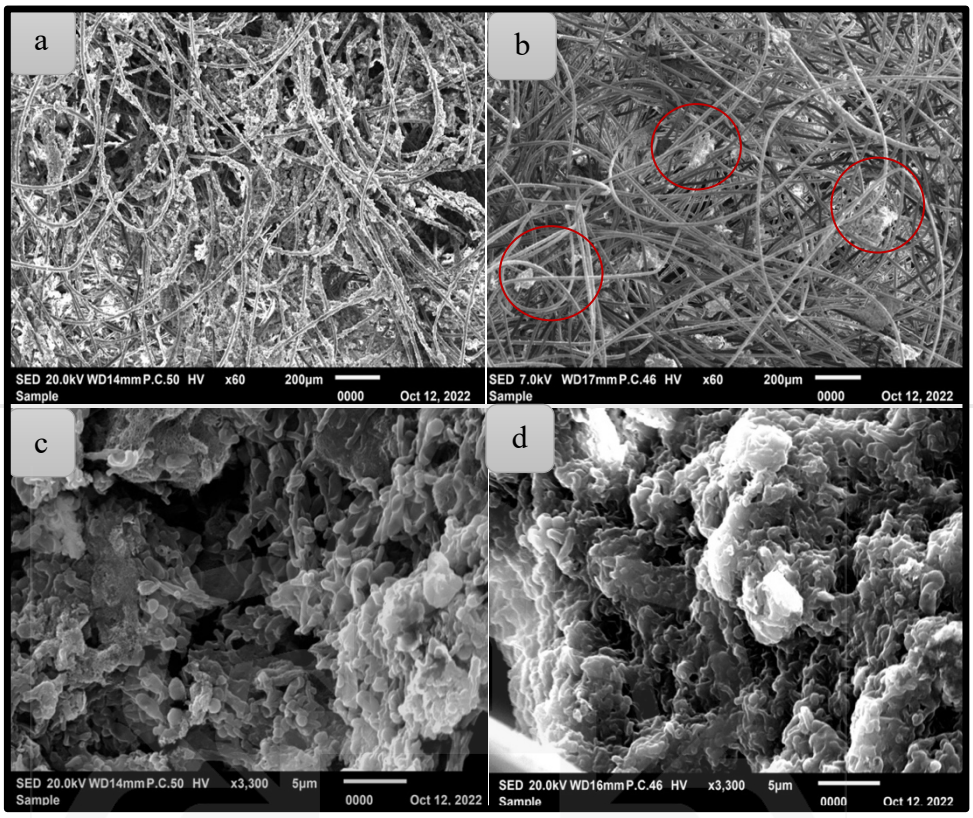

Figure 4.14 SEM Images of microbial attachment on modified (a&b) and unmodified (c&d) CF

Image (c) of modified CF shows the direct growth of microbes on an MWCNT-covered surface, offering a higher surface area for microbial growth. In addition, MWCNT, a conductive material, has also affected the electron transfer behaviour of the microbes. From the same image, MWCNT facilitated the electron transfer directly from the microbe's surface to the electrode. A study by Kadier et al., (2016) reported that electrons generated from the oxidation of organic materials by a single microbe are directly transferred to the anode, as observed from image (c) of the modified electrode. On the other hand, the microbial community growing on unmodified electrodes, as shown in the image (d), had a different electron transfer mechanism, namely electron transfer through conductive biofilm. Microbes in unmodified electrodes secret certain compounds called extracellular polymer matrix(EPS) to help them attach themselves

to the electrode and facilitate electron and substrate transfer.In modified CF, the extracellular polymeric substance density was lesser compared to unmodified electrodes; this has been reported previously by Salvador et al., (2017) owing to CNT, microbes have lesser secretion of substance in reactors equipped with CNT. The illustration below shows the electron transfer mechanism:

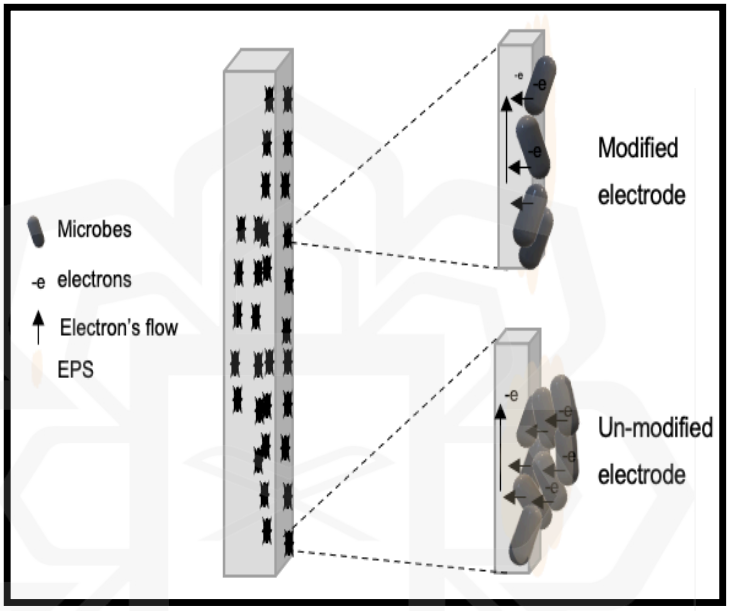

Figure 4.15 Electron transfer mechanism in unmodified and modified electrode

4.7.2 Stainless Steel Mesh

The microbial growth on modified and unmodified stainless-steel mesh had similar behaviour to microbial growth in modified and unmodified CF electrodes. In modified electrodes, microbes grew directly on the surface of the mesh and MWCNT, as shown in the image (a). While microbes in unmodified electrodes had a cluster growth behavior, as shown in the image (b). It can also be seen that image (c) and (d) had different microbial community growth and distribution. In the image (c) of modified SSTM, rod-shaped

microbes were of significant population, followed by cocci and di-cocci-shaped microbes. Hydrogenotrophic methanogenesis has rod-long shapes, while acetolactic methanogenesis has cocci and di-cocci shapes. Same results were reported by Babu, 2015; Sylvia et al., (2016), where long-rod shapes microbes were identified as hydrogenotrophics, and cocci, di-cocci shaped microbes were identified as acetolactic methanogens. This is evidence of the effect of MWCNT in enriching the methanogenic community, as reported previously by Salvador et al., (2017), the addition of CNT has accelerated the population of hydrogenotrophic methanogenesis culture in the digester In unmodified electrodes, a variety of different microbial shapes existed. Rod, long rods, cocci- and di-cocci-shaped microbes existed.

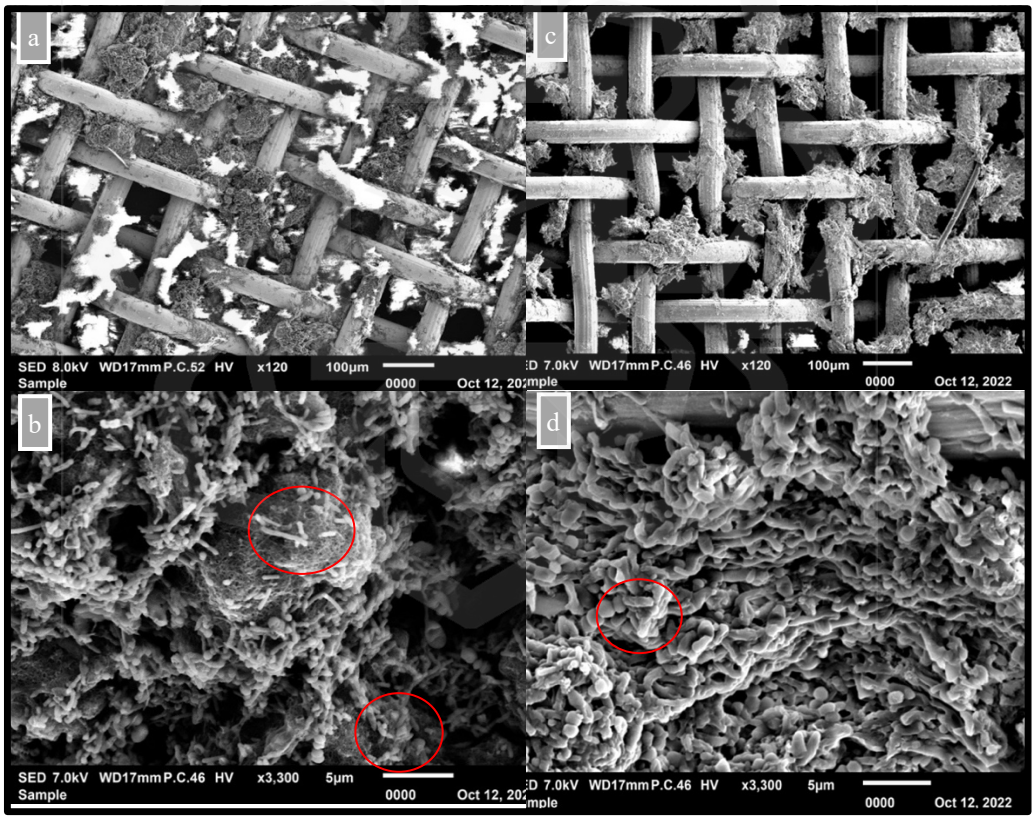

Figure 4.16 Different SEM images of microbial attachment Modified(a&b) and unmodified(c&d) stainless steel mesh

4.8 ANAEROBIC DIGESTER AND MEC-AD HYBRID SYSTEM WITH MODIFIED ELECTRODES

Three reactors were set-up for this experiment, conventional anaerobic digester, MEC-AD system with modified carbon felt anode, MEC-AD system with modified stainless steel mesh cathode. All reactors were fed with glucose as the main carbon source. The substrate degradation, biogas volume and composition, and current for the hybrid system were monitored.

4.8.1 Substrate Degradation

Fermentative and oxidative microbes grow directly on the anode, utilizing organic matter and producing VFA'S carbon and hydrogen. The substrate degradation rate was monitored in terms of glucose consumption. AD reactors showed no significant substrate degradation on the first cycle. The degradation value was lower than 55% throughout the cycles. The increase in degradation rate for the digester was faster in the first few days compared to the hybrid system with modified SSTM electrodes. However, the substrate consumption was higher in the hybrid system and increased throughout the first and second cycles with microbial adaptation to the anode. MEC-AD-SSTM achieved a high percentage of 83% towards the end of the second cycle. it can be attributed to the larger surface area for microbial growth hence, faster substrate consumption. In addition, this could be attributed to the enhancement of performance by degradative and oxidative microbes by carbon felt anode. A study by Luo et al., (2018) suggested that carbon felt anodes with an applied voltage above 0.5V highly enhance degradative microbes in MEC-AD hybrid systems, along with oxidative microbes. However, MEC-AD-CF showed the best substrate degradation performance throughout the first and second cycles, maintaining a value over 80% and achieving a maximum percentage of 92.55%. In addition to the enrichment effects of carbon felt, MWCNT modification has a wider porous surface area with high biocompatibility for oxidative and degradative microbes to grow as previously shown in

figure 4.14. The increase in substrate removal efficiency can also be attributed to the carbohydrate's bioconversion through the favorable redox potential between the electrodes, hence enrichment of functional degradative microbes (Zhao et al., 2021).

The results align with a similar study by Mansoorian et al., (2020) on the treatment of landfill leachate using MEC showed that the substrate degradation of systems equipped with MWCNT modified CF had a high substrate degradation value of 97%, compared to control with a value of 72% only.

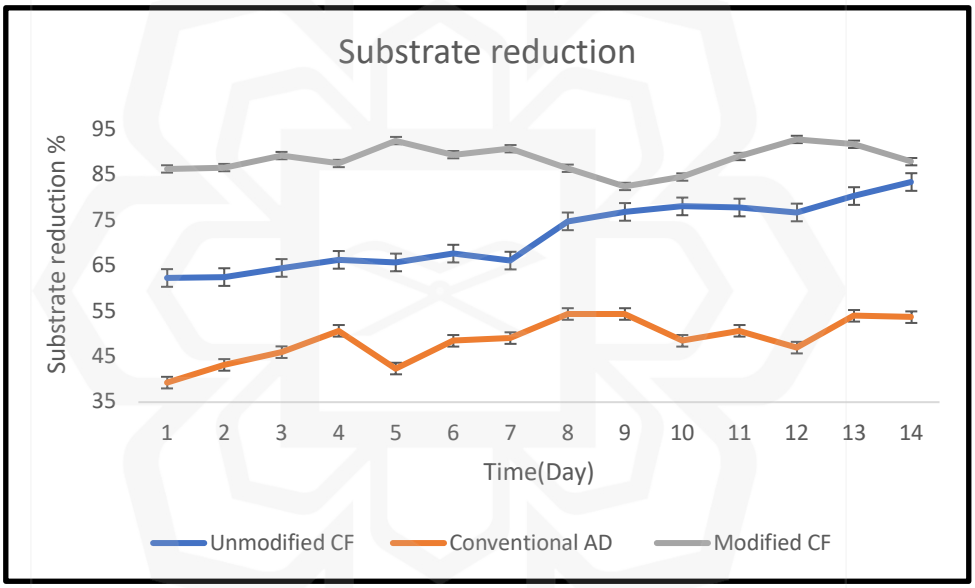

Figure 4.17 Glucose reduction in semi-batch systems of unmodified, modified CF, and conventional AD fed with 50ml/day substrate.

4.8.2 Current Generation

In the hybrid system of MEC-AD, electroactive microbes grow on both electrodes. On the anode, fermentable microbes utilize sugars, and fermentable matters oxidize organic to CO2, electrons, and protons, as shown in the equation 4.3. The electrons travel from the anode to the cathode, generating a current (Zakaria et al., 2020). The more organic matter is oxidized, the more electrons are generated, hence a higher current volume.

$$CH3COOH + 2H2O = 2CO2 + 8H+ +8e \quad (4.3)$$

The current density indicates the activity of electrogenic bacteria. Hence, the higher the current density is, the more active and the higher the population of electrogenic microbes are (Carrillo-Peña et al., 2022). Based on systems equipped with unmodified carbon felt anode, and modified stainless steel mesh cathode, it can be observed that no current was generated in the first few days as shown in figure 4.18. Starting from the sixth day, a small current volume was generated. The current volume increased up to day 10, and then a drop of 50% was observed on the following day. The increase in current volume refers to the growth and increase in the electroactive microbial community on the anode. This could be owed to the depletion of the substrate. The fluctuation in the current throughout the 20 days could also be owed to the microbes developing the extracellular polymer matrix on the electrodes (Salar-Garcia et al., 2020).

On the other hand, reactors equipped with a modified carbon felt anode, and unmodified stainless steel mesh cathode showed a relatively high current density on the first cycle with a current density of 2.67 mA/m2 compared to 0.0 mA/ m2 for reactors with unmodified carbon felt anode. A study by Jourdin et al., (2014)suggested that modifying porous electrodes with MWCNT increases carbon electrodes' biocompatibility, increasing the electrode's microbial density and thus generating the current density Coating with MWCNTs improves the electrochemical communication between the microbes and improves the conductivity of the materials (Aryal et al., 2017). Moreover, Sharma et al.,

(2014) reported that MWCNT modification reduces the inner resistance of the electrodes and increase the active surface area, which reduces the ohmic loss, hence improving the current density.

Moreover, the increase in current density can be attributed to a novel type of microbe called Geobacter which are electroactive that coexists with fermentable microbes(Walker et al., 2019). Geobacter produces high current densities in the MFC and MEC systems(Malvankar et al., 2012). They utilize VFAs like acetate using extracellular, insoluble Fe(III) and Mn(IV) oxides as terminal electron acceptors(Lovley et al., 2011a). A similar study with the anode of graphite felt modified with MWCNT to treat landfill leachate showed high current density production of 4.2mA/m^2(Mansoorian et al., 2020).

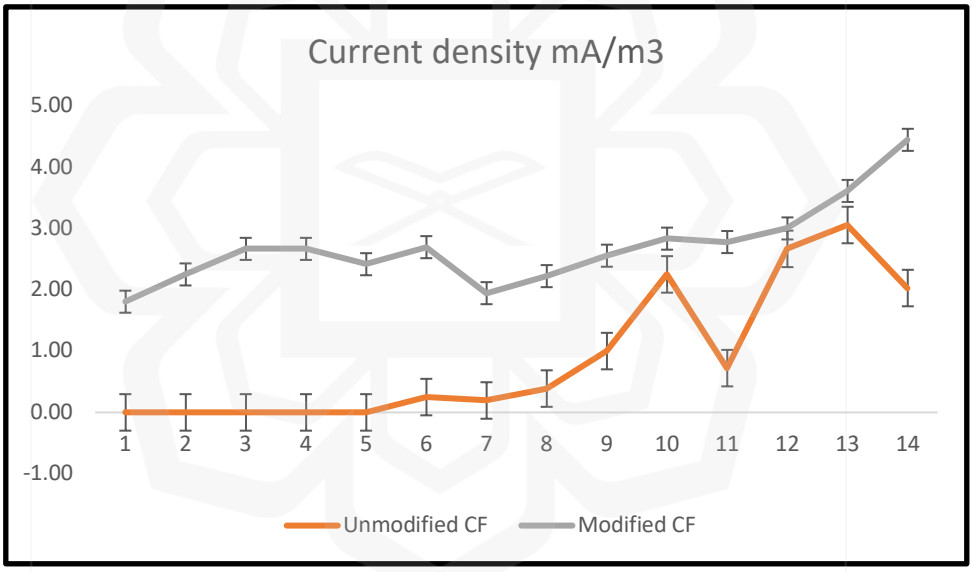

Figure 4.18 Current density of system equipped with unmodified and modified CF

4.8.3 Biogas Production

From the overall performance, the hybrid system with modified SSTM has substantially outperformed systems with unmodified SSTM and conventional digester. In the modified

reactor, the biomethane production substantially increased on the sixth day onwards, achieving a value of 287 CH4/g glucose while only producing 12 ml CO_2/g glucose. On the other hand, unmodified reactors gradually increased biomethane throughout the cycle, outperforming conventional digesters with a cumulative biomethane value of 57.7 ml/g glucose and 2.5 CO_2 ml/g glucose. The digester had the lowest biomethane production of 37 mL/ g glucose, yet the highest cumulative CO_2 with a value of 41 mL/g glucose. It was reported previously that conventional digesters' biomethane only accounts for 50-60%, and the remaining is CO_2 (Choi et al., 2017) compared to integrated systems. Integrating electrodes into the system gives a higher surface area for microbial growth. Hence, a higher volume of the substrate is available for faster consumption. Modifying the SSTM cathode with MWNT has increased the surface area and biocompatibility of the mesh, which was also reported and observed in the SEM images. In addition, MWCNT and conductive materials have been reported previously to improve DIET reactions between fermentative and methanogenic microbes (Baek et al., 2018).

Moreover, Salvador et al., (2017) reported that CNT increases the population and selectivity of the hydrogenotrophic and electroactive methanogenesis community. Unlike acetolactic methanogenesis, which consumes acetate to produce methane and carbon dioxide, Hydrogenotrophic methanogenesis produces methane through the consumption of hydrogen and CO_2 in the production of biomethane, thus, reducing the CO_2 concentration while increasing the biomethane volume. Modified stainless steel mesh reactors.

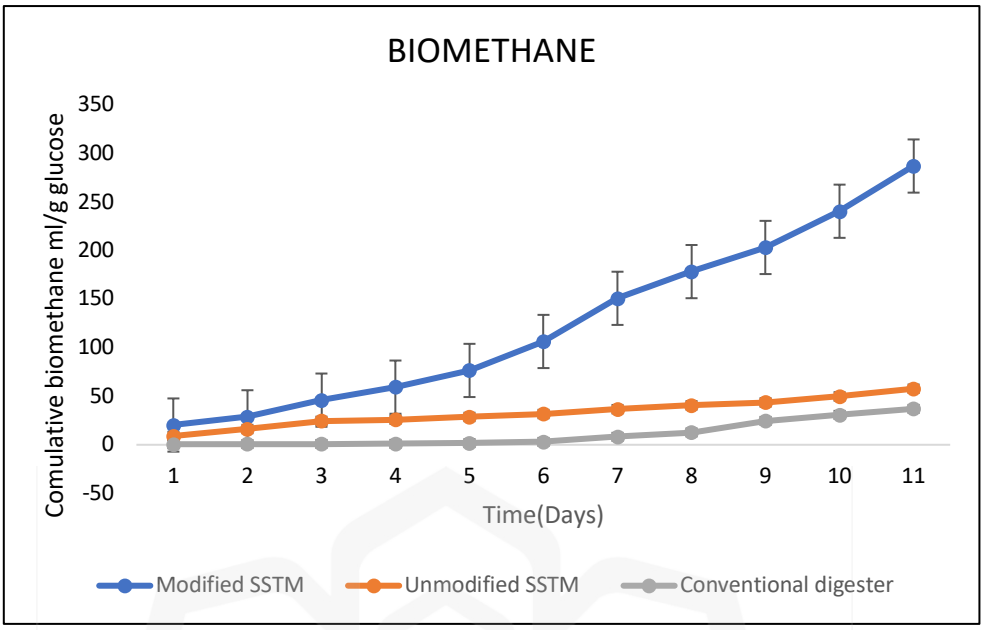

Figure 4.19 Cumulative biomethane production of conventional digester, Modified system, and unmodified system

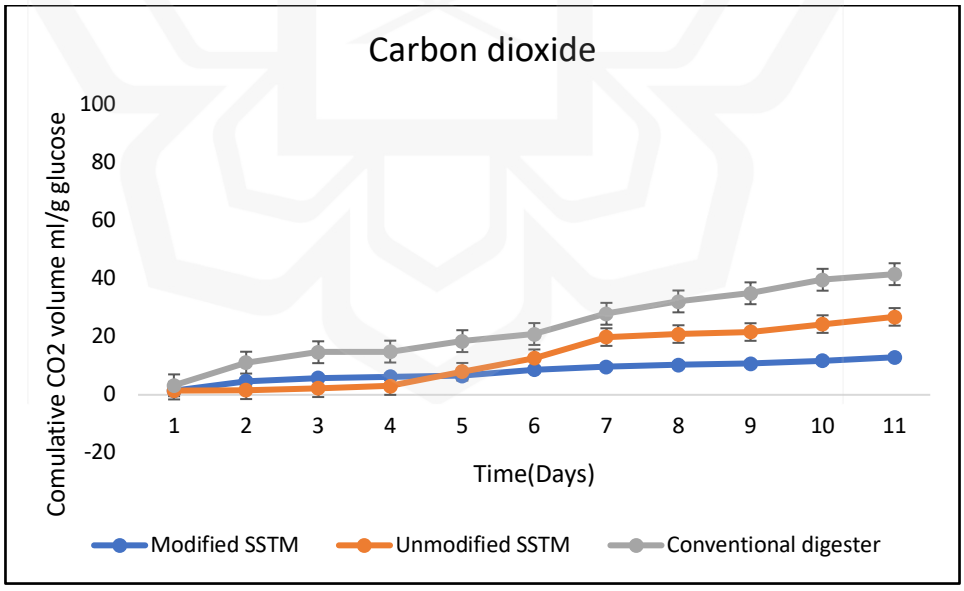

Figure 4.20 Cumulative Carbon dioxide production of conventional digester, Modified system, and unmodified system

4.9 The efficiency of different stages of the fermentation process of the modified mec-ad system

4.9.1 Hydrolysis Efficiency

The Hydrolysis of macromolecules into the soluble matter is deemed a rate-limiting step in the digestion process, limiting the activity of acidogenesis (Choi et al., 2021). The hydrolysis efficiency was measured for three reactors throughout 48h. Hybrid systems showed a substantial improvement in hydrolysis efficiency compared to conventional digesters. Unmodified electrode systems achieved an efficiency of 25% by the 8th hour, then remained constant towards the end of the cycle. While the modified electrode system's efficiency was the highest on the first day, with a value of 17%, it gradually increased to 38% on the 16th hour. This could be attributed to the enrichment of hydrolytic enzymes on the anode. Although Hydrolytic microbes are known to be very slow and perform incomplete degradation (Menzel et al., 2020). A study by Carrillo-Peña et al., (2022) reported that integrating the digester with MEC enriched hydrolytic microbes on the anode, and improved their performance, along with fermentative and VFA-consuming bacteria. AD showed the lowest yet the fastest increase in hydrolytic activity. The hydrolytic efficiency of the digester reached a maximum of 20%, then remained consistent towards the end of the cycle.

The findings are aligned with a similar study reported by Q. Huang et al., 2022 under the same voltage. Although the study's hydrolysis efficiency gradually increased with time, our findings showed that hydrolytic enzyme activity increased and reached a point of equilibrium for the three systems. To theoretically explain the difference and the behavior of hydrolytic enzymes with time, hydrolytic organisms secrete extracellular enzymes in the liquid phase, thus, attacking the soluble compounds first, increasing hydrolysis efficiency. With the depletion of soluble compounds, extracellular enzymes attack solid compounds. Carrere et al., (2016), described that when solid-liquid phase is significant, hydrolysis activity is slower, which explains the drop in efficiency with time. In addition, a different

source of inoculum offers different microbial consortia, hence different microbial performance, and behaviors.

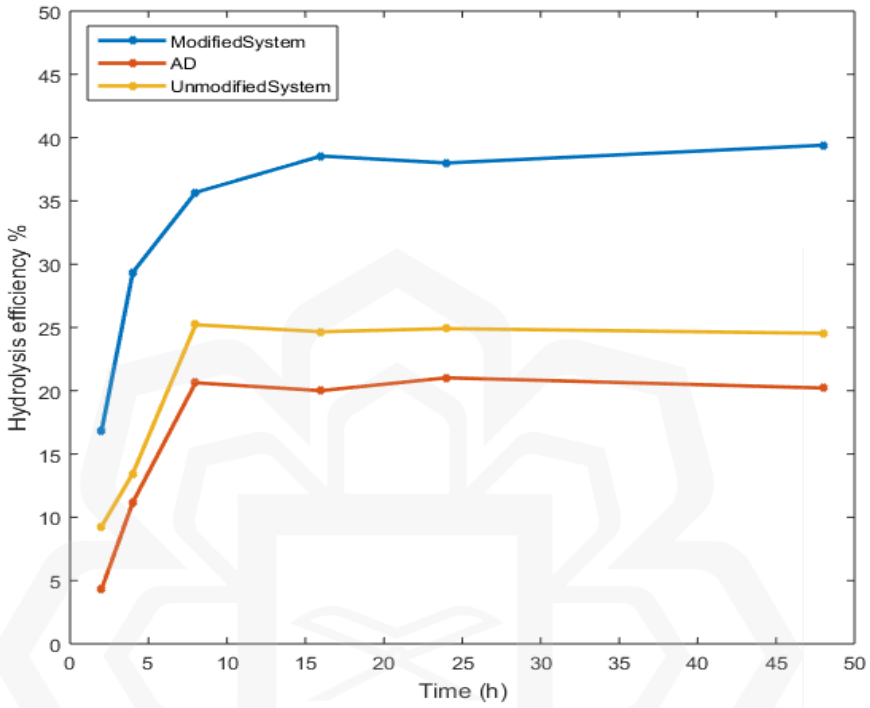

Figure 4.21 Hydrolysis efficiency of conventional digester, modified electrode system, and unmodified electrode system

4.9.2 Acidogenesis Efficiency

The acidogenesis efficiency shows the performance of fermentative microbes in utilizing and converting the substrate to volatile fatty acids, mainly acetic acid, butyric acid, and propionic acid (Agnihotri et al., 2022). Often, the acidogenesis efficiency is affected by the rate-limiting process of hydrolysis, which limits the activity of acidogenesis, hence slowing the fermentation process (Cai et al., 2013). Thus, hydrolyzed food waste was fed to the systems to avoid process limitations. Initial and final samples collected from three systems

were analyzed using RI-HPLC to determine the composition and quantity of volatile fatty acids and calculate each system's acidogenesis efficiency. However, due to unforeseen circumstances related low efficiency of the column used in separating certain volatile fatty acids, only acetic acid was spotted in the samples analyzed, as shown in the appendix. Hence, the acidogenesis efficiency calculated will need to be more accurate since different microbial communities in each system might exhibit different behavior and follow different metabolic pathways in the production of VFA (Khatami et al., 2021). Nevertheless, the initial and the final VFA concentration, biomethane produced, and the pH value could be correlated to explain the performance of acidogenesis, along with other VFA-consuming microbes. The Initial and final VFA concentration, final pH, and Biomethane concentration are tabulated in Table (4.5).

Table 4.5 Different analytical data on acidogenesis performance

System	VFA Initial concentration (mM)	VFA final concentration (mM)	Acetic acid COD (g/L)	pH	Final COD (g/L)	Biomethane (mL/g COD)
AD	14.5	45	2.8809	4.3	7	8.5
U-MEC	22.9	85.5	5.4417	4.5	7.4	13.8
M-MEC	90	106	6.8288	4.8	8.25	26.4

The anaerobic digester was referred to as AD. Systems equipped with unmodified electrodes were referred to as U-MEC, while systems equipped with modified electrodes were referred to as M-MEC. The available information on the concentration of acetic acid in terms of COD showed that more than 70% of the COD towards the end of the cycle was composed of acetic acid. Hence, the acetic acid pathway in U-MEC and M-MEC systems was the dominating pathway. Four hydrogen molecules are produced in the acetic acid pathway, as shown in the equation. In contrast, two molecules of hydrogen are consumed in the propionic acid pathway, as shown in the equation (Wattiaux et al., 2019). Thus, the

acetic acid pathway is favorable in the hybrid system since the CO_2 upgrade to biomethane requires four molecules of hydrogen, following the hydrogenotrophic methanogenesis pathway, which is enriched in the hybrid system.

Acetic acid production pathway:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2. \quad (4.4)$$

Propionic acid production pathway:

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O \quad (4.5)$$

Hydrogenotrophic methanogenesis:

$$CO_2 + 4\ H_2 \rightarrow CH_4 + 2H_2O \quad (4.6)$$

This result aligns with a previous study by Al-Sulaimi et al., (2022) on MEC-AD systems pre-acclimated with carbon-based material as acetic acid being the dominant pathway. However, this does not apply on the conventional digester, which might have been dominated by propionic or butyric acid's pathways.

Although M-MEC systems had the highest initial and final concentration of VFA's, followed by U-MEC, and AD, the accumulated VFA towards the end at hour 48 was 16 mM, compared to U-MEC with a value of 62.6 mM and 30.5 mM for AD. This could be attributed to the VFA's degrading microbes, namely Geobacter sulfurreducens, which oxidize VFAs, producing CO_2 and electrons (Fauque & Barton, 2012). To further support the statement, the previously reported results from sections (4.7.1 and 4.7.2) substrate degradation and current generation, where systems equipped with modified carbon felt anodes had the highest substrate degradation rates and current density compared to unmodified systems.

Referring to the biomethane-produced values in table 4.5, M-MEC outperformed U-MEC and AD by two and three folds respectively of biomethane per g COD consumed, which means that the two stages prior to methanogenesis were efficient in fermenting the substrate for methanogenesis consumption, namely acidogenesis and acetogenesis as they are highly interconnected to methanogenesis(Detman et al., 2021). In addition, the biogas production from U-MEC and M-MEC systems did not cease after 48h. However, the accumulated VFA were higher in these systems, unlike AD, in which the biogas production ceased at hour 32, which means methanogenesis activity was inhibited, which might have occurred due to a pH drop with a value of 4.3. This also proves that a pathway other than acetic acid dominated the conventional digester.

4.10 Modified Gompertz model of biomethane production from different inoculum

It has been reported that cattle manure has a high density and diversity of methanogenesis (Kim et al., 2014). Enhancing the original inoculum, which is rich in fermentative and degradative microbes, with cow manure rich in the methanogenic community offers the essential microbial consortia for high performance. Hence, in this section, different inoculation to the MEC-AD modified electrode system, fed with hydrolyzed food waste, was run on three different cycles. In the first cycle, the system was inoculated with 10% of the original inoculum, namely, sludge from an anaerobic digester of POME. The second cycle was inoculated with 10% cow manure fed with food waste for one month. The third cycle was inoculated with 10% of a mixture of the previous two cycles. The kinetic studies of these cycles were fitted into the modified Gompertz model. The modified Gompertz model is the best to describe the kinetic study of biogas fermentation related to bacterial behaviour and efficiency by determining the maximum biomethane yield(fd), maximum biomethane yield rate (Rm), and minimum time to produce biogas, also known as lag time (λ) (Etuwe et al., 2016).

Table 4.6 Dynamically fitted parameters according to Modified Gompertz model

Inoculum	Fd ml/g COD	Rm(mL/h)	λ (h)	$R_{squared}$
Original	29.1	0.8754	11.42	0.9922
Cow-manure	31.24	0.825	12.61	0.991
Mixed inoculum	38.68	1.2	11.95	0.9923

The Biomethane production on the span of 72h of three systems was plotted in figure (4.22). Data collected from the model fitting were tabulated in Table (4.6). The coefficient of determination and R2 values for the modified Gompertz model was about 0.99 for all regression, showing a strong correlation between the experimental data and the fitted curve.

The maximum biomethane production potential was of the system inoculated with mixed inoculum with a value of 38.68 ml, followed by cow-manure and original inoculum with values of 31.24 and 29.1, respectively. Although the third system was inoculated with the same microbial community of the two previously mentioned inoculums, Rajput & Sheikh, 2019 have explained that mixing inoculum offers high diversity of the microbial community, hence, simulates a diversity of multiple degradation pathways, which in return increases the biomethane yield and reduces the retention time. However, the lag phase for the mixed inoculum system was the longest, compared to systems inoculated with cow-manure and original inoculum, which had relatively close lag-phase duration. After mixing the inoculum, microbes should be given more time to adapt to the new environment and microbial community to they are introduced. This helps them have better performance (Rolfe et al., 2012).

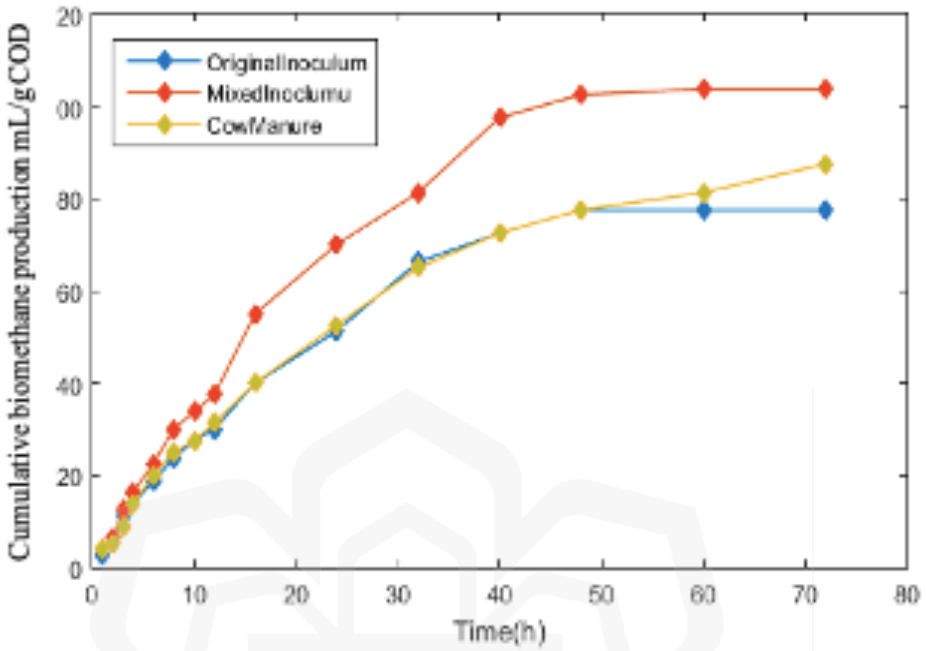

Figure 4.22 Cumulative biomethane production under different inoculation: Original inoculum, Cow-manure, and mixed of the previous inoculums

4.11 CHAPTER SUMMARY

In conclusion, it was confirmed that hydrolyzing food-waste helps increase the biogas production. However, acidic pre-treatment highly affects proteins and microbial community in the digester. Thus, enzymatic pre-treatment only is highly recommended over acidic-enzymatic pre-treatment.

Next, the electrode's modification with MWCNT highly improved the microbial attachement and behaviour. High current density and substrate degradation indicates the elevated performance of fermentative microbes. In addition, the increase in biomethane and decrease in carbon dioxide values compared to conventional digester and unmodified systems, shows that the biomethane upgrade within the system was successful.

Using the knowledge obtained from the first two objectives, into one system was reflected on the performance of the microbial community in hydrolyzing and fermenting the substrate, as discussed in the hydrolysis and acidogenesis efficiencies section. Lastly, mixing the inoculum elevates the performance and increases the competitiveness between the microbes, hence, improvement in the biomethane production potential and rate.

CHAPTER FIVE
CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

In conclusion, all the objectives of this study were achieved. The optimization of the enzymatic hydrolysis showed that an enzyme loading of 6%, substrate concentration of 10%, pH 7, and temperature 50 ºC were the best treatment conditions. The enzyme cocktail successfully hydrolysed up to 37% of the substrate for the first objective. Further chemical treatment was proposed to increase the release of reducing sugar. An acid concentration of 0.5% showed the best-reducing sugar release with a value of 49.2% and 256% compared to samples treated with only and without enzymes, respectively. However, the chemical treatment negatively affected biogas production compared to digesters fed with an enzymatically treated substrate. Digesters fed with enzymatically treated FW showed the best daily and cumulative biogas production performance, by 91% and 600% compared to acidic-enzymatic treated FW and untreated FW, respectively.

For the second objective, the electrodes were successfully modified with MWCNT, as shown previously in SEM images. The successfulness of the modification was reflected in the microbial behavioural attachment, density, and selectivity, along with the values of the current density with a value of 4.5 mA/m^2, substrate degradation of more than 80% and biomethane volume of 14.4 ml CH4/g glucose.

For the third objective, modified electrodes outperformed unmodified systems and conventional digesters regarding hydrolysis efficiency. Although the HPLC results for the analysis of volatile fatty acids only showed the concentration of acetic acid, using other available information, it could be observed that both hybrid systems were dominated by the acetic acid pathway, which is favourable for the upgrade of carbon dioxide to biomethane in the final digestion stage. Lastly, fitting the biomethane data from three different

inoculations to the modified Gompertz model has shown that mixing the inoculum showed the best biomethane production rate and potential.

5.2 RECOMMENDATIONS FOR FUTURE WORK

Multiple challenges were faced throughout the research regarding facilities available for running particular analysis, availability of certain materials needed, and microbial behaviour throughout the process. The recommendations for future development to improve the outcome and enhance the biomethane production process are as follows::

- •Enzymatic treatment helped speed up the process by tackling the rate-limiting stage, namely hydrolysis. However, the accumulation of VFAs still acts as a bottleneck. The optimization of the organic loading rate is highly recommended for the process to be successful.

- •Integrating the system helped reduce the accumulation of VFAs. It is recommended to study the separation of the processes as follows (Hydrolysis-acidogenesis) and (acetogenesis-methanogenesis) into two separate stages. This will help the system's stability, reduce inter-microbial competitiveness over the substrate, and inhibit methanogenesis, as they are highly sensitive.

- •Optimization of voltage is highly recommended, as several studies reported that voltage highly affects microbial consortia and performance. Different inoculums with different substrates have different behaviours.

•The screening of inoculum is highly recommended. One of the main challenges throughout the study is the poor methanogenic density and performance of the seeding inoculum.

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APPENDIX A

Standard curves:

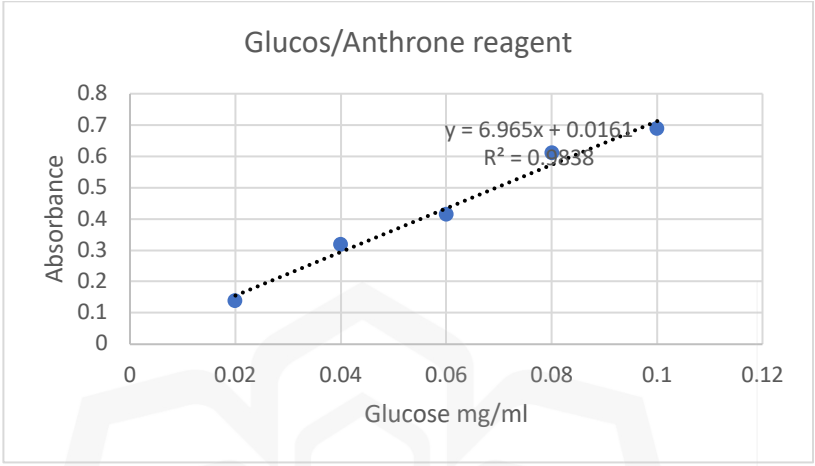

Figure A 1 Glucose standard curve using anthrone reagent

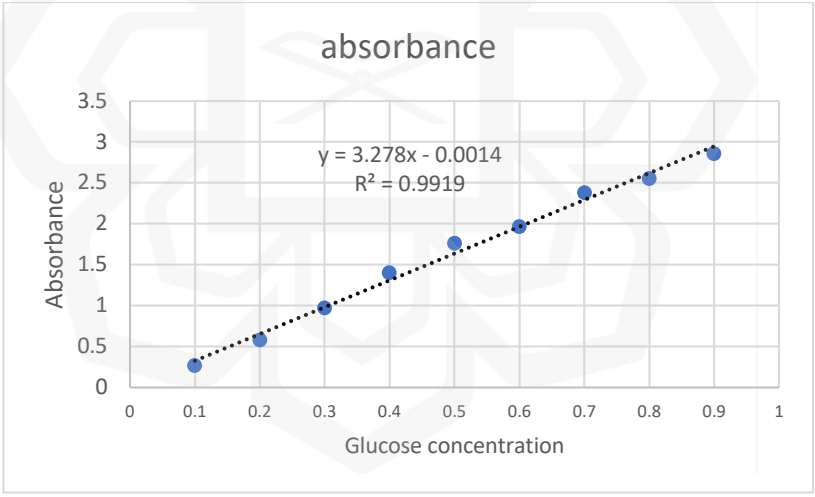

Figure A 2 Glucose Standard curve of DNS analysis

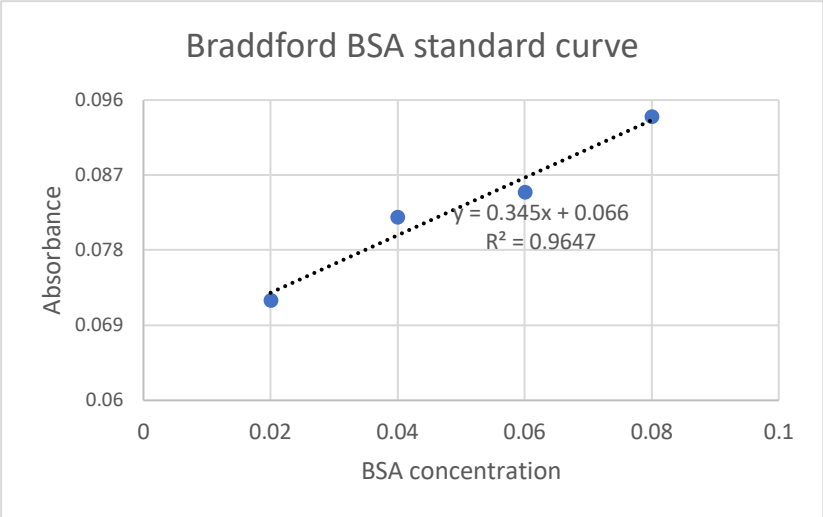

Figure A 3 Bradford BSA standard curve

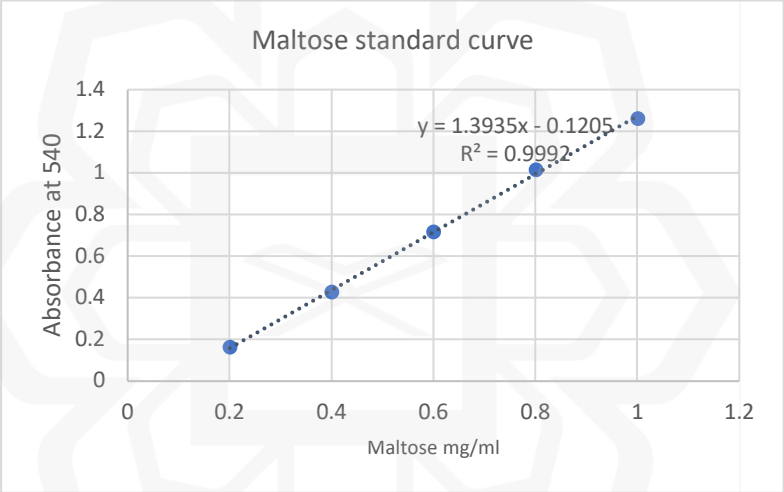

Figure A 4 Maltose standard curve

OFAT Results:

Table A 1 OFAT of substrate concentration

Substrate concentration TS %	Maltose concentration (mg/ml)	Maltose concentration (mg/ml)	Maltose concentration (mg/ml)	Average
2%	3.99	4.10	4.17	4.09
4%	7.33	8.05	7.33	7.57
6%	10.92	11.63	13.50	12.02
8%	12.71	14.15	14.22	13.69

Table A 2 OFAT of Enzyme loading

Enzyme Loading %	Maltose concentration (mg/ml)	Maltose concentration (mg/ml)	Maltose concentration (mg/ml)	Average
1%	3.74	3.02	3.09	3.28
2%	7.54	7.40	7.47	7.47
3%	12.35	10.92	11.63	11.63
4%	13.07	12.71	12.35	12.71
5%	11.63	12.35	12.71	12.23

Table A 3 OFAT of pH

pH	Maltose concentration (mg/ml)	Maltose concentration (mg/ml)	Maltose concentration (mg/ml)	Average
4	12.35	13.79	13.07	13.07
5	14.07	14.50	13.79	14.12
6	15.22	15.94	14.50	15.22
7	14.15	13.07	13.79	13.67
8	11.63	11.42	11.35	11.47
9	10.92	11.35	11.13	11.13

Table A 4 OFAT of Time

Time	Maltose concentration (mg/ml)	Maltose concentration (mg/ml)	Maltose concentration (mg/ml)	Average
8	8.5	8.1	8.6	8.4
16	13.3	13.2	13.2	13.2
24	13.5	12.7	13.2	13.1

Table A 5 OFAT of temperature

Temperature	Sample 1 absorbance	Sample 2absorbance	Sample 3 absorbance	Average
40	10.20	11.63	11.06	10.96
50	14.15	13.07	13.79	13.67
60	13.36	14.50	13.71	13.86

APPENDIX B: FIGURES

Figure B 1 Blended and diluted untreated food-waste

Figure B 2 enzyme cocktail mash

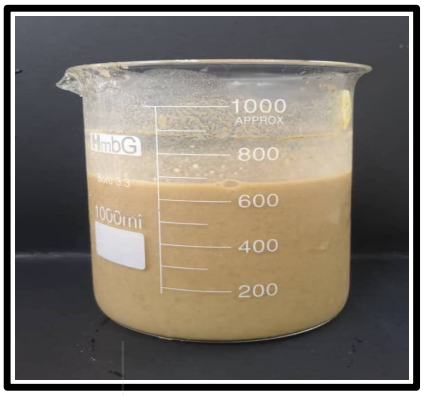

Figure B 3 Blended enzyme mash and food-waste

Figure B 4 Multiwall carbon nanotubes powder

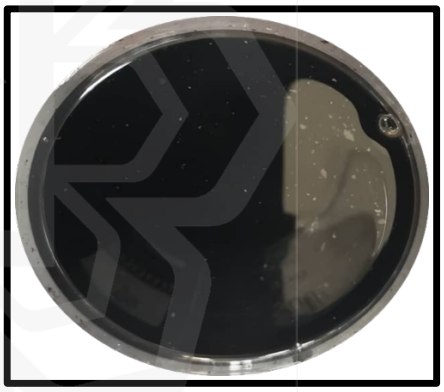

Figure B 5 MWCNT solution

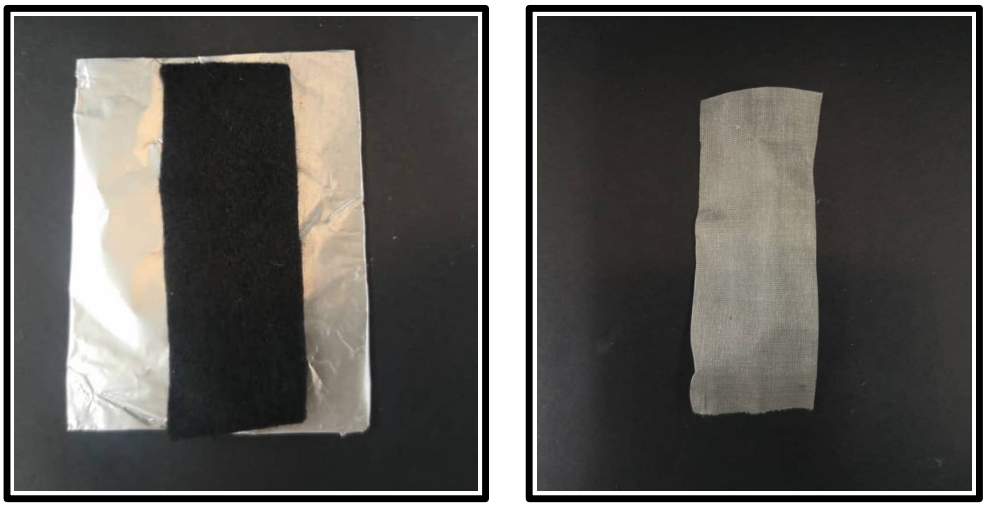

Figure B 6 Unmodified Carbon felt and stainless-steel mesh electrodes

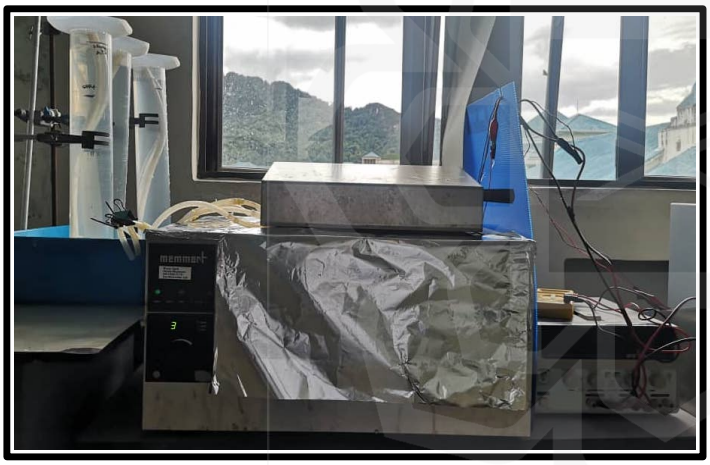

Figure B 7 Overall set-up of the hybrid system MEC-AD

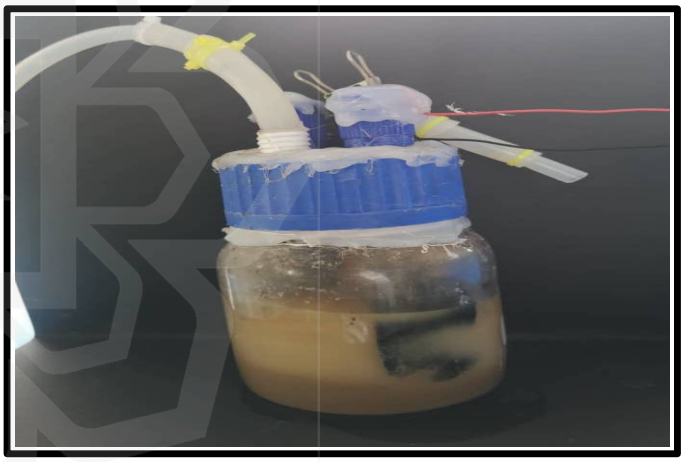

Figure B 8 Single system of the hybrid MEC-AD